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THE RAPID DETECTION OF SALMONELLA BY AN IMS-ELISA SYSTEM

By

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A thesis submitted in partial fulfilment of the requirements of
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ABSTRACT

Traditional methods of salmonella isolation and detection rely on the growth of an organism to detectable levels, requiring a minimum of 72h to achieve a presumptive salmonella colony.

An antigen-capture enzyme immunoassay was developed in order to provide a more rapid method of salmonella detection. Specific antisera, immobilised onto magnetic beads (Dynabeads® anti-Salmonella) were used to capture and isolate live salmonella cells from traditional pre-enrichment broths. The captured salmonellas were then post-enriched for 6h in GN broth plus novobiocin (nGN) followed by boiling. The heat treated cells were captured by a second batch of magnetic beads and detected by the *Salmonella* genus specific monoclonal antibody M105 in an immunomagnetic separation enzyme immunosorbent assay (IMS-ELISA). The total assay time was under 27h. The sensitivity limit of the IMS-ELISA in pure culture experiments was 10^5 cfu/ml. The IMS-ELISA detected the commonly isolated *Salmonella* serogroups B-E.

A preliminary food trial experiment compared salmonella detection of artificially inoculated raw chicken samples by traditional Rappaport Vassiliadis with selective plating on xylose lysine desoxycholate agar (RV-XLD), conventional IMS selective plating (IMS-XLD) and the IMS-ELISA method. Buffered peptone water (BPW)-raw chicken samples were artificially inoculated with five *Salmonella* strains (*S.typhimurium*, *S.virchow*, *S.enteritidis*, *S.ealing* and *S.arizonae*) at three inoculum levels (100, 10 and 1 cfu/ml). Traditional RV-XLD, IMS-XLD and IMS-ELISA methods detected *Salmonella* spp. in 14/18, 16/18 and 8/18 samples respectively. Salmonella was detected at all inoculum levels with the exception of *S.enteritidis* one cell inoculum size. Plate inoculation of nGN broths confirmed that the high number of false negative results for the IMS-ELISA was due to failure of salmonella numbers to reach the detection limit of the IMS-ELISA.

In addition the reactivity of LPS isolated from 42 *Salmonella* strains and 12 non-salmonella *Enterobacteriaceae* with the antibody M105 was analysed. Immunoblot assay of SDS-PAGE separated LPS molecules revealed that the M105 antibody reacted with (at the very least) the R-type LPS of 40 out of the 42 *Salmonella* strains tested. The lack of reaction of *S.djakarta* and a strain of *S.arizonae* (reference number 1803) implies an absence of the M105 epitope. Hence it is plausible that atypical salmonella core structures exist.

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ABBREVIATIONS

Abe	Abequose
Ac	Acetyl
AFNOR	Association Francaise De Normalisation
AOAC	Association of Official Analytical Chemistry
AP	Alkaline phosphatase
Ara	4-Amino-4-deoxy-L-arabinose
BCIP	Bromo chloro indolyl phosphate
BGA	Brilliant green agar
BIND	Bacterial Ice Nucleation Diagnostic Test
BPW	Buffered peptone water
BS	Bismuth sulphite agar
BSA	Bovine serum albumin
CDR	Communicable Disease Report
cfu	Colony forming unit
$CHCl_3$	Chloroform
CH_2N_2	Cyanamide
4-CN	4-Chloro-1-naphthol
3D	3 dimensional
DCA	Desoxycholate agar
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DNAH	Deoxyribonucleic acid hybridisation
EDTA	Ethylene diamine tetra acetic acid
EIA	Enzyme immunoassay
ELISA	Enzyme linked immunosorbent assay
Etn	Ethanolamine
FA	Fatty acid
FA'	Hydroxy fatty acid
Gal	D-galactose
Glc	D-glucose
GLC	Gas liquid chromatography
GlcN	D-glucosamine
GlNAc	N-acetyl-D-glucosamine
GN	Glucose nutrient broth
HCl	Hydrochloric acid
$HClO_4$	Perchloric acid
Hep	Heptose
HRP	Horseradish peroxidase
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulphuric acid
H_5IO_6	Periodic acid
IMS	Immunomagnetic separation
KB_4O_7	Potassium borate
Kdo	3-Deoxy-D-manno-octo-2-ulopyranosidic acid (2-keto-3-deoxyoctonic acid)
KH_2PO_4	Potassium phosphate, diabasic
LPS	Lipopolysaccharide
M105	Murine monoclonal antibody M105
Man	D-mannose
$MgCl_2$	Magnesium chloride
MLCB	Mannitol lysine crystal violet brilliant green agar
MPC	Magnetic particle concentrator
MPS	Magnetic particle separator
MS	Mass spectrometry
MSRV	Modified semi-solid Rappaport-Vassiliadis agar
NMR	Nuclear magnetic resonance
$NaAsO_2$	Sodium arsenite
$NaBD_4$	Sodium borodeutaride
$NaBH_4$	Sodium borohydride
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate

NaN_3	Sodium azide
$NaOCH_3$	Sodium methylate
NaOH	Sodium hydroxide
Na_2HPO_4	Sodium phosphate, diabasic
$Na_2PO_4.7H_2O$	Disodiumhydrogen phosphate
Na_2SO_4	Sodium sulphate
NBT	Nitroblue tetrazolium
nGN	Glucose nutrient broth with novobiocin
NTU	The Nottingham Trent University
OD	Optical density
OM	Outer membrane
P	Phosphate
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PBS	Phosphate buffered saline
PFA	Pooled antisera produced against *Salmonella* flagellins
PHL	Public Health Laboratory
PHLS	Public Health Laboratory Service
PK	Proteinase K
pNPP	p-Nitrophenylphosphate
RAM	Rambach agar
Rha	L-rhamnose
RIA	Radioimmunoassay
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RT	Room temperature
RV	Rappaport-Vassiliadis broth
SC	Selenite cystine broth
SD	Standard deviation
SDS	Sodium dodecyle sulphate
SEA	Spicer-Edwards Salmonella polyvalent H antisera
SMP	Skimmed milk powder
Spp	Species'
SS	Salmonella-Shigella agar
T6	Murine monoclonal antibody T6
TBS	Tris buffered saline
TBS-T	Tris buffered saline Tween 20
Tet	Müller-Kauffmanns tetrathionate broth
TMB	3',3',5',5' tetramethylbenzidine
Tris	Tris(hydroxymethyl)aminomethane
TSA	Tryptone soya agar
UV	Ultra violet
VIDAS	Vitek immunodiagnostic assay system
XLD	Xylose lysine desoxycholate agar

CHAPTER ONE

INTRODUCTION

1 INTRODUCTION

1.1 FOOD POISONING

Foodborne diseases are one of the most widespread problems of the contemporary world. Food poisoning or foodborne gastroenteritis results from the ingestion of contaminated food or water and is usually characterised by vomiting and/or diarrhoea and various abdominal disturbances. The causative agents may be microbial or non-microbial in origin. Microbial infection may be bacterial, viral, fungal or protozoal. While non-microbial causes of food poisoning include poisonous animals, poisonous plants, heavy metals, pesticides and herbicides or allergic or sensitivity reactions to certain foods.

The number of reports of food poisoning have increased dramatically over the last decade. In 1982 there were 14,253 reported cases of food poisoning in England and Wales. By 1988 the number had risen to 39,713 (PHLS 1998), 65% of which were bacterial in origin (Hayes 1995). Annual figures have continued to rise and by 1996 83,230 (provisional) cases of food poisoning were reported (PHLS 1998).

Reports of bacterial food poisoning exhibit a seasonal incidence which peak during the summer and early autumn. There is no explanation for this, but increased temperature abuse of foods during the summer may be at least one reason for the increase. Both sexes can be affected more or less equally, with all ages potentially at risk. The highest reported laboratory confirmed incidence occurs in the age groups who are more likely to have been referred to their family doctor, such as infants or pre-school children.

When they occur, episodes of food poisoning may be present in the form of sporadic cases (with no apparent association with any other cases), family outbreaks (affecting two or more members within a household) or general community outbreaks (affecting groups of persons who have eaten in the same restaurants or canteens or who have eaten contaminated foods obtained from the same retail outlets).

Examples of bacterial food poisoning organisms are: *Salmonella*, *Campylobacter*, *Escherichia coli* O157:H7 and *Listeria*. Other food-borne pathogens that contribute to the total number of cases include *Clostridium perfringens*, *Staphylococcus aureus* and *Bacillus cereus*.

Food poisoning caused by *Campylobacter* (campylobacteriosis) is consistently the most common cause of foodborne gastroenteritis in the UK. In 1995 there were almost 44,000 laboratory reports of confirmed cases of *Campylobacter* (Anon. 1996). Two strains of *Campylobacter* are responsible for approximately 95% of food poisoning incidences: *C.jejuni* and *C.coli*. Symptoms include severe abdominal pain and profuse diarrhoea. The incubation period varies from 2-11 days and symptoms may last up to three weeks. The primary habitat of *Campylobacter* is the gastrointestinal tract of birds (particularly poultry) and animals, cattle and domestic pets. Hence *Campylobacter* food poisoning is associated with contaminated raw or undercooked meat, unpasteurised milk and bird pecked milk on doorsteps.

Listeriosis is caused by *Listeria monocytogenes*. Healthy adults are not normally infected, the susceptible include pregnant women, the very young or elderly, and the immunocompromised. The symptoms of listeriosis can vary from a mild flu-like illness to meningitis and meningoencephalitis and is associated with spontaneous abortions. Confirmed cases of food transmission have involved unpasteurised milk and milk products such as soft cheeses. The ability of *L.monocytogenes* to grow at refrigeration temperatures (8°C) means that normal cooling of food does not inhibit the growth of the organism. Listeriosis is an important food-borne disease not because of a high incidence rate, only 130 cases were reported in 1991, but because it has a mortality rate of 31% (Newton *et al.*, 1992).

Although *Escherichia coli* normally inhabits the gastrointestinal tract of man and animals, some strains can cause intestinal infections, for example *E.coli* 0157:H7. Infection by *E.coli* 0157:H7 can cause haemorrhagic colitis where patients stools contain red blood. Severe complications may arise leading to renal failure (especially in children) and thrombolic thrombocytopenic purpura which involves brain damage and therefore the mortality rate is very high. Studies have shown that cattle are a main reservoir of the organism. Hence meat and meat products can be contaminated. Undercooked ground beef, raw milk, cold sandwiches (contaminated cooked meats), vegetables and water have all been implicated as sources of outbreaks (Vernozy-Rozand 1997). The incidence of *E.coli* 0157:H7 infection in England and Wales has increased over the past two decades with one laboratory confirmed case in 1982 to 660 cases in 1996 (PHLS 1998).

Food poisoning induced by *Clostidium perfringens* is caused by enterotoxins and is

produced after the ingestion of the contaminated food. Symptoms include diarrhoea and abdominal pain, commonly occurring 12-18h after ingestion of contaminated food. The reservoir of the organism includes the gastrointestinal tract of food animals, soil and dust. Transmission is by contaminated cooked meat and poultry dishes.

Staphylococcal food poisoning (staphyloenterotoxicosis) results from the consumption of a food in which *Staphylococcus aureus* has grown and formed toxins. Symptoms include vomiting and diarrhoea, occurring 2-8h after ingestion of an enterotoxin-containing food. Sources of *Staphylococcus aureus* are skin lesions, nostrils or fingers of foodhandlers. Transmission occurs when cooked foods such as ham, meat, poultry, fish, prawns and cream cakes are handled, stored at room temperature for more than two hours and eaten cold.

Bacillus cereus is widespread in the environment, being found in soil, sediments, dust and vegetation. The ability to produce spores resistant to factors such as drying and heat means that the organism is widely distributed in foods. There are two main types of poisoning caused by *Bacillus cereus* - diarrhoeal syndrome and emetic syndrome. The symptoms of diarrhoeal syndrome include diarrhoea, abdominal pain and nausea which develop 8-16h after ingestion. This type of poisoning has been particularly associated with meat and meat products. The second type of poisoning is accompanied by vomiting and mostly occurs upon ingestion of boiled or fried rice that has been allowed to stand at elevated temperatures for several hours after cooking (spore germination).

1.2 SALMONELLA FOOD POISONING

In this report all references to 'salmonella' with respect to genus, strain, serotype, serogroup and species (sp./spp.) are italicised i.e. *Salmonella enteritidis* as opposed to salmonella as a general term. The researcher is aware of variations in nomenclature style in scientific literature.

1.2.1 *DISEASE*

Food poisoning caused by the ingestion of *Salmonella* spp. is termed salmonellosis. The symptoms include diarrhoea, vomiting and abdominal pains, which occur 12-36h after infection and last 2-5 days. The severity of the symptoms varies depending on the host's immunocompetence and many other factors. Severe complications of *S.enteritidis* which have been reported include a small number of cases of acute renal failure, osteomyelitis

and meningitis. A mortality rate of 0.1%-0.2% has been calculated (Doyle and Cliver 1990). Those fatally afflicted are principally the elderly and the very young.

Opinions differ on the number of salmonella cells which need to be ingested to form an infective dose. It appears to be dependent on food type, the *Salmonella* strain (differences in virulence), as well as differences in individual tolerances or susceptibility and stomach contents (Doyle and Cliver 1990). Thus numbers varying from ten cells to a million have been reported to have caused foodborne infections (D'Aoust 1989).

1.2.2 *INCIDENCE*

Over the past 35 years there has been a dramatic increase in the incidence of salmonella food poisoning in industrialised countries. In recent years the number of incidences of salmonellosis have reached a peak. From 1989-94 annual figures have been relatively constant; around 30,000 cases have been reported per annum (Table 1.1). In contrast the 1980's witnessed a remarkable increase in the number of reported cases of salmonella food poisoning. In 1980 there were 9,540 laboratory reported cases of salmonella food poisoning in England and Wales which by 1989 had risen to 29,998.

Almost any of the 2,400 recognised *Salmonella* serotypes (see Section 1.32 for definition) may on occasion cause food poisoning. Although about 95% of the strains causing disease in man belong to serogroups A-E (see Section 1.32 for definition) and comprise of less than 40 serotypes (Davis *et al.*, 1990). The ten most commonly reported serotypes for the years 1989-97 are shown in Table 1.2. These accounted for over 90% of the total reports received.

Although the relative isolation rates of *Salmonella* serotypes have undergone change; two serotypes in particular have predominated. Up to 1986/87 *S.typhimurium* was the most common isolate, and this has since been displaced into second place by *S.enteritidis* (Table 1.1). By 1988 the incidence of food poisoning caused by *S.enteritidis* (15,427) had risen to a level more than double that of *S.typhimurium* (6,444), accounting for more than 50% of the total number of reports. Presently these serotypes account for 75% of reported cases of food poisoning in the UK.

The dramatic increase in the number of cases of human salmonellosis caused by *S.enteritidis* in the 1980's was due to phage type 4 (PT4; Table 1.1). In 1988 *S.enteritidis*

Table 1.1: Reported cases of salmonella food poisoning in England and Wales 1981-1997 (source PHLS 1998; CDR reports)

Year	S.typhimurium	S.enteritidis		Other serotypes	Total
		(all)	(PT4)		
1981	3,992	1,087	395	5,171	10,251
1982	6,089	1,101	413	5,132	12,322
1983	7,785	1,774	823	5,596	15,155
1984	7,264	2,071	1,362	5,392	14,727
1985	5,478	3,095	1,771	4,757	13,330
1986	7,094	4,771	2,971	5,111	16,976
1987	7,660	6,858	4,962	6,014	20,532
1988	6,444	15,427	12,522	5,607	27,478
1989	7,306	15,773	12,931	6,919	29,998
1990	5,451	18,840	16,151	5,821	30,112
1991	5,331	17,460	14,693	4,902	27,693
1992	5,401	20,094	16,987	5,860	31,355
1993	4,778	20,254	17,257	5,618	30,650
1994	5,523	17,370	13,782	7,535	30,428
1995	6,743	16,044	12,482	6,527	29,314
1996*	5,573	18,296	13,184	5,242	29,111
1997*	4,498	22,617	15,140	4,431	31,546

* Provisional data

Table 1.2: Top ten *Salmonella* serotypes reported to Communicable Disease Surveilance Centre 1995-97* (source CDR reports)

Serotype	Ranking		
	1995	1996	1997
S.enteritidis	1	1	1
S.typhimurium	2	2	2
S.virchow	3	3	4
S.hadar	4	4	3
S.newport	5	6	6
S.heidelberg	6	5	5
S.infantis	7	7	7
S.indiana	8	8	-
S.agona	9	9	9
S.braenderup	10	10	8
S.java	-	-	10

* Provisional data

PT4 accounted for over 80% of the total number of reported cases of salmonella food poisoning in England and Wales. However since its peak in 1993 the number of human isolations of *S.enteritidis* PT4 have declined annually and have been replaced by other phage types. While *S.typhimurium* definite phage type 104 (DT 104), carrying multiple antibiotic resistance, has been increasing steadily and is now the second most prevalent *Salmonella* strain isolated from human sources (Wall *et al.*, 1997).

1.2.3 *THE ORGANISM*

The genus *Salmonella* are Gram-negative facultative anaerobic rod shaped bacteria (0.7-1.5 x 2-5μm). They are usually motile with peritrichous flagella with the exception of *S.gallinarum*, *S.pullorum* and one type of *S.arizonae*. They can grow between 7-48°C (optimum 37°C), at pH 4-8 and at water activities above 0.93. They are readily killed by heat and acid and are resistant to both freezing and drying, particularly in the presence of proteins and other protectants (Baird-Parker 1991).

1.2.4 *RESERVOIRS AND TRANSMISSION*

The salmonella organism is widely distributed in nature with humans and animals being their primary reservoirs. Their primary habitat is the intestinal tract of animals such as birds, reptiles, farm animals, humans and occasionally insects. As intestinal forms, the organisms are excreted in faeces and can be subsequently transmitted to a large number of places by horizontal and vertical routes. The following section gives examples of the possible routes of infection with respect to poultry associated *S.enteritidis*.

The horizontal spread of *S.enteritidis* has been facilitated by modern intensive agricultural practices of the poultry industry. Cross-infection from infected to uninfected poultry by faecal contamination can occur in the housing unit (where large numbers of poultry are grouped together) and on the way to slaughter (where faecal discharge is increased by the stress of transportation). Furthermore, carcass to carcass cross-contamination can occur during the slaughter, dressing and preparation of raw meats.

Another possible source of environmental contamination is poultry feed. Inedible materials and other parts of the animals not used for human consumption are sent to the rendering plant. Meat and bone meal produced by this process is used in compounded animal feeds. Although rendering should destroy any salmonella present in the raw materials, cross-contamination may occur (Baird-Parker 1991).

The vertical transmission of *S.enteritidis* from parent flock to progeny can occur via transovarian infection of the egg and contents or the egg shell during, or shortly after, laying. Thus eggs may be contaminated with salmonella.

Poor hygiene practices in the home and food service industries have perpetuated and exacerbated the problem of salmonella infection. If raw and cooked foods are prepared on the same surfaces using the same equipment or if they are stored in close proximity, salmonella may spread to the foods which will receive no further heat treatment before consumption. Additionally when salmonella infected poultry is undercooked or inadequately thawed with cooking time too short and temperatures too low, salmonellas may survive. Moreover the organism can survive normal light cooking; soft-boiled, lightly fried or scrambled eggs may still contain salmonella.

1.2.5 *FOOD VEHICLE*

Some serovars appear to show a degree of host adaption and primarily infect one species of animal. For example *S.cholerasuis* and *S.abortusovis* occur in pigs and sheep respectively. While *S.pullorum/gallinarum* infect poultry and *S.dublin* infect cattle. In contrast *S.typhimurium* affects all species of animals.

Hence, previous increases in the incidence of human salmonellosis have been attributable to infection in particular types of animals and their entry into the food chain (Philips and George 1994). For example an increase in *S.typhimurium* in the late 1970's and early 1980's was presumed to have been the result of infected cattle (Cooke 1990). While the increase in *S.enteritidis* between 1989 and 1994 has been attributable to infected poultry and eggs. During the period 1989-91, 23% of outbreaks were associated with egg and egg products. While poultry meat (including chicken and turkey) accounted for 20% of outbreaks (Sockett *et al.*, 1993).

Imported foods are another possible source of salmonella as they may not meet the bacterial quality requirements of the UK. For example in 1993 a survey into the level of salmonella contamination in a range of food samples was carried out by the Public Health Laboratory Service (PHLS) Food Surveillance Group (Anon. 1993a). Among the food samples tested was a small range of imported foods. A staggering 64% of imported raw poultry (compared with 18% of UK poultry) and 54% of imported peppercorns were positive for *Salmonella* spp.. Although it was emphasised that the samples examined were

not necessarily representative of the food market, it highlights the fact that imported foods were a notable possible source of infection. Previous food poisoning outbreaks caused by imported foods include bean sprouts from Australia, mustard cress from the Netherlands, Irish soft cheese made from unpasteurised milk and pâté from France (D'Aoust 1994).

1.2.6 *ECONOMIC COST*

Foodborne diseases are an important cause of economic loss. For example, the costs of salmonella infection in the UK are estimated to run into millions of pounds annually for reported cases alone (Sockett and Roberts 1989). Direct or tangible costs of salmonellosis are easily measured in monetry terms. They include loss of earnings, medical costs, costs of diagnosis and investigation of an outbreak. When a common food-source is identified as the cause of an outbreak, additional costs arise from the recall and destruction of the implicated food. The tangible costs associated with seven salmonella outbreaks reported in the UK since 1981 are summarised in Table 1.3. The largest society related costs were recorded in outbreaks which were due to manufactured foods having wide distribution nationally. For example an outbreak in the UK which was due to baby milk powder, gave total direct costs of £14.6 million (Roberts *et al.*, 1989). Major costs to the manufacturer resulted from the recall and destruction of the product. Additional costs included capital expenditure on plant clearing, renovation and equipment replacement. Furthermore indirect costs to the manufacturer involved resulted from a £22 million loss in the sale value of the company, a decline in public confidence in the product and the eventual closure of a production unit with the loss of about 200 jobs (Sockett 1991).

1.2.7 *CONTROL MEASURES*

It is practically impossible to eradicate salmonellas from the environment. Therefore control measures are necessary to limit the recurrence of the disease. Control measures include education about the possible sources of salmonella infection, reduction and subsequent control of salmonella infection in farm animals, surveillance of food poisoning incidences and epidemiological investigation of outbreaks which will be discussed below. Additional control measures include microbiological investigation and food testing, which will be discussed in detail in Sections 1.3 and 1.5 respectively.

Table 1.3: Direct costs associated with seven salmonella outbreaks reported in the UK since 1981 (from Sockett 1991)

Year	Serotype (no. of cases)	Food	Public Sector Cost (£)	Society Cost (£)	Total Cost (£)
1981	*S.typhimurium* (654)	Raw milk	43,493	39,737	83,230
1982 #	*S.napoli* (245)	Chocolate	104,312	274,646	378,958
1982	*S.typhimurium* (31)	Not known	3,234	3,280	6,514
1985 *#	*S.ealing* (76)	Baby milk powder	163,012	14,500,000	14,663,017
1985	*S.thompson/ infantis* (242)	Turkey	35,612	79,240	113,852
1987	*S.heidelberg* (19)	Poultry	23,146	2,197	25,343
1987	*S.typhimurium* (19)	Raw milk	11,392	>6,500	>17,892

*, direct costs only; #, outbreaks due to manufactured food

1.2.7.1 *Education*

The education of the food services industries include implimentation of the Hazard Analysis Critical Control Point (HACCP) system. HACCP is a systematic approach to food safety management, encompassing the entire process from farm to table (Hall 1994). It evaluates the hazards of raw materials including those that may appear throughout processing and those that may occur from consumer abuse. Once the hazards have been identified they can be wholly or partly eliminated. HACCP therefore controls organisms at the point of production and preparation. Thus proper implementation of the HACCP system ensures microbiologically safe foods.

Education of the public have included a publication by the Department of Health in 1988 which gave advice on the infection of salmonella from raw eggs (DHSS 1988). People were advised to avoid eating raw eggs or uncooked foods made from them such as mayonnaise. Additionally, vulnerable individuals such as the elderly, the sick, babies and pregnant women were urged to consume only eggs which had been cooked until the white and yolk were solid.

1.2.7.2 *Reduction and control of salmonella infection in farm animals with reference to poultry*

Between 1989 and 1993 infected flocks were slaughtered in an attempt to establish salmonella-free breeding stocks. However these measures were abandoned because new laying flocks were still liable to be contaminated with salmonella.

There are codes of practise which aim to control the spread of infection by reducing cross-contamination in hatcheries, in the transport of animals and in rendering plants (Altekruse 1993; Baird-Parker 1991). Moreover in the UK there is a legal requirement to carry out routine bacteriological monitoring of poultry flocks including environmental, faecal and carcass samples (MAFF 1989).

Alternative methods of reducing faecal shedding of salmonella by poultry have included the use of antibiotics (Griffin 1991), competitive exclusion (Silliker 1982; Doyle and Cliver 1990), vaccination (Hassan and Curtiss 1990; 1996; Griffin 1991) and irradiation of poultry carcasses (Szczawińska *et al.*, 1991) and egg shells (Feung-Lin *et al.*, 1997; Wallner-Pendleton *et al.*, 1994).

Processed animal protein that is to be used in animal feed is also monitored and applies both to domestic and imported products (MAFF 1989). This is supported by codes of practise including bacteriological monitoring for the handling and storage of raw materials and the production and handling of final feed (Dawson 1992). Other attempts to produce salmonella-free feedstuffs have included heat, chemical treatment and irradiation (Board 1983; Simonsen *et al.*, 1987).

1.2.7.3 *Surveillance and epidemiological investigation*

The Public Health Laboratory Service (PHLS) Salmonella dataset records the incidence of salmonella food poisoning in England and Wales. It is essential for detecting geographically widespread outbreaks of infection and facilitates swift epidemiological investigation. Epidemiological and microbiological typing techniques include phenotypic and molecular methods and enables the fine differentiation of salmonellas into types (for details see Section 1.32). This in turn increases the precision and certainty with which the sources and methods of spread of infection can be identified. Thus if the source of infection is continuous and can be classified, action can be taken to limit the outbreak. For example, a widespread outbreak of infection with *S.napoli* occured in the Midlands

and the South of England between April and August 1982 (Roberts *et al.*, 1989). Fifty-one (21%) patients were admitted to hospital and more than one third of these had serious complications, including 16 with bacteraemia. Epidemiological and microbiological investigations identified imported Italian chocolate bars as the vehicle of infection. A public warning was issued and wholesale distribution stopped. Four fifths of the consignment of three million chocolate bars were recalled and destroyed. In the absence of epidemiological investigation all contaminated chocolate bars would have been distributed and eaten. Under these circumstances it has been estimated that the outbreak would have been five times as large and would have caused five deaths.

At this point it should be emphasised that under reporting is still a significant problem. Hence the data obtained by the PHLS dataset is only a indication of the trends of salmonella food poisoning. It is estimated that only ten percent of cases of food poisoning are actually reported (Scott 1996).

1.3 MICROBIOLOGICAL INVESTIGATION

1.3.1 *CLASSIFICATION AND BIOCHEMICAL CHARACTERISTICS*

The genus *Salmonella* belong to the family *Enterobacteriaceae*. They can be differentiated from closely related genera by biochemical tests (Table 1.4). For example citrate is usually utilised as a sole carbon source and they rarely ferment lactose or sucrose. With the exception of *S.typhi* salmonellas regularly ferment glucose with the evolution of gas. Additionally *Salmonella* spp. can grow both aerobically and anaerobically.

1.3.2 *DIFFERENTIATION OF THE GENUS SALMONELLA*

1.3.2.1 *Species and subspecies*

Salmonella nomenclature has undergone a number of changes. Under the taxonomic treatment of Le Minor and Popoff (1987) the genus was assigned to a single species designated *S.enterica* which was divided into seven subspecies and was defined on the basis of biotype and genomic DNA hybridisation. These included *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae*, *bongori*, and *indica*; which were numbered I, II, IIIa, IIIb, IV, V and VI respectively. Under this system *S.typhimurium* would be reported as *S.enterica* subspecies *enterica* serovar *typhimurium*. Following work carried out by Reeves *et al.* (1989) the subspecies *bongori* (group V) was elevated to species status. A recent study by Boyd *et al.* (1996) used molecular genetic analyses to identify an eighth taxonomic group designated VII. This group is composed of several strains that were

Table 1.4: Differentiation of genus *Salmonella* from other members of the family Enterobacteriaceae

Biochemical test	Genus			
	Salmonella	*Shigella*	*Citrobacter*	*Edwardsiella*
β-Galactosidase	-[a]	+/-	+	-
Arginine dihydrolase	+/-	-	+/-	-
Lysine decarboxylase	+	-	-	+
Ornithine decarboxylase	+	-[b]	+/-	+
Simmons citrate	+	-	+	-
H_2S production	+	-	+/-	+
Acid from				
lactose	-[a]	-[c]	+/-	-
dulcitol	+/-	-	-/+	-
melobiose	+	-/+	-	-
sorbitol	+	-+	+	-
xylose	+	-	+	-
Motility	+	-	+	+

[a], *S.arizonae* and strains of some other serotypes are positive; [b], *Shigella sonnei* is usually positive; [c], delayed fermentation is a feature of some strains of *Shigella sonnei*; H_2S, hydrogen sulphide

previously assigned to group IV on the basis of biotype.

1.3.2.2 *Serogroup and serotype*

The differentiation of the genus *Salmonella* into serogroups and serotypes is based on a system known as the Kauffmann-White Scheme (Edwards and Ewing 1972). The scheme relies on differences of somatic (O), flagella (H) and capsular (Vi) antigens (Figure 1.1). The designation H is an abbreviation of the German word '*Hauch*' meaning breath. This term was first used to describe the swarming growth of the flagellated *Proteus* spp. on the surfaces of moist agar plates which resembled the light mist caused by breathing on glass. The desination O an abbreviation of the German word '*Ohne*' meaning without is used as a generic term for the lipopolysaccharide somatic antigens of all enteric bacilli. The designation Vi refers to the additional surface (capsular) antigen of *S.typhi* which was originally thought to be responsible for virulence.

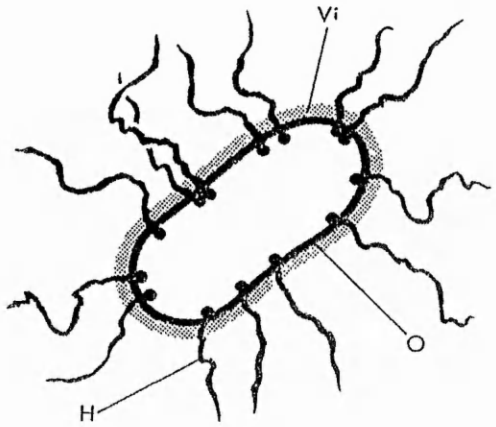


Figure 1.1: Schematic diagram of the cellular locations of flagellar (H), somatic (O) and capsular (Vi) antigens of enteric bacilli (from Davis *et al.*, 1990).

The O-antigens form part of a structure known as lipopolysaccharide which will be discussed in detail in Section 1.4. Each salmonella possesses a distictive O-antigen. This is made up of two or three distinguishable factors (determinants). These factors are numbered consecutively from 1 to 67 by means of arabic numerals, although this series is not completely continuous as there is no factor in the scheme represented by the numerals 29, 31, 32, 33, 49, 62 and 63.

Each O-antigen has a strongly reacting major factor and on this basis the *Salmonella* spp. have been classified into serogroups. The first 26 groups are designated by letters (A-Z), the subsequent ones are designated by numbers (50, 51, etc.). For example, *Salmonella* serogroup E are classed together because they all possess the major O-factor number 3 (Table 1.5). Additional divisions within a group are made on the basis of minor O-factors. Therefore *Salmonella* serogroup E can be further divided into subgroups E_1, E_2, E_3 and E_4.

The members of each serogroup may be differentiated still further into serotypes (serovar) on the basis of their flagellar (H) antigens (Table 1.5). A given strain may form either one or two kinds of H-antigen. They are referred to as phase 1 and phase 2. Phase 1 flagellar antigens are designated with letters of the alphabet. However the limitation of the alphabet made it necessary to use subscript numbers. Thus flagellar antigens that are labelled z_1, z_2 etc are not subdivisions of z. Although there are flagellar antigens which may be subdivided, for example b_1, b_2, b_3 etc. While phase 2 flagellar antigens are identified by arabic numerals 1-7. Another important point to note is that some specific phase antigen complexes originally designated to phase 1, were later found to be phase 2 antigens. These were not re-named and therefore remain as letters. Although the organism in a given culture may be entirely in one phase (monophasic culture), they frequently can give rise to mutants in the other phase (diphasic culture), especially if the culture is incubated for more than 24h. This phenomenon is known as phase variation.

The capsular Vi (virulence) antigen occurs only in three serotypes: *S.typhi*, *S.paratyphi-C* and in rare strains of *S.dublin*. It is usually too thin to be seen as a capsule but often masks the O-antigens and can be removed by boiling. The Vi antigen also serves as a phage receptor.

There are in excess of 2,400 *Salmonella* serotypes. Each is referred to by a Linnean name or simply by antigenic formula. In the case of uncommon serotypes, no further subdivision may be necessary. However for those serotypes commonly encountered, such as *S.typhimurium* and *S.enteritidis*, serotyping alone is not sufficiently discriminatory for epidemiological investigation. A means of subdivision is therefore required for common serotypes. Various means used are, biotyping based on the ability to ferment different sugars, resistance to antibiotics (R-typing), phage types and plasmid profiles.

Table 1.5: *Salmonella* differentiated by the Kauffman-White Scheme (from Rowe and Hall 1989)

Serogroup	Serotype	Antigenic formula		
		O-antigen	H-antigen	
			Phase 1	Phase 2
B	*S.agona*	1,4,[5],12	f,g,s	[1,2]
	S.bredeney	1,4,12,27	l,v	1,7
	S.gallinarum	1,9,12	-	-
	S.saint paul	1,4,[5],12	e,h	1,2
	S.typhimurium	1,4,[5],12	i	1,2
C_1	*S.livingstone*	6,7	d	1,w
	S.virchow	6,7	r	1,2
	S.infantis	6,7,[14]	r	1,5
C_2	*S.hadar*	6,8	z_{10}	e,n,x
	S.manchester	6,8	l,v	1,7
	S.newport	6,8	e,h	1,2
C_3	*S.kentucky*	8,20	i	z_6
D_1	*S.enteritidis*	1,9,12	[f],g,m,[p]	[1,7]
	S.panama	1,9,12	l,v	1,5
	S.dublin	1,9,12,[Vi]	g,p	-
E_1	*S.give*	3,10	[d],l,v	1,7
E_4	*S.senftenberg*	1,3,19	g,[s],t	-
L	*S.minnesota*	21	b	e,n,x
O	*S.adelaide*	35	f,g	-
	S.ealing	35	g,m,s	-

Somatic antigens that are underlined indicate that they are phage determined; [], may be present or absent

1.3.2.3 *Phage type*

Bacteriophage (phage) typing has been developed as a means to discriminate between *Salmonella* serotypes that are serologically or biochemically indistinguishable. Strains are distinguished on the basis of their sensitivity to lysis by specific bacteriophage. Specific sets of typing phages are required to type *Salmonella* spp.. For example there are set of phages for those salmonellas that posses the Vi antigen. Other phage are used to type *S.typhimurium*, *S.enteritidis*, *S.virchow* and *S.bareilly* (Varnam and Evans 1991). However it is not possible to develop typing schemes which are sufficiently discriminatory for some salmonellas.

1.3.2.4 *R type (drug resistance)*

Antibiogram screening tests for resistance to a range of commonly-used antimicrobial drugs, for example ampicillin, chloramphenicol, gentamycin and tetracycline (SAB 1994).

1.3.2.5 *Biotype (biovar)*

Biotypes or biovars are identified by differences in the biochemical characteristics and may be used either to subdivide a species or to discriminate within a serotype as with the xylose$^+$:xylose$^-$ character of *S.typhi*.

1.3.2.6 *Genetic and molecular typing methods*

Salmonella strains can be further differentiated by deoxyribonucleic acid (DNA) based methods. These DNA-molecular typing methods involve the analysis of plasmid DNA and chromosomal DNA. Techniques of plasmid analysis include plasmid profile typing, plasmid fingerprinting and identification of plasmid-mediated virulence genes. While chromosomally-based methods include ribotyping, random cloned chromosomal sequence typing, insertion sequence typing, pulse-field gel electrophoresis and random amplified polymorphic DNA typing (Threlfall *et al.*, 1994).

1.3.2.7 *Lipopolysaccharide typing schemes*

The lipopolysaccharide of Gram-negative bacteria exhibit marked structural heterogeneity. This affects their physical properties and in particular their molecular mass (Towner and Cockayne 1993). Extracted lipopolysaccharide can be differentiated by electrophoretic means to produce a characteristic fingerprint (for more detail see Section 1.4.4.2). Direct visualisation of lipopolysaccharide banding patterns offers a rapid alternative method of differentiation of species and strains of a number of Gram-negative bacteria (Towner and

Cockayne 1993).

1.4 LIPOPOLYSACCHARIDE

Lipopolysaccharide (LPS) molecules are located in the outer leaflet of the outer membrane of the Gram-negative bacterial cell (Nikaido and Vaara 1987), where they form the outermost layer of a bilayered structure. An estimated 1.2 million LPS molecules (Tsang and Nielsen 1992) cover 40-50% of the bacterial cell surface (Iglewski and Clark 1990) and account for 3-8% of the dry weight of the cell (Rietschel *et al.*, 1984). In this exposed position LPS are involved in the interaction of the cell with the environment. Since LPS are immunogenic, contact of the bacterium or isolated LPS with the immune system elicits the formation of antibodies specific for distinct epitopes on the molecule. Thus LPS are major cell surface antigens.

LPS serves as a bacteriophage receptor, e.g. affords recognition by the virus and subsequently adsorption on the cell surface (Knirel and Kochetkov 1993a).

LPS are heat stable endotoxins inducing fever and shock in a host on entering the bloodstream (Raetz 1993). For a summary of the progress of investigations into the chemical nature of endotoxins and the mechanisms of their biological action see 'The Handbook of Endotoxin' (Protor 1984; 1985a; 1985b; 1986).

This thesis is concerned with salmonella LPS and its detection using specific antibodies. Therefore an understanding of the composition and structure, biosynthesis and properties of LPS is necessary. The following section will review these aspects and where appropriate details of salmonella LPS will be given.

1.4.1 *COMPOSITION OF LPS*

The structure of LPS varies among species of bacteria and even among strains, but all Gram-negative bacteria possess it in some form or another. Despite their extensive structural diversity, distinct architectural elements are common: a hydrophobic lipid A residue linked to an oligosaccharide core region and a hydrophilic O-polysaccharide or O-chain (Figure 1.2). A number of non-enteric Gram-negative organisms lack O-chain. These include *Haemphilius influenzae*, *Bordetella pertussis*, *Neisseria meningitidis* and *N.gonorrhoaeae* (Hancock *et al.*, 1994).

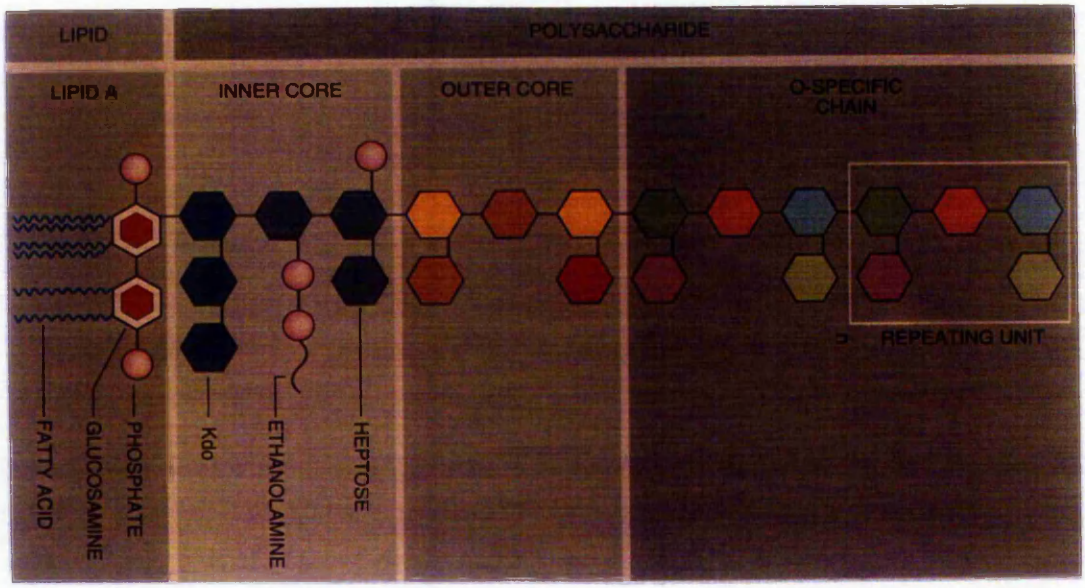


Figure 1.2: A schematic representation of salmonella lipopolysaccharide (from Rietschel and Brade 1992)

1.4.1.1 *Lipid A*

Lipid A anchors the LPS in the outer membrane. It is the least structurally variable region of LPS. Its structure is virtually constant among *Enterobacteriaceae* (Rietschel *et al.*, 1984), although the fatty acid composition may vary (Kenne and Lindberg 1983). The general structure of lipid A consists of a disaccharide β-D-glucosaminyl-(1-6)-α-D-glucosamine backbone, which may be substituted by phosphate groups, ethanolamine phosphate or 4-amino-4-deoxy-arabinose-1-phosphate. Also attached to the disaccharide are usually ester and amide linked fatty acids with chain lengths often between 10 and 16 carbon atoms long. Mild acid hydrolysis techniques cleave lipid A from the core and O-chain generating free lipid A and degraded polysaccharide. In soluble form, free lipid A possesses most of the characteristics of intact LPS (Hitchcock *et al.*, 1986). Thus LPS is the endotoxic principal of LPS (Brade *et al.*, 1988). For a summary of the biological activities of LPS and further references see Poxton and Arbuthnott (1991).

1.4.1.2 *Core region*

The core region is an acidic oligosaccharide which binds the lipid moiety of LPS to the O-chain. It is a short series of sugars consisting of an inner and outer element (Figure 1.3). Partitioning of these two regions is based on differences in the composition and

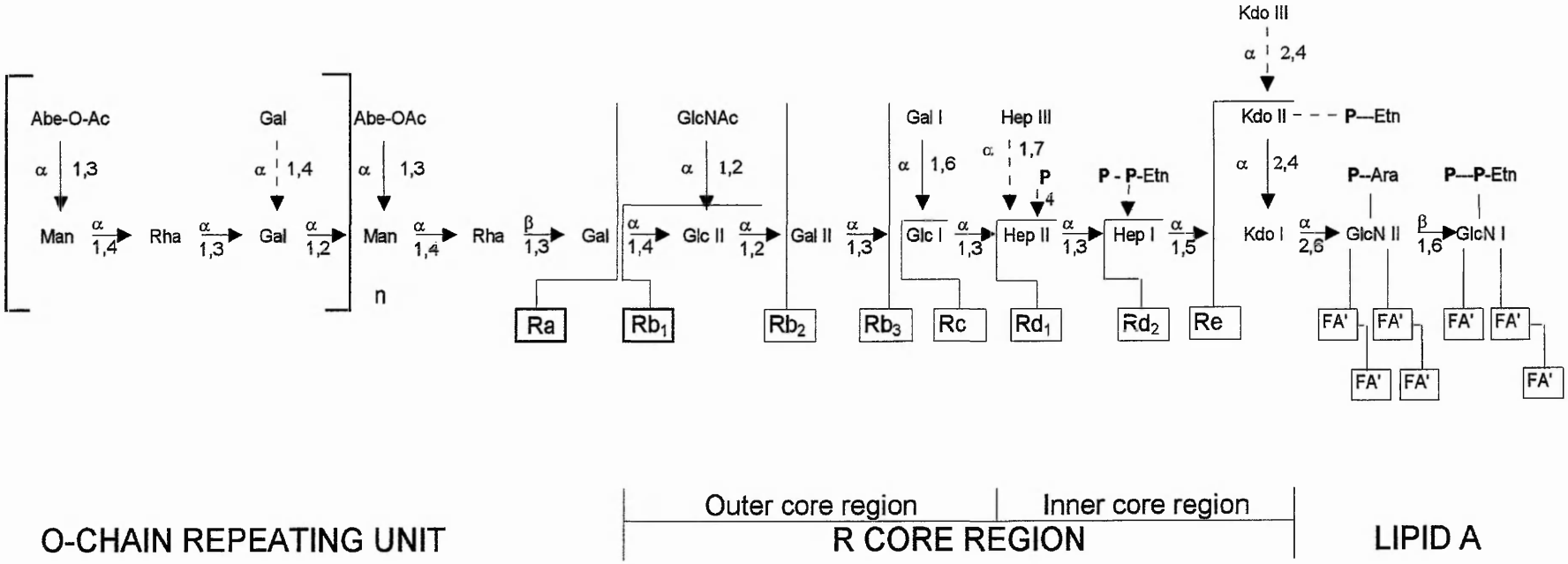


Figure 1.3: The chemical structure of salmonella lipopolysaccharide (Dotted lines indicate incomplete substitutions)

Abe, abequose; Ac, acetyl; Ara, 4-amino-4-deoxy-L-arabinose; Eth, ethanolamine; FA, fatty acid; FA', hydroxy fatty acid; Gal, D-galactose; Glc, D-glucose; GlcN, D-glucosamine; GlcNAc, N-aceyl-D-glucosamine; Hep, heptose; KDO, 3-deoxy-D-manno-octo-2-ulopyranosonic acid; Man, D-mannose; P, phosphate; Rha, L-rhamnose

variability of the structures.

The inner core region is attached to lipid A and usually contains the sugars 3-deoxy-D-manno-octo-2-ulopyranosonic acid (Kdo) and L-glycero-D-manno-heptose. Sometimes the inner region contains some other monosaccharides and often charged non-carbohydrate substituents such as phosphate, pyrophosphate and ethanolamine which are attached to the sugars in varying degrees (Knirel and Kochetkov 1993b). It is the next least structurally variable region after lipid A and is relatively constant among Gram-negative bacteria (Lüderitz *et al.*, 1982).

The outer core region of Enterobacteriaceae contains the sugars D-glucose, D-galactose, N-acetyl-D-glucosamine and N-acetyl-D-galactosamine. It is more variable than the inner core (Knirel and Kochetkov 1993b). The outer core may be subject to small structural variations. For example five structural types have been elucidated for *E.coli*, denoted by R1-R4 and K-12 (Westphal *et al.*, 1983; Brade *et al.*, 1988; Rietschel *et al.*, 1990). In *Proteus* five core types have been found and in *Citrobacter* four common core types have been described (Rietschel *et al.*, 1991). However the outer core region of the genus *Salmonella* is generally believed to have a single structure (Jansson *et al.*, 1981; Westphal *et al.*, 1983; Jann and Jann 1984). This is shown in Figure 1.3 and comprises of a pentasaccharide of D-glucopyranose (D-glucose), D-galactopyranose (D-galactose) and 2-acetamido-2-deoxy-D-glucopyranose (N-acetyl glucosamine). Although recently there have been a number of reports of variations within the core structure (Tsang and Schlecht 1990; Tsang *et al.*, 1991a; Tsang *et al.*, 1991b; Tsang *et al.*, 1992; Hoofvak and Olsthoorn 1995).

1.4.1.3 *O-chain*

The outer most portion of the LPS molecule contains repeating carbohydrate units of identical oligosaccharides termed O-chains (synonymous with the term O-antigen). The types of sugars present, their arrangement within the repeat units and the linkage between repeat units vary. In some cases the repeating units may contain an oligomer of a single sugar type but in a distinct linkage sequence e.g. mannan in *E.coli* 09. However in most cases the repeating oligosaccharide contains units of 3-6 different sugars in specific linkages giving rise to heteropolysaccharides (Hancock *et al.*, 1994). The constituent components of these units include neutral pyranosidic and furanosidic sugars (hexoses, pentoses, and deoxy- and O-methyl derivatives) and charged sugars (aminohexoses and

aminopentoses, hexuronic acids, and hexosaminuronic acids), which may carry a variety of substitutents such as amino acyl, phosphoryl, glyceryl, lactyl, and acetyl groups (Lüderitz *et al.*, 1982; Rietschel *et al.*, 1991).

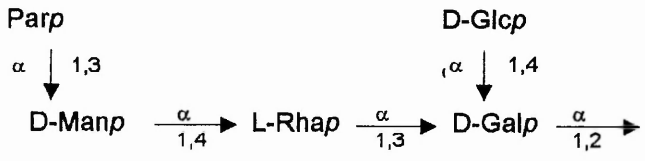
A review article by Knirel and Kochetkov (1994) details the structure of the O-chain repeat unit. Examples of the chemical structures of selected *Salmonella* serotypes are listed in Figure 1.4. Investigations of the chemical composition of the O-chain repeat units of salmonella have revealed that serologically distinct serogroups of the Kauffmann-White Scheme (Section 1.3.2) essentially can be grouped together on the basis of structurally related polysaccharides. For example the O-chain repeat unit of serogroups A, B, and D_1 are characterised by a branched structure with the same main chain. This is built up of a trisaccharide repeating unit containing one residue each of D-mannose, D-galactose and L-rhamnose. The differentiation into the three serogroups is defined by the configuration of a monosaccharide side chain - a 3,6-dideoxyhexose. In serogroup A it is D-ribo (paratose), in serogroup B, D-xylo (abequose) and in serogroup D_1 it is D-arabino (tyvelose).

While subgroups C_2 and C_3 have identical main chains built up of tetrasaccharide repeating units and two lateral monosaccharides substituents (abequose and glucose). They differ in the site of the attachment of a side chain glucose residue. Subgroups C_1 and C_4 have main chains with pentasaccharide repeating units and similarly differ in the position of the glucose side chain. Modifications may be stoichiometric as with the substitution of the glucose in *S. livingstone* or nonstoichiometric as with *S. thompson*. Both serotypes belong to subgroup C_1.

The subspecies *arizonae* are in some respects different since the O-chain of many of its representatives are acidic due to the presence of uronic acids (D-glucuronic acid in serogroup O45 and N-acetyl-D-galactosaminuronic acid in serogroup O62) or aldulosonic acids (N-acetylneuraminic acid in serogroup O21 or an N-acetyl derivative of 5,7-diamino-3,5,7,9-tetradeoxy-D-glycero-L-galacto-nonulosonic acid in serogroup O61; Knirel and Kochetkov 1994).

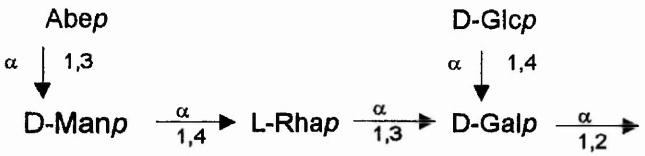
Serogroup A

S.paratyphi



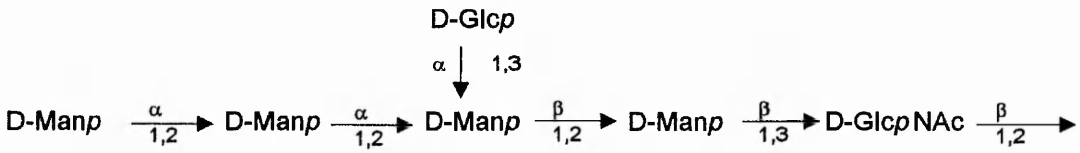
Serogroup B

S.typhimurium



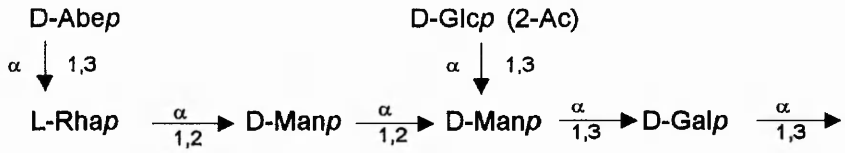
Serogroup C_1

S.livingstone



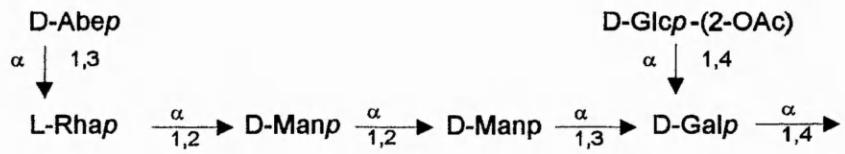
Serogroup C_2

S.newport



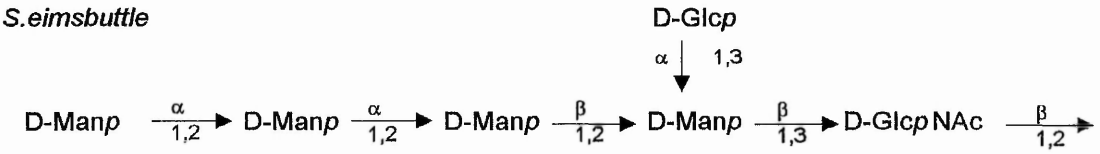
Serogroup C_3

S.kentucky



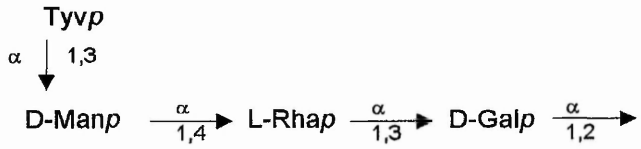
Serogroup C_4

S.eimsbuttle



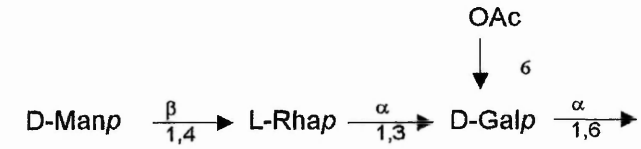
Serogroup D_1

S.enteritidis



Serogroup E_1

S.anatum



Serogroup O

S.adelaide

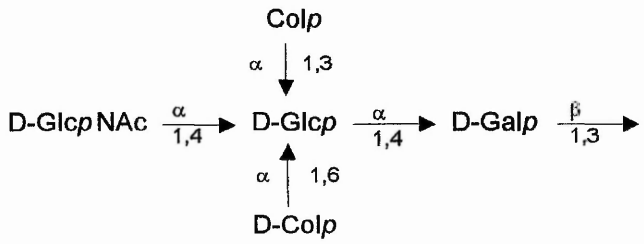


Figure 1.4: Known structures of the O-chain repeat units of some *Salmonella* serotypes (from Knirel and Kochchetkov 1994)

1.4.2 *OVERVIEW OF THE BIOSYNTHESIS AND GENETICS OF LPS*

Our understanding of the biosynthetic pathways and genetics of LPS is based largely on the analysis of a limited number of specific LPS mutants of *S.minnesota* and *S.typhimurium* (Hitchcock *et al.*, 1986). However it should be kept in mind that there are over 2,400 *Salmonella* serotypes.

A great deal of research effort has been focused on the biosynthesis and genetics of LPS. It is not the aim of this report to relay details of the individual processes. Instead the following section will simply give an overview of the biosynthesis and genetics of LPS. For more comprehensive reviews see Rick (1987), Raetz (1992), Schnaitman and Klena (1993), Reeves (1994) and Whitfield (1995).

1.4.2.1 *Biosynthesis of LPS*

Many individual enzymatic steps involved in the LPS synthesis have been characterised. However detailed information regarding the topology of specific events is lacking. The steps involved in the biosynthesis of the outer core and O-chain regions of LPS are now reasonably established (Whitfield 1995). The main feature of the biosynthesis of LPS is the independent synthesis of lipid A-core and the O-chain unit. They are synthesised on the inner face of the cytoplasmic membrane as two separate components. Lipid A is synthesised by the addition of the fatty acids and Kdo to the glucosamine disaccharide. With its hydrophobic chains embedded in the membrane, lipid A serves as the primer and the carrier for building up the core polysaccharide. The outer core region is synthesised by sequential addition of the individual sugar residues. Any defect in the synthesis of the core results in R mutants termed *rfa* (see below).

The repeat units of the O-chains are built up on another carrier, undecaprenol-P (UPP) to give a UPP-O-chain unit (UPP-O). It is believed that lipid A-core and UPP-O unit are then separately translocated to the outer (periplasmic) face of the cytoplasmic membrane where the UPP-O unit is polymerised. Each step in polymerisation involves transfer of the growing polysaccharide chain from UPP-O_n to the non-reducing end of the UPP-O to give UPP-O_{n+1}. When the chain reaches the designated length the separate components are ligated by the enzyme ligase to give the complete molecule. Details of the ligation reaction have not been fully resolved (Whitfield 1995). The molecule may be further modified by the addition of glucosyl branches to the O-side chain. Although devoid of O-chain rough mutants generally produce and polymerise, but cannot attach, the O-chain

sugars. The molecular mechanisms of LPS translocation and integration into the outer membrane remain to be established (Rick 1987).

1.4.2.2 *Genetics of LPS and rough mutant bacteria*

The enzymes responsible for the biosynthesis of LPS are determined by a number of genes or gene clusters. They are defined as rough mutants and are therefore termed *rf*. The mutations are mapped at three loci named *rfa*, *rfb* and *rfc*. Simply the *rfa* codes for synthesis of core oligosaccharide; *rfb* codes for O-chain unit synthesis and *rfc* codes for the polymerase joining of O-chain repeat units. Anomolies in this nomenclature have been recognised: mutants affecting lipid A or Kdo were not detected in initial investigations probably because they were lethal. Those found later were named after the pathway affected and not the *rfa* mutants. The existence of a separate *rfc* locus is now seen to be atypical as in most cases the O-polysaccharide gene is in the *rfb* cluster although they are still termed *rfc* (Schnaitmann and Klena 1993).

Naturally occuring wild type bacteria grow in the form of smooth (S) colonies on agar. The LPS from these S form strains is made up of S- and R-type LPS (Section 1.4.4.2.1). Sometimes these bacteria may undergo a spontaneous mutation into rough (R) mutants which are characterised by the formation of rough (R) colonies on agar. This morphological mutation is combined with the disappearance of the O-chain. R mutants as they are known consist only of the core region bound to lipid A. The LPS from these R forms is termed R-type LPS. Rough mutants do not occur frequently in nature and are usually obtained with the aid of experimental mutagenesis, in which the biosynthesis of the core oligosaccharide is blocked at different stages, and selection procedures. These highly deficient R mutants have been used for the elucidation of the structures of the complete core oligosaccharides and for the understanding of the complex pathways of the bacterial polysaccharide biosynthesis (Kato 1993). Two main groups of R mutants can be distinguished. The first Ra mutants, often with a mutation in the *rfb* locus, are defective in O-chain biosynthesis and therefore contain the complete core linked to lipid A. The second group, often with a mutation in the *rfa* locus, are defective in core biosynthesis and contain only a portion of the core linked to lipid A. These so called deep mutants are termed Rb, Rc, Rd and Re. The Re-type LPS is considered to be the minimum requirement to maintain the integrity of the Gram-negative outer membrane. The R mutants form serologically distinct groups, with each terminal sugar residue representing the immunodominant sugar unit. The structures of *Salmonella* R mutant strains Ra-Re are

shown in Figure 1.3.

One additional mutant should be mentioned, although it is not a core mutant, since the resulting LPS phenotype is not rough. A defect in the *rfc* cluster results in the absence of O-chain polymerase activity. The resulting semi-rough (SR) LPS has lipid A, complete core and one covalently bound O-chain unit.

1.4.3 *ORGANISATION AND PROPERTIES OF LPS*

1.4.3.1 *Bacterial cell envelope*

The distribution of LPS in cells has been probed using immunoelectron microscopy, freeze fracture studies and enzyme accessibility studies (Hancock *et al.*, 1994). Lam *et al.* (1987) demonstrated, by the use of O-chain specific antibodies and a protein A-dextran-gold conjugate, that the LPS of *Pseudomonas* extends outward up to 10-12nm beyond the outer membrane. While immunoelectron microscopic examination of the reaction of anti-O serum with the somatic O-antigen of *Klebsiella pneumoniae* revealed that the O-chains were fine fibres which formed brushlike structures on the cell surface (Meno and Amako 1990).

LPS are essential for maintaining the integrity and structural stability of the cell wall. Bacteria that, due to a mutational defect, are unable to synthesise entire LPS molecules are no longer able to reproduce and cease growing (Lüderitz *et al.*, 1982). Moreover LPS assist in the functioning of the membrane. Studies have suggested that mixtures of long and short chain LPS produce cross-overs which form a mechanically stable, felt-like network (Peterson *et al.*, 1986; Kastowsky *et al.*, 1992). This creates a barrier against the permeation of harmful substances. Wild type strains of *E. coli* and *S. typhimurium* show a strong resistance to hydrophobic antibiotics (e.g. macrolides, novobiocin, rifamycin, actinomycin), detergents (SDS, bile salts, Triton X-100) and dyes (eosin, methylene blue, brilliant green). In contrast rough mutant strains (devoid of O-chain) are exceptionally sensitive to a wide range of hydrophobic compounds (Nikaido and Vaara 1987; Seltmann 1990; Rietschel and Brade 1992).

1.4.3.2 *Isolated LPS*

Biological investigations often require isolated pure LPS free of proteins, murein, and nucleic acids in order to produce reproducible results (Lüderitz *et al.*, 1982). This can be achieved by different extraction methods (Westphal and Jann 1965; Galanos *et al.*, 1969;

Kabir *et al.*, 1978; Hitchcock and Brown 1983). However it should be kept in mind that in nature LPS does not occur in pure form and that the lysis of bacteria leads to either water-soluble or insoluble complexes containing LPS, proteins, phospholipids and other components of the bacterial cell.

Isolated purified LPS has a poor solubility in aqueous solutions, it spontaneously associates to form aggregates which are visualised as colloidal solutions. This phenomenon can be explained by the amphiphilic nature of LPS. Their hydrophobic regions (lipid A) are organised such that they are isolated from water by a layer of polar (polysaccharide) heads. Studies have shown that LPS isolated from wild type bacteria consist principally of flat ribbon-like and spherical structures covered with hairy (polysaccaride) projections (Kato 1993). The ribbon-like structures may be readily reduced to discs or doughnuts by a variety of procedures (Shands 1971). For example self aggregation may be prevented by addition of surfactants such as Tween 80 which breaks the long ribbon-like structures into short segments (Shands 1971). While sodium desoxycholate has been reported to disperse LPS into dimer form (Shnyra *et al.*, 1993).

1.4.3.3 *The 3D structure of an LPS molecule*

In a study by Kastowsky *et al.* (1992) molecular modelling techniques were applied to calculate the possible 3D architecture and conformational flexibility of complete bacterial S-type LPS. The core region was shown to exhibit a well defined cylindrical shape. Its long axis was assumed to run parallel to the membrane normal (Figure 1.5). This was however in contrast to the O-chain, which did not represent a regular helix over longer chain distances. It was suggested that the O-chain would bend at specific sites. From this study it can be concluded that the conformational flexibilty of S-type LPS depends on the O-chain linkage to the core, the length of the O-chain and the sugar types and linkages that make up the O-chain.

1.4.3.4 *The release of LPS from the outer membrane*

The release of outer membrane (OM) complexes (vesicles) containing LPS, phospholipids and proteins have been described by several authors. These so-called blebs are released under variable conditions. Reports have described that fragments are released during normal cell growth (Devoe and Gilchrist 1973; Hoekestra *et al.*, 1976) and that the majority of which represented newly synthesised OM (Mug-Opstelten and Witholt 1978).

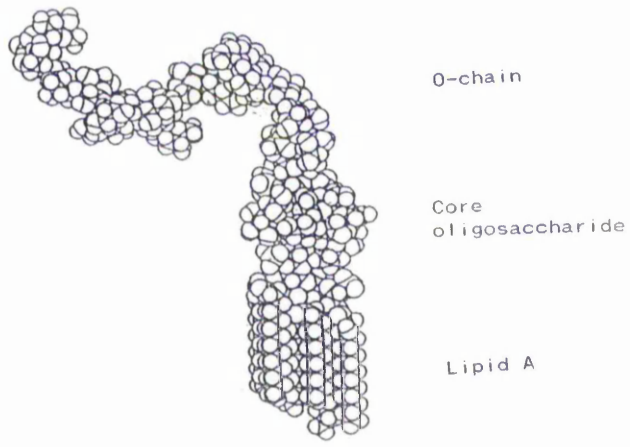


Figure 1.5: A three dimensional model of bacterial S-type LPS consisting of a hexaacyl lipid A identical to *E.coli* lipid A, a complete *S.typhimurium* core oligosaccharide portion and four repeating units of *Salmonella* serogroup B O-chain (from Kastowsky *et al.*, 1992)

Increased blebbing has been demonstrated by heat and chemical treatment. Tsuchido *et al.* (1985) reported that heat treatment (55°C) of *E.coli* in 50mM tris hydrochloride buffer with or without 10mM magnesium sulphate or HEPES buffer at pH 8.0 led to the release of outer membrane fragments and the disruption of the barrier function. Furthermore treatment with tris enhanced the effect of heat damage. Leive and Shovlin (1968) reported that up to 50% of the LPS from *E.coli*, *S.annatum* and *S.minnesota* was released by treatment with 10mM EDTA in tris buffer. Blais and Yamazaki (1989a) examined the effect of combined heat and EDTA treatment on the reactivity of anti-salmonella LPS antibodies. They found that boiling cells in 50mM EDTA for 10min caused a fourfold increase in antibody reactivity as compared to the untreated cells.

1.4.4 *HETEROGENEITY OF LPS*

1.4.4.1 *Chemical characterisation and structural heterogeneity*

Chemical characterisation and structural analysis techniques have been used to elucidate the structure of the O-chain repeat units of some *Salmonella* serotypes (Lowry *et al.*, 1954; Reissig *et al.*, 1955; Hakamori 1964; Karkhaus *et al.*, 1978; Wollenweber and Rietschel 1990; Haishima *et al.*, 1992; Holst *et al.*, 1994). Examples of which have already been mentioned in Section 1.4.1.3.

The composition and arrangement of the sugar residues present in the O-chain results in an almost infinite degree of variation. Some examples are shown in Figure 1.4 (Section 1.4.1.3). Thus each bacterial serotype synthesises a unique LPS characterised by a specific composition and structure of the O-chain.

1.4.4.2 *Characterisation visualised by SDS-PAGE and immunoblot assay*

The heterogeneity of LPS can be visualised by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) as reproducible ladder-like structures. Resolution of bands can be achieved by radio-isotope labelling (Goldman and Leive 1980; Palva and Mäkelä 1980), ethidium bromide (Kido *et al.*, 1990) or by silver staining (Tsai and Frasch 1982; Kittleberger and Hilbink 1993). The characterisation of LPS can be improved further by transfering LPS profiles from SDS-PAGE onto nitrocellulose membrane, for reaction with specific antibodies. The electrophoretic transfer technique and immunoblotting procedure first described for protein analysis (Towbin *et al.*, 1979; Burnette 1981) has been successfully applied to the analysis of LPS (Bradbury *et al.*, 1984; Vullo *et al.*, 1987; Nelson *et al.*, 1992; Chart 1995). Once transferred to nitrocellulose, LPS can be probed with specific antibodies and detected by autoradiography (Bradbury *et al.*, 1984) and enzyme linked immunosorbent assay (Jongh-Leuvenink *et al.*, 1985; Vullo *et al.*, 1987).

1.4.4.2.1 *Size heterogeneity*

LPS exhibit considerable variation in O-chain length i.e. they express size heterogeneity which has been observed using SDS-PAGE as characteristic ladder-like patterns. The fastest moving band is believed to be lipid A-core devoid of O-chain (R-type LPS), the band above this in the profile is the lipid A-core plus one repeat unit of the O-chain (SR type LPS), the band above this, lipid A-core plus two O-chain repeat units (S-type LPS) and so forth (Figure 1.6; Goldman and Leive 1980; Palva and Mäkelä 1980). Typically the chain consists of 20-40 repeating units (Rietschel and Brade 1992). The highest molecular weight bands in the LPS profiles of some Gram-negative bacteria may represent contaminating capsular polysaccharide (Cousland and Poxton 1983).

1.4.4.2.2 *Intra- and inter-strain heterogeneity*

Salmonella intra-strain heterogeneity is a very unusual phenomenon since the expression of LPS is usually consistent within a given strain (Chart 1995). However strains of *S. virchow* have been shown to express LPS which produces two distinct LPS profiles

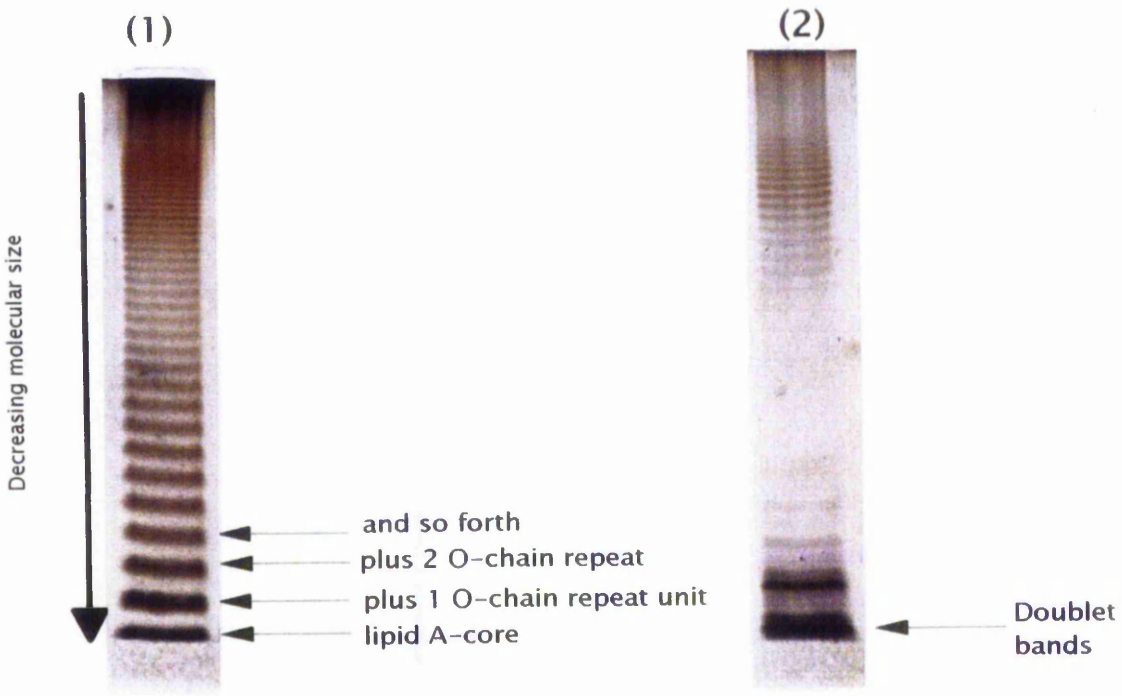


Figure 1.6: Silver stained SDS-PAGE gel of *S.typhimurium* (Lane 1) and *S.infantis* (Lane 2) LPS

characterised by a single or a double rung profile (Chart and Rowe 1995).

Considerable heterogeneity has been reported in the LPS profiles of different strains of *C.freundii* (Chart *et al.*, 1993). Thirty-six strains of *C.freundii* were isolated from samples of beefburgers, patients with diarrhoea, blood or milk. Sixteen of the strains gave LPS profiles that could be divided into five distinct patterns (based on similarities in LPS profile) termed A(3), B(7), C(2), D(2) and E(2) (Figure 1.7; Lanes 1-5). Eighteen of the remaining strains each expressed LPS that gave unique patterns with LPS chain lengths ranging from predominantly long-chain (Lane 6) to LPS with generally shorter chains (Lane 7). Two strains did not express long-chain LPS.

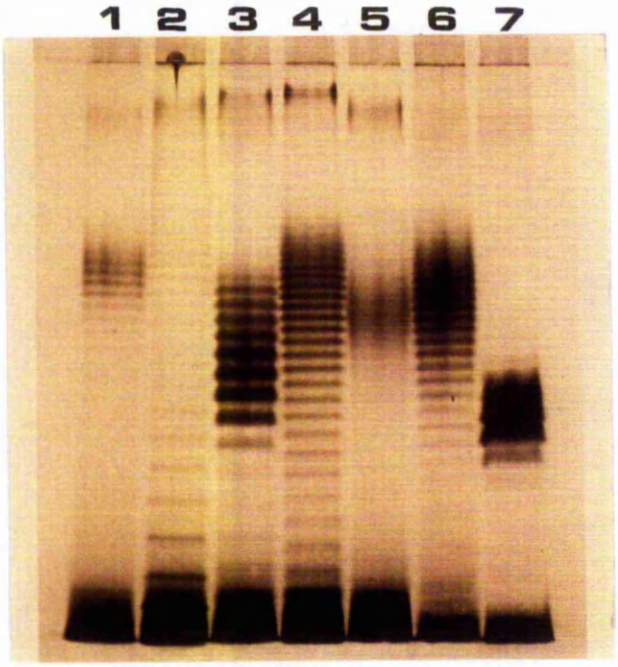


Figure 1.7: Silver stained LPS profiles of C.freundii (Original kindly supplied by Dr H.Chart as quoted in Chart *et al.*, 1993 and is printed with the permission of the *Journal of Applied Bacteriology*)

1.4.4.2.3 *Heterogeneity in the amount of S- and R-type LPS*

Schlecht *et al.* (1992) noted that amount of R-type LPS isolated from wild type *Salmonella* strains representing serogroups B, K, N, O, U, V, X and Y varied between 5 and 50% of the total LPS amount. Moreover LPS production with regard to the amount of S- and R-type LPS is influenced by incubation temperature (Schlecht 1986; Schlecht and Mayer 1994) and culture conditions (Nelson *et al.*, 1991; Schlecht and Galanos 1994). Nelson *et al.* (1991) investigated the influence of culture conditions on the expression of *E.coli* LPS. Researchers reported that the expression of R-type LPS was increased under magnesium-depleted conditions. Moreover increased iron-depletion led to a greater expression of R-type LPS at the expense of high molecular mass S-type LPS. Whereas nitrogen-deficient/ high-carbon conditions led to an increase in chain length and production of the O-chain.

1.4.4.2.4 *Heterogeneity in the core-lipid A region*

SDS-PAGE analysis of LPS often reveals doublet bands (Figure 1.6). Some researchers believe that this is related to phosphate heterogeneity in the lipid A-core region and that the subsequent change in the charge alters mobility slightly and hence doublet bands are formed (Goldman and Leive 1980; Palva and Mäkelä 1980; Hitchcock and Brown 1983).

However other researchers suggest that intermolecular interactions occur between LPS molecules with approximately the same O-chain length (Peterson and McGroarty 1985; Solovjeva and Ovodov 1992). This results in the formation of aggregates or dimers which are stable even during polyacrylamide gel electrophoresis in the presence of a detergent and are detected as distinct, anomolous slow migrating bands (the doublet). Solovjeva and Ovodov (1992) suggest that the presence of deoxy and dideoxy sugars in the O-chain polysaccharides can facilitate this interaction.

Schlecht *et al.* (1992) reported that the R-type LPS present in wild type *Salmonella* strains is not exclusively made up of Ra type core. Instead these researchers demonstrated that R-type LPS of various *Salmonella* serotypes (including the more frequently isolated serogroup B *S.typhimurium*) constitutes different R-classes of Ra, Rb_1, Rb_2 and Rc. These are visualised on SDS-PAGE by one long diffuse band.

As previously mentioned the Ra core structure is generally believed to be common to all *Salmonella* spp. (Kato 1993). However, recent reports have questioned the universal validity of these claims. It has been proposed that in addition to the typical Ra core structure, that atypical core sructures exist (Tsang and Schlecht 1990; Tsang *et al.*, 1991a; Tsang *et al.*, 1991b; Tsang *et al.*, 1992; Hoofdvak and Olsthoorn 1995; Mansfield *et al.*, 1996).

1.5 SALMONELLA FOOD TESTING

1.5.1 *PROBLEMS OF DETECTING SALMONELLAS IN FOOD*

1.5.1.1 *Physical nature and antimicrobial effect of food*

The physical nature of food may make it difficult to recover target cells. For example fatty foods can interfere with the recovery of salmonella by trapping cells in lipid micelles.

Some foods contain naturally occurring substances that have antimicrobial properties. For example certain spices posess essential oils which have antimicrobial activities (Hall 1969; Wilson and Andrews 1976; Silliker *et al.*, 1980). Among these are eugenol in cloves and allspice, altyl isothiocyanate in mustard seed and thymol in oregano. While egg albumen contains lysozyme, enzyme inhibitors such as ovomucoid, avidin, conalbumin which together with a high pH limit the growth of *Salmonella* spp. (Silliker *et al.*, 1964; Stephenson *et al.*, 1991). Moreover the presence of a wide variety of volatile and non-volatile compounds such as formic, acetic, propionic and butyric acid inhibit the growth

of *Salmonella* spp. in milk (Levine and Fellers 1940; Goepfert and Hicks 1969; Sorrell and Speck 1970). Kulshresthal and Martin (1974) demonstrated that concentrations of 10ppm of some fatty acids significantly inhibited the growth of *S.typhimurium*. Other foods that inhibit bacterial growth include onion and garlic juice (Pivnick 1980) and cocoa (Price *et al.*, 1972).

1.5.1.2 *Sublethally injured salmonellas*

Cells can be sublethally injured by food processing methods such as heat, cold, freeze-thawing, freeze-drying, moisture reduction, irradiation and exposure to sanitisers or preservatives. In addition exposure to and storage in the food environments themselves (low pH or low water activity) can induce injury.

All forms of injury involve membrane damage with the loss of internal solutes (Hurst 1977). However the type (structural or metabolic) and extent of injury is related to the environmental stress imposed on the cell. Heat injury, for example causes disruption of the cell membrane which alters the glucose transport system of *S.typhimurium* (Pierson and Ordal 1971). Different levels of injury may exist. Van Schothorst and van Leusden (1975) classified three degrees of injury based on different resuscitation times when they studied the isolation of injured salmonellas from milk powder during prolonged storage. The more severly damaged cells could only be recovered after an 18h pre-enrichment. Whereas the more lightly injured cells could be recovered after a 2h resuscitation. Those cells designated into the final class of injury required no resuscitation at all. Cell injury often manifests itself as an increased sensitivity to selective media (see below).

The term 'sublethal injury' describes cells which are in a debilitated state and are capable of restoring a normal physiological state of growth when placed in an unrestricted environment. Thus sublethally injured cells remain viable and this has important implications for the food industry. If microorganisms remain undetected, a process may be thought of as producing a commercially acceptable product and yet retain food poisoning organisms (Hurst 1977).

1.5.1.3 *Low numbers of salmonellas and the effect of competing microorganisms*

Recovering organisms from foods is a challenging task. The level at which *Salmonella* spp. naturally contaminate foods is generally low.

When the number of competing bacteria present in a food sample is high, the chance of isolating *Salmonella* spp. is significantly reduced (Beckers *et al.*, 1986b). The number and type of competing flora is dependent on the food tested. This can have a direct effect on salmonella growth. For example van Schothorst and Renauld (1985) reported high levels of Gram-positive organisms in samples of milk powder. Growth of these organisms caused a dramatic drop in the pH of the BPW pre-enrichment broth from 7 to 4.5 which retarded the growth of salmonellas. However, Beckers *et al.* (1987a) found no such fall in the pH of minced meat samples which contained *Enterbacteriaceae*.

1.5.2 *TRADITIONAL CULTURE METHODS OF SALMONELLA ISOLATION AND DETECTION*

The presence of salmonellas in food that is 'ready-to-eat' is considered significant regardless of the level of contamination. Hence the UK 1990 Food Safety Act requires an **absence** of *Salmonella* spp. from 25g of ready-to-eat foods (Roberts *et al.*, 1995). Methods of salmonella isolation and detection should therefore be able to detect one cell in 25g foodstuff.

Traditional cultural methods of salmonella isolation and detection are based on four stages: pre-enrichment, selective enrichment in broth, selective enrichment on agar (which produce presumptive positive samples) and confirmatory tests. This procedure takes 72-96h for a presumptive salmonella result (Figure 1.8). The rationale behind these enrichment steps are explained below.

A multitude of pre- and selective enrichment media have been developed and are discussed in detail by D'Aoust (1981). However only a limited range of media are currently recommended for use by national and international organisations; the British Standards Institution (BSI), the International Standard Organisation (ISO), and the Food and Drug Administration (FDA). In the following report particular emphasis has been placed on the BSI method of salmonella isolation and detection (BSI 1993).

1.5.2.1 *Pre-enrichment*

Salmonella isolation and detection procedures originally designed to isolate salmonellas from faeces were used as a basis for the analysis of food samples. These were principally

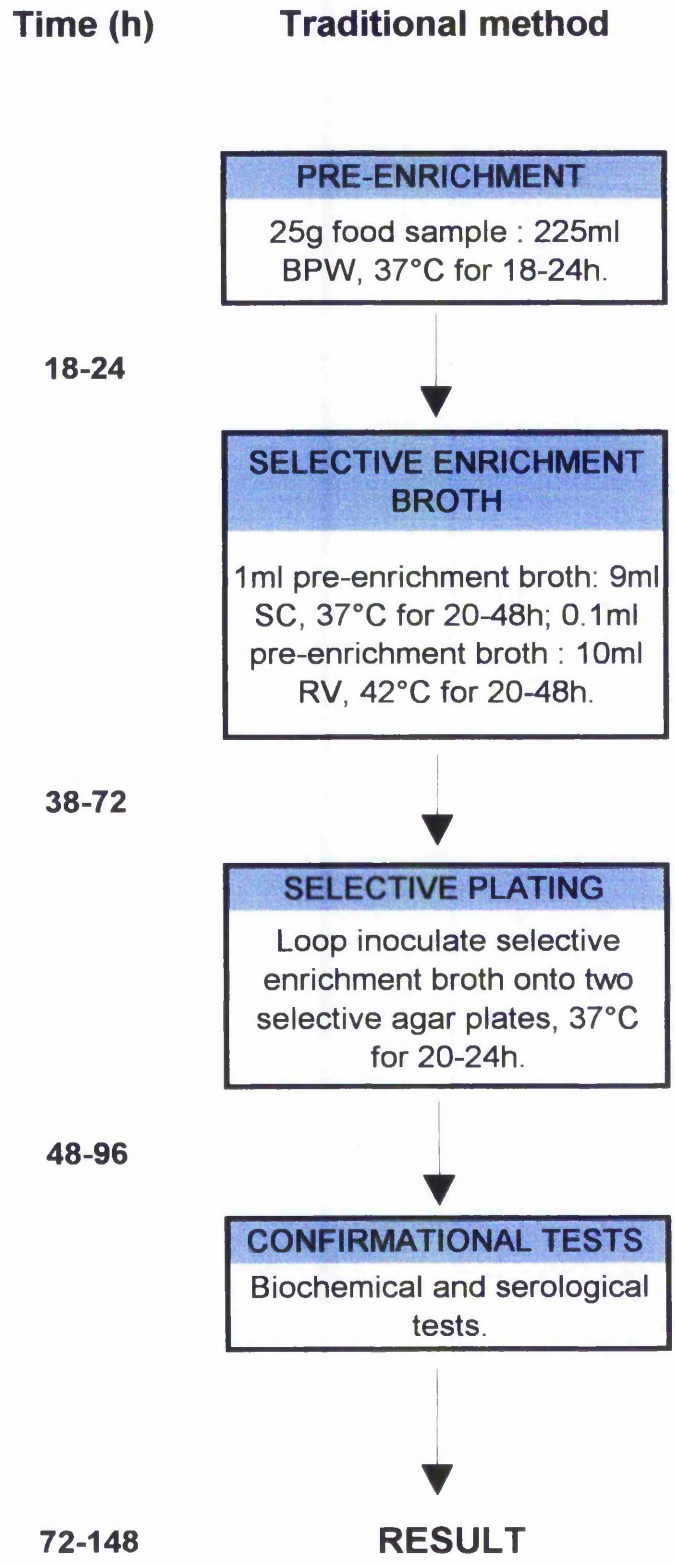


Figure 1.8: British Standards Institute (BSI) approved traditional culture method

methods of selective enrichment. However if this approach is adopted for naturally contaminated food samples, it often results in failure to recover the organism of interest. The concentration of selective agents present is often toxic to some healthy bacterial cells (Vassiliadis *et al.*, 1974) but because clinical specimens habour large numbers of physiologically active organisms, the sacrifice of a small number of cells poses no problem. However in food samples salmonellas may be present in low numbers and may be sublethally damaged, so the death of an entire population may result. Thus the first stage of traditional culture methods for the isolation and detection of salmonella from foods is pre-enrichment.

Pre-enrichment provides a non-toxic environment for the resuscitation of stressed or injured cells. Once these cells have been restored to a normal physiological condition the pre-enrichment medium may serve as a source of nutrients for the proliferation of the rejuvenated cells. The use of pre-enrichment media has been shown to improve the frequency of isolation of injured salmonellas (North 1961; Edel and Kampelmacher 1973; Litchfield 1973; van Schothorst *et al.*, 1977). The heterogenous distribution of organisms within a given sample is a well recognised phenomenon. Various methods are used to obtain a more homogeneous distribution of microorganisms within samples, including grinding, 'stomaching', rinsing and blending. The choice of method for sample preparation is very much dependent on the material being examined, but in general the gentlest method consistent with effective dispersal of bacteria should be used (Fricker 1987). For example swirling is generally adequate for powdered or granulated foods. However foods such as chocolate, vegetables, meats and shellfish are usually blended or stomached (Andrews 1985).

A wide range of pre-enrichment media have been reported (D'Aoust 1981). While studies into their effectiveness have produced conflicting results lactose broth and buffered peptone water (BPW) are probably the most widely used pre-enrichment media (Fricker 1987); BPW is recommended for use by the BSI.

Due to the nature of the food matrix, certain foods require an adjustment of the ratio of sample to broth or the addition of various substances. For example onion and garlic samples require the addition of potassium sulphite to a final concentration of 0.5%. This reduces the natural bactericidal properties which would inhibit the growth of any salmonellas. Other examples of the additions, adjustments and their purpose are listed in

Table 1.6.

Table 1.6: Additions and adjustments to salmonella pre-enrichment broths (from Roberts *et al.*, 1995)

Product	Addition and adjustment	Purpose
High fat foods e.g cheese	Surfactant (e.g. Tergitol 7 1.0% with lactose broth, 0.22% with BPW)	Aids dispersion of food
Onion and garlic	Potassium sulphite (0.5% final concentration)	Reduces natural bactericidal properties
Cocoa powder	Casein (5% final concentration)	Reduces natural bactericidal activity
Cocoa powder and chocolate confectionery	10% (w/v) non-fat dry milk	Reduces bacterial activity
High salt/sugar	Reduce sample:broth ratio (amount will depend on initial salt/sugar concentration)	Maintains salt or sugar foods concentration at <2%
Oregano, cinnamon, cloves, all spice	Reduce sample:broth ratio to 1:100 or 1:1000	Reduces inhibitory properties
High acid/alkaline products	Adjust pH to 6.6 - 7.0 prior to incubation	Neutralises effect of acid/alkali

Although elevated incubation temperatures have been studied with mixed success (D'Aoust *et al.*, 1992). It is generally accepted and the BSI recommends that pre-enrichment cultures are incubated at 37°C.

The BSI method recommends that pre-enrichment broth is incubated for 18-24h. Shortened pre-enrichment periods have been evaluated with mixed success. Van Schothorst and van Leusden (1975) reported good recovery rates of heat-injured salmonellas isolated from milk powder samples pre-enriched for 2-6h in BPW. In contrast D'Aoust and Maishment (1979) reported that a 6h pre-enrichment of food and feed samples resulted in a high number of false negative results. This can be explained by the duration of the lag phase of growth of sublethally damaged cells which vary according to the severity of damage (Fricker 1987). So that severely damaged cells have extended lag times. Thus in general shortened (4-8h) periods of pre-enrichment fail to provide sufficient time for the repair of stressed or injured salmonellas encountered in raw or processed foods (D'Aoust *et al.*, 1992). Hence a standard incubation time has been set

in order to resuscitate potentially severely damaged cells.

1.5.2.2 *Selective enrichment*

Following pre-enrichment replicate portions of broth culture are generally inoculated into two or more selective enrichment broths to ensure greater recovery of *Salmonella* spp. from different food types. A number of selective enrichment broths have been developed and evaluated (Carlson and Snoeyenbos 1974; Harvey and Price 1981; Northolt *et al.*, 1985; Patil and Parhad 1986; Beckers *et al.*, 1987b; Vassiliadis *et al.*, 1987; D'Aoust *et al.*, 1992; Arroyo and Arroyo 1995; June *et al.*, 1995). Those specified by the BSI are Rappaport-Vassiliadis (RV) and Selenite Cystine (SC). These and other selective enrichment broths contain selective ingredients that are toxic to non-salmonellas and at the same time allow the proliferation of salmonellas. The performance of RV broth is based on its high osmolarity ($MgCl_2$), low pH and the presence of malachite green, to which salmonellas can adapt better than most competing bacteria (Maijala *et al.*, 1992). The toxic ingredient in SC broth is sodium selenite. While the addition of cystine enhances the growth of salmonellas (Fricker 1987).

The concentration of selective agents is often toxic to some healthy salmonellas (Vassiliadis *et al.*, 1974). Chen *et al.* (1993) determined that (depending on the serotype) a minimum number of 10^2-10^5 cfu/ml of salmonellas was required in the pre-enrichment medium to generate variants resistant to RV which can subsequently grow in this selective media.

Generally the ratio of pre-enrichment to selective enrichment broth is 1:10 (usually 1ml:9ml; Fricker 1987). Transfer volumes greater than 1ml do not increase method sensitivity (Price *et al.*, 1972). Vassiliadis (1983) reported that the isolation of *Salmonella* spp. using RV broth is more effective when the inoculum is diluted 1:100 . Hence the BSI approved method recommends that 1ml and 0.1ml of pre-enrichment culture should be inoculated into 9ml of SC and 10ml RV broths respectively.

Incubation of selective enrichment media at an elevated temperature of 43°C has been reported to enhance recovery of *Salmonella* spp. from egg products, pork sausage, meat products and ground meat filtrate (Andrews 1985). This enhanced recovery of salmonella is thought to be due to increased sensitivity of competitive organisms to elevated temperatures as well as a greater sensitivity to toxic selective agents, which means that they die off more rapidly, thus facilitating the recovery of *Salmonella* spp. on selective

plate media. In contrast studies which recovered *Salmonella* spp. from dairy products, soya and meat, fish or bone meal samples demonstrated no significant difference in their recovery from enrichments incubated at 35°C or 43°C (Andrews 1985). The BSI recommends that SC and RV broths are incubated at 37°C and 42°C respectively.

The BSI approved method recommends that selective enrichment media is incubated at the appropriate temperature for 20-24h. Shorter incubation periods have been investigated with mixed success (Mulindwa and Pietzsch 1979; D'Aoust *et al.*, 1983; 1990; Allen *et al.*, 1991a; 1991b; D'Aoust *et al.*, 1992). While prolonged incubation periods of up to 48h are sometimes required. This is a result of the number and type of competing flora. Van Schothorst and Renauld (1983) concluded that salmonellas did not start to multiply until the number of competitors were significantly reduced.

1.5.2.3 *Selective plating*

Selective plating media are formulated to encourage the growth of salmonellas (resulting in the formation of discrete colonies) and at the same time inhibit the growth of non-salmonellas. They consist of selective and differential agents incorporated within a nutritive agar base. Selective agents include bile salts, dyes (chiefly brilliant green), sulphonamides, heavy metals and antibiotics (Moats 1981; Fricker 1987). A wide number of selective agar media are available. The BSI method recommends the use of two or more selective media from those listed in Table 1.7.

Selective agar plates are examined for typical colonies and screened biochemically using triple sugar iron agar or lysine iron agar slopes in conjunction with urease and sucrose/lactose media and incubated overnight at 37°C. Presumptive positive salmonella are further characterised by biochemical and serological tests.

1.5.2.4 *The identification of Salmonella spp. by biochemical and serological tests*

Numerous biochemical tests are available for the characterisation of cultural isolates obtained from food products. A comprehensive list of biochemical and nutritional characters of the family Enterobacteriaceae is found in *Bergey's Manual of Systematic Bacteriology* (Kreig and Hold 1984). The characters that distinguish the genus *Salmonella* have already been mentioned in Section 1.3.1.

Table 1.7: BSI recommended selective plating media

Selective enrichment media	Description of salmonella colony	Comments
BGA	Red colonies surrounded by bright red medium	
XLD	Pink colonies with or without black centres. Strong H_2S-producing strains may yeld colonies with large glossy black centres or appear as completely black colonies	Atypical lactose-positive and/or sucrose positive strains produce yellow colonies with or without black centres; *Proteus mirabilis* and slow lactose fermenting *Citrobacter* strains also produce black colonies
DCA	Slightly opaque, domed-shaped with a central black dot	
SS	Transparent colonies usually with black centres	
BS	Grey, brown or black colonies; with or without metalic sheen; black halo effect surrounding colony	The prepared medium is unstable and its selectivity changes during its period of storage
MLCB	Large purple-black colonies	Atypical *Salmonella* strains grow as mauve-grey colonies and may develop a central black bulls-eye. Some strains of *Citrobacter* spp. may grow sufficiently well to mimic the appearance of the *Salmonella* spp.. *Proteus* spp. may swarm

BGA, brilliant green agar;
XLD, xylose lysine desoxycholate agar;
DCA, desoxycholate citrate agar (Hynes modification);
SS, salmonella-shigella agar;
BS, bismuth sulphite agar
MLCB, mannitol lysine crystal violet brilliant green agar
H_2S, hydrogen sulphide

1.5.2.5 *Advantages and disadvantages of the traditional culture technique*

The major advantage of traditional methods of salmonella isolation and detection is their high sensitivity. It is accepted that traditonal methods can detect one salmonella cell in 25g of foodstuff. In addition traditional methods have a long history of development and application which has led to their wide acceptance by food testing laboratories. Moreover traditional culture methods require low technology in the form of standard microbiological techniques, involve non-specialised equipment such as incubators, balances and waterbaths. This results in a low capital outlay and a relatively low cost per sample.

The major disadvantage of the traditional culture technique with respect to the food industry is time. Media preparation time is extensive. A great volume of media is required to analyse large numbers of samples. Moreover media preparation is complicated by the requirements of some media. RV broth (Oxoid) according to the manufacturer's instructions should be boiled to dissolve the ingredients, then autoclaved at a lower temperature than is normally required for sterilising purposes. While XLD and BGA cannot be autoclaved.

The total test time is extended because culture techniques rely on the growth of the target organism to detectable levels. Hence it takes 3-4 days to identify a presumptive salmonella positive sample. During this time large volumes of food will have been produced and stored. Short shelf life products may have been distributed, bought and consumed before results are available. Hence some microbiological food testing is retrospective and results are obtained too late to be effective. Products with a longer shelf life can be stored until results are obtained, but storage is costly, distribution of products is delayed and supermarket shelf life is reduced.

Variation in the composition of commercially available RV broth adversely affects media performance. A number of studies have suggested that differences in the concentration of $MgCl_2$ and the type of peptone used (soya peptone and tryptone) affect the growth rate of *Salmonella* spp. (Fricker 1987; Peterz *et al.*, 1989; Maijala *et al.*, 1992).

Traditional media have limitations with respect to their ability to isolate all contaminating *Salmonella* spp. in food samples. Fricker (1987) reported that some salmonellas were in fact inhibited by sodium selenite. However *Proteus* spp. and *Pseudomonas* spp. appeared to be relatively resistant to its effects. Moreover freshly prepared BSA plates have been

reported to inhibitory to *Salmonella* spp. (Flowers *et al.*, 1992). False negative samples can result, since sublethally injured cells (including salmonellas) are more sensitive to the toxicity of selective enrichment broths. While a number of studies has suggested that elevated temperatures increase the recovery of *Salmonella* spp. from selective enrichment broths (Eckner *et al.*, 1992). The control of the elevated temperature appears to be critical. A study by Peterz *et al.* (1989) showed that *S.dublin* was almost completely inhibited at 43°C in RV broth but was not inhibited any more than other serotypes tested when incubated at 42°C. Thus in order to provide greater recovery of salmonella from different food products and overcome the limitations of the selective enrichment media, the use of a number of selective enrichment broths and agars are recommended (Bailey *et al.*, 1991; Manafi and Sommer 1992).

The toxicity of selective media can be compromised. SC broth is sensitive to overheating. Over-heated broth (characterised by a bright red deposit) has a low toxicity and will allow commensal organisms to grow (Bridson 1994). Furthermore prepared XLD and BGA media are unstable and their selectivity changes during storage (Bridson 1994). Hence storage of these media is not generally recommended by manufacturers.

An additional problem with SC broth is that it contains the heavy metal selenium. Animal studies have shown that selenium is embryotoxic, tetratogenic and neurotoxic (June *et al.*, 1995). Thus the use of such hazardous materials should be eliminated, if not greatly reduced.

The aim of selective enrichment is to reduce the high ratio of non-*Salmonella* to *Salmonella* spp. established during the non-selective pre-enrichment of foods. The ability to do this is dictated by the number and type of competing flora, as well as the number of salmonella initially present in the food sample (Beckers *et al.*, 1987a; Arroyo and Arroyo 1995). Thus cultural analysis of some food types, together with the toxicity limitations of selective enrichment results in the presence of mixed cultures on selective agar. This can make it impossible to identify presumptive positive salmonella colonies. Van Schothorst and Renauld (1983) reported that when numbers of non-salmonellas exceeded 10^7 cfu/ml the isolation of salmonellas became virtually impossible as high levels of competitors plated out onto solid selective enrichment media would obscure any salmonella colonies present. While van Leusden *et al.* (1982) suggested that in order to detect salmonella colonies on BGA, the numbers of salmonellas in selective enrichment

broth needed to be at least 10^3 cfu/ml. Hence traditional culture methods are also labour intensive and require trained personnel to isolate and distinguish between *Salmonella* and non-*Salmonella* spp..

1.5.3 *ALTERNATIVE METHODS OF SALMONELLA ISOLATION AND DETECTION*

The disadvantage of the traditional culture methods has prompted a considerable effort to develop more rapid techniques. A number of strategies have been proposed which attempt to reduce the time of detection of presumptive *Salmonella* spp. by reducing media preparation time, reducing sample incubation time and testing multiple samples. Collectively these have been termed 'rapid'. This title is somewhat misleading, since the majority of techniques require a minimum of two days from sample preparation to presumptive result. Thus in the following report they will be termed 'alternative'.

There have been numerous reports on alternative methods of salmonella isolation and detection including different culture techniques, measurements of metabolism, flow cytometry, the use of bacteriophage, immunoassays, nucleic acid based methods and separation and concentration techniques. Indeed there are too many to cover in this report; therefore the following section reviews some of the alternative methods for the isolation and detection of *Salmonella* serotypes in foods. Particular emphasis has been placed on commercially available systems. In some instances researchers have combined methods in an attempt to further reduce the total test time (Davies and Wray 1994; Molla *et al.*, 1996; Poppe *et al.*, 1996; Giammanco *et al.*, 1997). With the exception of IMS, these combined methods will not be reported on here.

1.5.3.1 *Culture techniques*

1.5.3.1.1 *Solid selective media*

New solid selective media have been developed which are designed to give better differentiation, so that salmonella colonies are more easily distinguishable. These include novobiocin, brilliant green, glycerol, lactose agar (Poisson 1992); lysine-mannitol-glycerol agar (Cox 1993) and Rambach agar (Rambach 1990). Rambach (RAM) agar is now commercially available. Salmonella colonies can be differentiated from non-salmonella colonies by their red/scarlet or red/crimson colour. A number of reports have evaluated the agar. However none could achieve 100% characteristic colony formation of salmonella claimed by developers (Greunwald *et al.*, 1991; Manafi and Sommer 1992; Pignato *et al.*, 1995), instead pink, blue or colourless colonies were produced. Garrick and Smith (1994)

professed that RAM agar had potential as a supplementary agar in testing foods for salmonella but remarked on its limitations of non-characteristic colony formation of some salmonellas and typical salmonella appearance of a strain of *C.freundii*.

1.5.3.1.2 ***Semi-solid media (selective motility enrichment technique)***

Selective motility enrichment techniques have utilised the fact that most *Salmonella* serotypes are motile. *Salmonella* strains grow and migrate on the semi-solid media producing an enrichment and differentiation between the samples that produce migrating growth and those that do not. Non-motile salmonellas are not detected. Two examples which are commercially available are (1) modified semi-solid Rappaport-Vassiliadis (MSRV) medium (Goossens *et al.*, 1984) and (2) the Oxoid Salmonella Rapid Test.

The MSRV procedure involves the inoculation of the MSRV plate with 0.2ml drop of pre-enrichment culture to the centre of the plate. If flagellated salmonellas are present they migrate across the surface of the agar creating a migration zone around the inoculation spot. Suspected salmonella colonies are plated onto MacConkey agar for further tests. Thus presumptive results are available after 48h. Several studies have been carried out to compare MSRV agar containing novobiocin with traditional methods of salmonella isolation and detection from artificially and naturally contaminated foods, waters and related materials. Comparable results were obtained (De Smedt and Bolderdijk 1990; De Zutter *et al.*, 1991; O'Donoghue *et al.*, 1992; Barrell *et al.*, 1996). Although O'Donoghue *et al.* (1992) reported that *Enterobacter cloacae* was capable of causing false positive reactions. Additionally a study by Cherrington and Huis in't Veld (1993) reported that as much as 4% of the motile strains isolated from foods by motility enrichment on MSRV were not salmonellas.

The Oxoid Salmonella Rapid Test takes place in self-contained units (culture vessel). Following traditional pre-enrichment a sample is added to the culture vessel containing elective medium. Salmonellas migrate from the elective broth into tubes containing selective and diagnostic media. Cultures in the tubes in which the biochemical tests are positive are tested by serological agglutination with antibody latex. Results are available within 42h. Holbrook *et al.* (1989) tested the performance of this motility enrichment technique and found it to be highly specific and sensitive. It gave an efficiency of 96.8% when tested against traditional methods using >800 food samples.

1.5.3.1.3 *Shortened liquid enrichment*

Attempts have been made to utilise a combined pre-enrichment and selective enrichment. One such example is the commercially available Salmosyst Enrichment technique. This system replaces BPW with Salmosyst broth base. After a 6-8h incubation at 35°C, a 10ml volume of Salmosyst broth base is removed and a selective supplement tablet is added which converts the broth into a selective enrichment broth. The sample is then incubated at 35°C for a further 18-24h, after which it is streaked onto solid selective media. Thus, both media preparation time and pre-enrichment incubation time are reduced.

1.5.3.1.4 *Hydrophobic grid membrane filtration*

A method for the isolation of salmonella which included a hydrophobic grid membrane filter (HGMF) step was described by Entis (1982). Following traditional pre-enrichment and a shortened 6h selective enrichment, a portion of the broth is filtered through a HGMF. The HGMF is then placed onto traditional selective enrichment agar plates. Results were obtained within 48-72h with a sensitivity equal to that of traditional cultural techniques. However the performance of the HGMF method has not been fully assessed, being limited to the analysis of six food types (nonfat dry milk, powdered eggs, cheese powders, pepper, chocolate and raw poultry) (Blackburn and Patel 1991).

1.5.3.2 *Measurements of metabolism by impedance or conductance*

Salmonella detection by impedance or conductance microbiology involve instruments which monitor microbial metabolism. Commercially available systems include the Bactometer 123 (bioMérieux Ltd, Basingstoke, UK), Bactrac (Sy-Lab, Purkerdorf, Austria), Malthus 2000 (Radiometer Ltd., Crawley, UK) and the Rapid Automated Bacterial Impedance Technique (RABIT; Don Whitley Scientific Ltd.,Shipley, UK).

Microbial metabolism can be monitored either directly or indirectly. The direct impedance technique involves the inoculation of pre-enriched samples into individual units containing non-traditional selective enrichment broth. The units are capped and incubated at the required temperature within the impedance-conductance instrument module. Metabolising organisms utilise nutrients in the medium, converting them into smaller, highly charged molecules which results in an increase in the conductivity of the medium. Traditional salmonella selective enrichment media are not suitable for use. They have high salt concentrations that result in high impedance readings, which are outside the normal working range of this direct impedance technique. Therefore alternative selective media

have been developed including selenite cystine/trimethylamine oxide/ribose, lysine/iron/cystine/neutral red and modified ornithine decarboxylase broths. For references on the development of these and other media see review by Silley and Forsythe (1996).

The indirect impedance procedure developed by Owens *et al.* (1989) allows the use of traditional selective enrichment media. Potassium hydroxide (KOH)-agar is placed into the unit, then a separate chamber or tube containing the traditional selective enrichment broth is added and the unit stoppered. Sealed units are then incubated at the required temperature. Any carbon dioxide gas produced by the metabolising organisms is absorbed by the KOH-agar causing a resultant decrease in impedance. The efficacy of RV and SC enrichment broths have been investigated by Donaghy and Madden (1993) and Madden *et al.* (1997).

The impedance techniques require a minimum of 10^6 cfu/ml microorganisms to produce a measured change in conductance or impedance. This so called 'detection time' is inversely proportional to the number of organisms present initially; so that the higher the level of contamination, the faster the result. Presumptive positive *Salmonella* spp. are confirmed by traditional means.

1.5.3.3 *Bacteriophage*

Bacteriophage are routinely used to discriminate between *Salmonella* strains that are serologically or biochemically indistinguishable. This specificity has been utilised by a number of salmonella detection methods to detect *Salmonella* spp. in raw milk samples (Hirsh and Martin 1984), eggs (Chen and Griffiths 1996) and environmental samples (Stewart 1990; Turpin *et al.*, 1993).

The Bacterial Ice Nucleation Diagnostic (BIND) test is based on bacteriophage which have been modified by the introduction of a bacterial gene responsible for an ice-nucleating protein (Wolber and Green 1990). After incubation with the modified bacteriophage, samples are chilled to 5°C. Any salmonella-positive samples will freeze while uncontaminated samples will remain in a liquid state. *Salmonella* spp. could be detected within 2-6h, with a sensitivity of <10 bacteria/ml. Non-salmonellas, including *Citrobacter* and *E.coli* did not give false positive results and food material offered no interference.

1.5.3.4 *Immunoassays*

The term immunoassay covers a wide range of methods that are based upon reactions between antigens and antibodies. The antigen may be hapten conjugates, immunoglobulins, polysaccharides, proteins, viral particles, or any other substance against which antibodies can be raised (Keith 1997). Immunoassays comprise the largest group of alternative methods. Some have been developed into commercially available systems which are routinely used in the food testing laboratory. Examples of the fluorescent antibody technique, radioimmunoassays and enzyme immunoassays including the widely used enzyme linked immunosorbent assay are described below.

1.5.4.3.1 *Fluorescent antibody technique*

A method of salmonella detection by the fluorescent antibody technique was developed by Insalata *et al.* (1972), improved and evaluated by Fantasia *et al.* (1975). Traditional pre- and selective enriched samples are post-enriched in fresh selective enrichment broth for 4h. Aliquots are removed, stained with polyvalent OH reagent (to detect O and H antigens) and observed by fluorescence microscopy. The presence of fluorescent cells morphologically typical of *Salmonella* spp. constitutes a presumptive-positive result. Confirmation is by traditional cultural methods. A semi-automated system was developed but examination of unprocessed food samples led to procedural and mechanical difficulties and a high rate of false-negative results (Blackburn 1993).

1.5.3.4.2 *Radioimmunoassays*

Immunological assays using radioactive components are termed radioimmunoassays or RIAs. The assay is based on competition between unlabelled and radioactively labelled antigens to bind to specific antibodies. The RIA technique has been used for the detection of salmonella in foods. Ibrahim *et al.* (1985) developed a RIA for the detection of *Salmonella* spp. using titanous hydroxide to immobilise a wide range of Gram-negative and Gram-positive bacteria. *Salmonella* spp. were detected by specific antibodies. Ibrahim *et al.* (1986) evaluated this technique for the rapid detection of salmonellas in food samples and compared their efficacy with traditional cultural methods. Immobilised *Salmonella* spp. were detected by either commercial Spicer-Edwards salmonella polyvalent H antisera (SEA) or pooled antisera produced against ten salmonella flagellins (PFA). The RIAs detected as many salmonella positive samples as traditional methods. For the SEA detection, two false negatives were produced, with a false positive rate of 0.9%. While the PFA detection produced no false negatives and with a 3% false positive rate. In

addition the RIAs could detect 77 *Salmonella* serotypes when grown alone or in association with other species of Enterobacteriaceae in mannitol selenite cystine broth.

1.5.3.4.3 *Immunodiffusion*

The BioControl 1-2 Test™ for salmonella (BioControl Systems Inc., Bothwell, WA, USA) is a self-contained clear plastic vial consisting of two interconnected chambers. An inoculation chamber contains selective enrichment media, while a motility gel contains a non-selective semi-solid media. A 0.1ml aliquot of pre-enriched sample (low level contaminated products) or 1.5ml pre- and selectively enriched sample (highly contaminated products) are inoculated into or replace the selective enrichment media. The unit is then incubated for 14-30h at 35°C. During which selectively enriched motile salmonellas migrate through the motility gel in a process which is visualised by polyvalent anti-salmonella flagellar antibodies. A positive result is indicated by a white, U-shaped precipitation band; and is confirmed by loop inoculation of the selective enrichment media onto selective enrichment agar plates, followed by traditional confirmation. Thus presumptive positive results are obtained within 32-72h depending on the food type tested.

Warburton *et al.* (1995) carried out a study and a multilaboratory collaborative study to test and validate the BioControl 1-2 Test™ immunodiffusion method. Naturally and artificially contaminated foods were analysed and compared with traditional culture methods. In the precollaborative study 320 raw and highly contaminated food samples were analysed. An overall agreement rate of 96.9% was achieved. While in the collaborative study 612 raw shrimp, animal meats and raw ground poultry samples achieved 93.1% agreement.

1.5.3.4.4 *Enzyme Immunoassays*

A number of enzyme immunoassays (EIAs) have been developed. An example of the immunodiffusion method is described below. Other examples include the use of membrane filters (HGMF; Faber *et al.*, 1985; Cerqueira-Campos *et al.*, 1986; Blais and Yamazaki 1989a; 1989b), flow cytometry (McClelland and Pinder 1994; Pinder and McClelland 1994), polymyxin coated polyester cloth (Blais and Yamazaki 1990; Wang *et al.*, 1995) and sponges (Davies and Wray 1997). For a more comprehensive review of EIAs see Ibrahim and Fleet (1985) and Blackburn (1993).

Enzyme linked immunosorbent assay

Although a large number of salmonella specific antibodies have been developed for use in enzyme linked immunosorbent assay (ELISA) systems, they are based on similar ELISA techniques. Therefore to avoid repetition only the commercially available ELISAs will be detailed below.

Most commercially available ELISAs use the antibody sandwich technique for the capture and detection of target bacteria (with the exception of the LOCATE *Salmonella* immunoassay which does not use a capture antibody). The antibody sandwich technique involves antibodies which capture and detect salmonella. The capture antibody is frequently attached to a solid surface, usually the wells of microtitre plates, but more recently other solid support media have been introduced such as magnetic particles, dipsticks or paddles and hollow spurs (Table 1.8). The captured salmonella is then detected by an enzyme labelled antibody.

The majority of commercially available ELISAs incorporate traditional pre- and selective enrichment broths in their protocol. In addition many include a post enrichment step, prior to analysis by ELISA, in order to increase antigen to a detectable level. For example there is evidence to suggest that M-broth enhances flagellar production more than other media (Blackburn 1993).

A large number of evaluation studies have been carried out, in fact too many to discuss here, which compare ELISA techniques with traditional methods. A variety of naturally and artificially contaminated food samples have been tested. In general the ELISA techniques are affected by the food type and choice of selective enrichment producing 90-100% agreement with traditional methods. For the sake of clarity and to reduce repetition, the enrichment protocols and references of pertinent evaluation studies have been summarised in Tables 1.9 and Table 1.10 respectively. Where appropriate explanations of the techniques have been described.

Table 1.8: Commercially available salmonella ELISA detection methods

Solid support system	Product Name	Company/ Address
Manual		
Microtitre plate	TECRA™ Salmonella Visual Immunoassay (VIA)	Bioenterprises Pty Ltd, Roseville, NSW, Australia
	Salmonella-Tek™	Organon Teknika, Cambridge, UK
	Transia-*Salmonella*	Transia-Diffchamb S.A., Lyon, France
	Mastazyme™-*Salmonella*	Mast Diagnostic Ltd, Bootle, UK
	Assurance Enzyme Immunoassay (EIA)	BioControl Systems, Inc., Bothell, USA
	Locate *Salmonella* Immunoassay	Rhône-Diagnostics, Glasgow, UK
Dipstick	Path-Stik Rapid Salmonella Test	Celsis. Lumac, Cambridge, UK
Semi-automated		
Magnetic bead	EiaFoss system	Foss Electric, Wheldrake, UK
Automated		
Hollow spur	Vitek Immunodiagnostic Assay System (VIDAS) Salmonella Assay	bioMérieux Ltd, Basingstoke, UK

Table 1.9: Enrichment protocols for commercially available salmonella ELISA detection methods

Product name	Expected contamination level	Enrichment (h)			ELISA (h)	Treatment (°C/min)	Total test time (h)	Detection limit (cfu/ml)
		Pre-	Selective	Post				
TECRA™ VIA	Low	18-22	6-8	16-20	2	100°C/15	42-52	10^5-10^6
	High	18-22	16-20	6				
Salmonella-Tek™	Low	18-24	6-8	14-18	2	100°C/20	40-52	10^5-10^7
	High	22-26	18-24	4-6				
Transia- *Salmonella*	Low	16-20	6-8	14-18	2	100°C/20	38-52	10^5
	High	16-20	18-24	4-6				
Mastazyme™- Salmonella	Low and high	16-24	16-24	NONE	2	100°C/15-20	36-50	-
Assurance EIA	Low	24	6-8	14-18	2	100°C/20	47-59	-
	High	24	18-24	6-8				
Locate *Salmonella* Immunoassay	Low	18-24	6-8	18-24	2	100°C/20 or 121°C/20	48-54	10^3-10^7
	High	18-24	18-24	4-6				

Product name	Expected contamination level	Enrichment (h)			Treatment (min)	ELISA (min)	Total test time (h)	Detection limit (cfu/ml)
		Pre-	Selective	Post				
Path-Stik	Low and High	16-24	16-24	6-8[a]	Untreated or 100°C/	10	28-56	10^5-10^6
EiaFoss	Low	-	19-28[b]	3	100°C/15	75	24-33	10^5-10^6
	High	-	19-24[c]	3				
VIDAS Salmonella Assay	Low	24	18	6-8	100°C/15	45	42-50	10^4-10^6
	High	24	6-8	18				

[a], post enrich in BPW;
[b], enrichment in a novel Salmonella enrichment broth number 1 (25g/225ml) at 42°C;
[c], enrichment in novel Salmonella enrichment broth number 2 (25g/400ml) first at 37°C for 4h, then at 42°C for 15-24h

Table 1.10: References for commercially available salmonella ELISA detection methods

Product name	References
TECRA™ VIA	Jay and Comar 1988; Flowers *et al* 1988; Lambiri *et al.*, 1990; Blackburn 1993
Salmonella-Tek™	van Pouke 1990; Bailey *et al.*, 1991; Eckner *et al.*, 1992; Huis In'T Veld and Notermans 1992; Eckner *et al.*, 1994
Transia-Salmonella	Transia-Diffchamb 1996
Mastazyme™-Salmonella	Bolton and Franklin 1996
Assurance EIA	Andrews 1992, Feldsine *et al.*, 1992
Locate *Salmonella* Immunoassay	Anon. 1993b; 1993c; Bobbit and Betts 1993a; Eckner 1992; 1993; 1995; Gangar *et al.*, 1996
Path-Stik	van Beuden and Mackintosh 1992; Brinkman *et al.*, 1995
EiaFoss	Krussell and Skovgaard 1993; Jones and Betts 1994; Fierens and Huyghebaert 1996; Masso and Oliva 1997
VIDAS Salmonella Assay	Blackburn *et al.*, 1994; Keith 1997; Curiale and Gangar 1997

The Path-Stik Rapid Salmonella test is based on immunochromatographic technology. All the required reagents are contained within a dip-stick (Path-Stik). The Path-Stik is dipped into the pre-, selective and post enriched (Table 1.9) sample for a few seconds and placed onto a tissue. The presence of a wick in the top part of the dip-stick generates and maintains a flow by which the sample is transported through the devise. The sample is drawn across a reagent pad containing latex coated anti-salmonella antibodies. The subsequent antibody-antigen complex is captured by further antibodies immobilised on the membrane strip. This procedure takes 10min after which the result can be read from the reaction window. A positive salmonella reaction is indicated by the presence of a pink/purple line in the test area. A control line will always be visualised to indicate that the test has worked properly.

The EiaFoss system uses salmonella specific antibody coated paramagnetic spheres (Dynabeads®) referred to as immunospheres as the solid support matrix. Enriched, heat treated samples are manually transferred, together with an aliquot of immunospheres into test tubes within the EiaFoss analyser. Any salmonella antigen present will form complexes with the immunospheres. Application of a magnet allows non-bound material to be discarded. The captured salmonella is then detected by an enzyme labelled flagellar specific antibody. Traditional pre-, selective and post enriched (M-broth) samples or samples which have undergone VIDAS Immuno-concentration are boiled and manually introduced into the Vitek Immunodiagnostic Assay System (VIDAS; an automated instrument for performing ELISAs). Salmonella antigens are captured by antibodies coated onto the internal surface of a hollow spur. The subsequent ELISA stages are carried out automatically by the VIDAS in disposable plastic strips containing all the required reagents for the ELISA protocol.

1.5.3.5 *Nucleic acid based amplification and detection methods*

Nucleic acid based detection methods involve the techniques of DNA-hybridisation and polymerase chain reaction. The following section explains the principle behind the methods and details some commercially available salmonella detection systems. For a more comprehensive review of nucleic acid based detection methods in food microbiology and further references on the technical aspects of these techniques see Olsen *et al.* (1995a).

The essential principal behind nucleic acid based detection methods is the specific formation of double stranded nucleic acid molecules from two complementary, single

stranded molecules under defined physical and chemical conditions (termed hybridisation). One of the strands is produced in the laboratory in the form of a probe molecule (DNAH) or primer molecule (PCR), while the other strand is provided by the target organism (Olsen *et al.*, 1995a).

1.5.3.5.1 *DNA hybridisation technique*

Two methods of DNA hybridisation (DNAH) have been developed (i) colony hybridisation where DNA is fixed to a solid support filter and (ii) single phase hybridisation in aqueous solution. A number of *Salmonella* spp. specific probes have been described (Fitts *et al.*, 1983; Fitts 1985; Gopo *et al.*, 1988; Tsen *et al.*, 1989; Tsen *et al.*, 1991; Cohen *et al.*, 1993; Olsen *et al.*, 1995b; Soumet 1997). These reports indicate the potential of DNAH as a rapid screening method for *Salmonella* spp. in foods.

The Gene-Trak® system colour DNAH (cDNAH) *Salmonella* assay (Gene-Trak Systems, Framingham, USA) is a commercially available single phase hybridisation system. The technique is based on the identification of regions of ribosomal RNA (rRNA) that are unique to the genus *Salmonella*. Pre-, selective and post enriched samples (Table 1.11) are treated to lyse target organisms and release rRNA. Sample treatment is followed by the addition of two specific probes which hybridise to adjacent regions on the same target rRNA. One probe is used to capture target rRNA, while the other (which is labelled with fluorescein) is the detector probe. A plastic dipstick coated with polymers of deoxythymidinylic acid is used to capture the entire complex, which is detected by the addition of anti-fluorescein antibody labelled with horseradish peroxidase.

Food validation studies have been carried out which demonstrated that the cDNAH method was as effective as traditional methods of salmonella isolation and detection (Chan *et al.*, 1990; Curial *et al.*, 1990; Mozola *et al.*, 1991; D'Aoust *et al.*, 1995) and more sensitive for meat and poultry products (Rose *et al.*, 1991).

The cDNAH technique is a DNA/RNA detection method not an amplification method and as such requires the presence of at least 10^3-10^4 target bacteria in the sample to elicit a positive signal (Jones *et al.*, 1993).

1.5.3.5.2 *Polymerase chain reaction*

Polymerase chain reaction (PCR) technology is a DNA amplification method. The

Table 1.11: Enrichment protocols for commercially available nucleic acid detection procedures

Product name	Expected contamination level	Enrichment (h)			Treatment time (min)	Procedure time (h)	Total test time (h)
		Pre-	Selective	Post			
Gene-Trak®	Low	22-24	6	12-18	Lysis	2.5	50
	High	22-24	16-18	6			
Bax™	Low and high	20	-	3	Lysis	5	28
Probelia™	Low and high	18	-	-	Heat in ionic resin	6	24

technique can be used to specifically amplify target DNA (RNA) sequences (unique to target microbial pathogens) from minute quantities of heterogenous starting material, by more than a billionfold (Joshi *et al.*, 1991). Target DNA is combined with polymerase, nucleotides and primers that are specific for a given nucleotide sequence. This mixture then undergoes a series of timed heating and cooling cycles: two strands of target DNA sequences are separated by heat denaturation; then as the mixture cools the primers anneal (hybridise) to the target DNA sequence. DNA polymerase then uses nucleotides to extend the primers, thus creating a copy of the target DNA fragment. The newly synthesised PCR products then serve as templates in the following rounds of amplification: repeated cycles of denaturing, annealing and extending produces an exponential increase in the number of target DNA fragments, within a matter of hours.

The BAX™ system for screening *Salmonella* (FOMS Du Pont Wilmington, USA) and Probelia™ *Salmonella* species kit (Sanofi Diagnostics Pasteur; France) are commercially available methods of PCR. Following traditional pre-enrichment, food samples are enriched in brain heart infusion broth for 3h before introduction into the BAX™ system for PCR detection by gel electrophoresis (Table 1.11). Results are indicated by the presence or absence of a single band in each lane. The BAX™ system has been reported to detect 10^2-10^3 cfu/ml (Sobities *et al.*, 1997). A food trial investigation carried out on 142 naturally and artificially contaminated food samples showed that the BAX™ system gave results comparable to those of the traditional culture method (Sobities *et al.*, 1997).

In the Probelia™ system DNA is released from traditionally pre-enriched samples and detection is achieved by spectrophotometric means by a microtitre plate hybridisation technique (Table 1.11). The detection limit of the system was reported to be 10-10^2 cfu/ml (Dilasser *et al.*, 1997). Fifty-two out of 52 *Salmonella* strains tested were detected by the Probelia™ system. Moreover all 51 non-*Salmonella* strains tested were negative. A food trial investigation carried out on 285 naturally, artificially and non-contaminated food samples showed 99.6% agreement with traditional culture methods (Dilasser *et al.*, 1997).

1.5.3.6 *Separation and concentration technique*

Separation and concentration techniques aim to remove target organisms from food debris and competing flora and concentrate them into a smaller volume. This allows target cells to be concentrated above the detection threshold of alternative methods without relying

upon microbial multiplication (Bennett *et al.*, 1997). A number of separation and concentration techniques have been reported including filtration (Truscott and Lammerding 1987; Bobbit and Betts 1993b), centrifugation (Stannard and Wood 1983; McElroy *et al.*, 1995) and ultrasound (Miles *et al.*, 1995). The most frequently studied separation and concentration techniques have used antibodies and antibody-based biosorbents. A biosorbent is a structure with an affinity for a particular analyte immobilised to a solid phase such as a dipstick or magnetic particles (Bennett *et al.*, 1997). Three commercially available systems: the TECRA™ Unique Salmonella test, the TECRA™ Salmonella Immunocapture and the VIDAS Immuno-Concentration *Salmonella* are explained below. Dynabeads® anti-Salmonella is another salmonella separation and concentration technique. It is based on immunomagnetic separation (IMS). Since both IMS and Dynabeads® anti-Salmonella are fundamental to this project, they are dealt with in a separate section (1.6) and in considerably more detail than other alternative methods.

The TECRA™ Unique Salmonella test consists of a ready to use module composed of six tubes. Incubation steps are carried out at 42°C. Food samples are pre-enriched in a modified BPW. After incubation a volume of pre-enriched sample is placed into tube 1, along with a salmonella specific antibody coated paddle. If any salmonellas are present, it is captured by the antibodies. The paddle is then washed in tube 2 and transferred to M-broth plus novobiocin (tube 3). This allows the salmonella to replicate. Tube 4 contains conjugate, tube 5 is a wash stage and finally tube 6 contains substrate. A sample is considered positive when a purple colour covers the lower three-quarters of the paddle and a white band (negative control) covers the upper quarter. A negative sample is produced when the entire paddle is white except for a small purple cross on the base (the positive control).

The TECRA™ Unique method has been evaluated with respect to sensitivity, specificity and ability to detect *Salmonella* spp. from a range of artificially inoculated foods (Jones *et al.*, 1995a). The detection limit of the system was reported to be 10^4 cfu/ml. All 42 *Salmonella* serotypes tested were detected, although the reactions of *S.pullorum* and *S.gallinarum* were only weakly positive. The food trial experiment showed that the TECRA™ Unique method could detect *Salmonella* spp. inoculated at low levels (<20 cfu/25g). However four positive samples were produced which could not be confirmed by traditional methods. A further study by Jones *et al.* (1995b) evaluated the TECRA™ Unique method for the detection of salmonella from naturally contaminated poultry. The

TECRA™ Unique method detected *Salmonella* spp. in 29 more samples than traditional methods.

The TECRA™ Salmonella Immunocapture method is based on the first three steps of the TECRA™ Unique test (see above). After enrichment in M-broth, an aliquot is removed for confirmation purposes and the rest is heat treated to release antigens from the paddle. Detection is then performed with the TECRA™ Salmonella VIA.

The VIDAS™ Immuno-capture *Salmonella* (ICS) method replaces the traditional selective enrichment stage with a 40min automated concentration step. Food samples are pre-enriched in modified BPW for 16-24h. A 0.8ml aliquot is removed for ICS enrichment: *Salmonella* spp. are captured by an antibody coated hollow spur. After several washing steps (to remove non-specifically bound material) the salmonellas are released by an enzymatic cleavage method. The captured bacteria are non-selectively enriched in ICS broth for 5-6h (41-42°C). After which an aliquot is removed and heat treated (100°C, 15min). Antigen is detected by VIDAS™ Immunoassay. An evaluation study was carried out which tested 564 naturally contaminated meat products. The VIDAS™ ICS/ VIDAS™ *Salmonella* assay was compared with traditional culture methods (Lescure *et al.*, 1997). Sixty-two (11%) of the 564 meat samples tested positive for *Salmonella* spp.. All were detected by the VIDAS™ system. Traditional culture methods detected 42 (68%) of samples. Although five samples positive by VIDAS™ could not be confirmed.

1.6 MAGNETIC SEPARATION TECHNIQUE

The specific magnetic separation of target organisms from a sample or pre-enrichment medium replaces the selective enrichment step with a non-growth related procedure. This section explains the magnetic separation technique for the detection of target bacteria from foods with particular emphasis on salmonella isolation and detection. For a more detailed review on the applications of immunomagnetic separation in applied microbiology with special reference to applications in the food industry see Safarík *et al.* (1995).

1.6.1 *EQUIPMENT*

1.6.1.1 *Magnetic particles and affinity ligands*

There are a variety of magnetic or magnetisable carriers available, most of the particles used are superparamagnetic. Superparamagnetic particles express their magnetic properties in the presence of an external magnetic field. This property means that they can be

suspended into a homogenous mixture in the absence of any external magnetic field and easily removed from the suspension by the application of a magnetic separator. Magnetic carriers are commercially available: Dynabeads® (Dynal A/S, Oslo, Norway) are superparamagnetic, polystyrene-based particles with diameters of 2.8μm (Dynabead M-280) and 4.5μm (Dynabead M-450). Bio-mag (Advanced Magnetics, USA) particles are formed by silanized magnetic iron oxide with diameters ranging from 0.5μm to 1.5μm. Another magnetic sorbent used for microbiology work is Magnisort M, based on chromium dioxide particles, which exhibits high protein uptake capacity (40mg/g) allowing rapid capture kinetics. Alternatively, microbial cells can be made magnetisable by the direct adsorption of submicron particles of magnetic iron oxides to their surface (Dauer and Dunlop 1991). Microbial cells can also be made magnetisable by coating them with rare metals (Zborowski *et al.*, 1991). Magnetic separation of microbial cells can be useful for the determination of the total amount of microorganisms, for example during analysis of urine or water samples.

To achieve specific binding of magnetic particles to the target microbial cell, affinity ligands are used. Lectins (Payne *et al.*, 1992), agglutins (Payne *et al.*, 1993) and bacteriophage (Bennett *et al.*, 1997) have been used as affinity ligands but more frequently specific monoclonal or polyclonal antibodies are employed, hence the term 'immuno' magnetic separation. Polyclonal antibodies often exhibit unwanted cross-reactions and it is difficult to prepare them in a reproducible form. Monoclonal antibodies however often exhibit preferable characteristics.

Dynabeads® anti-Salmonella combine polyclonal and monoclonal anti-salmonella antibodies which bind a wide range of serotypes (>1400 serotypes), but also exhibit some cross-reaction with a few non-target organisms, for example *Citrobacter freundii* binds to anti-Salmonella Dynabeads® (Parmar *et al.*, 1992).

1.6.1.2 *Magnetic particle separators*

Strong permanent magnets or electromagnets can be used for the separation of magnetic particles and their complexes with adsorbed (immobilised) cells. Magnetic particle separators, employing strong permanent magnets (based on the rare earth metals) are most suitable due to very simple manipulation. High gradient magnetic separators, enabling the separation of very small magnetic particles on a laboratory scale, can be obtained, e.g. from the German company Miltenyi Biotec (Miltenyi *et al.*, 1990). Examples of two

magnetic particle concentrators (MPC; Dynal) are shown in Figure 1.9. The first picture shows the Dynal MPC®-M which is used in conjunction with Eppendorf tubes. Up to ten samples can analysed at any one time. The Dynal MPC®-96 (shown in the second picture) is for use with ELISA trays. This allows the co-analysis of up to 96 samples. A large-scale magnetic separator for selective cell separations has also been described by Hardwick *et al.* (1990). This can remove 10^{10} microbeads from a suspension processed at a flow rate of 9ml/min.

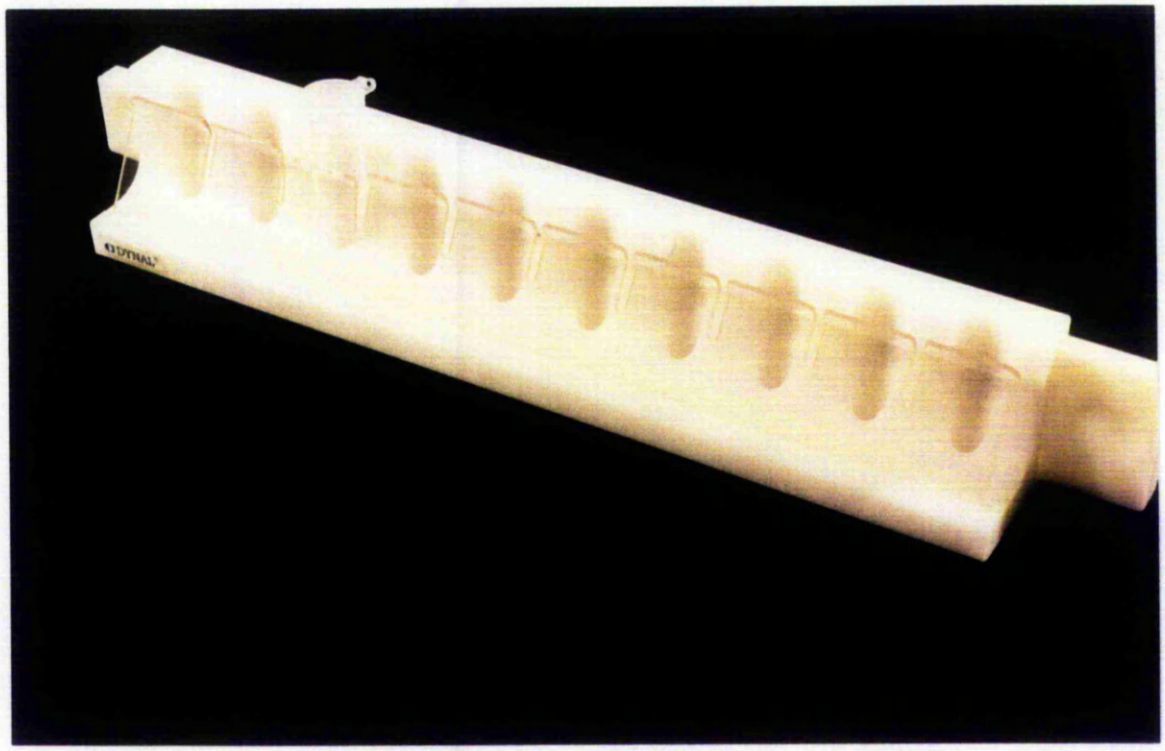
1.6.2 *APPLICATIONS*

The immunomagnetic separation (IMS) of target bacteria is based on the reaction of specific antibodies. These are either bound to magnetic particles (direct IMS) or free in solution (indirect IMS). The antibody coated beads and free antibodies are suspended in an aliquot of mixed cell suspension for an appropriate incubation time, during which antibodies bind to specific target cell structures. The resultant antibody-target organism complex is removed from the suspension. In the direct IMS technique this is achieved by application of a magnetic particle separator (MPS) which concentrates the particles to allow removal of unbound non-target organisms. In the indirect IMS technique antibody coated magnetic particles (specific for primary antibody) are added before application of the MPS. Organisms that are not bound to the magnetic particles are removed by washing with a suitable solution. Target organisms are detected by a number of detection procedures.

The IMS technique has been used to isolate a number of bacteria from food and environmental samples. Anti-salmonella beads are central to this project and therefore will be discussed in detail later with respect to the commercially available Dynabeads® anti-Salmonella. Examples of other beads include anti-*E.coli* O157:H7 beads (Okrend *et al.*, 1992; Chapman *et al.*, 1994; Upton and Coia 1994; Wright *et al.*, 1994; Bennett *et al.*, 1995; Vernozy-Rozand 1997), anti-*Listeria monocytogenes* beads (Skjerve *et al.*, 1990; Fluit *et al.*, 1993), anti-*Yersinia enterocolitica* beads (Kapperud *et al.*, 1993), anti-*Vibrio haemotylicus* beads (Tomoyasue 1992) and anti-*Staphyloccocus aureus* beads (Johne *et al.*, 1989).

The amount of immunomagnetic particles required for capturing the target cells differ according to the application. For example it has been reported for *Salmonella* spp. and *E.coli* O157:H7 that the highest recovery of the isolated target cells can be achieved with

(1)



(2)

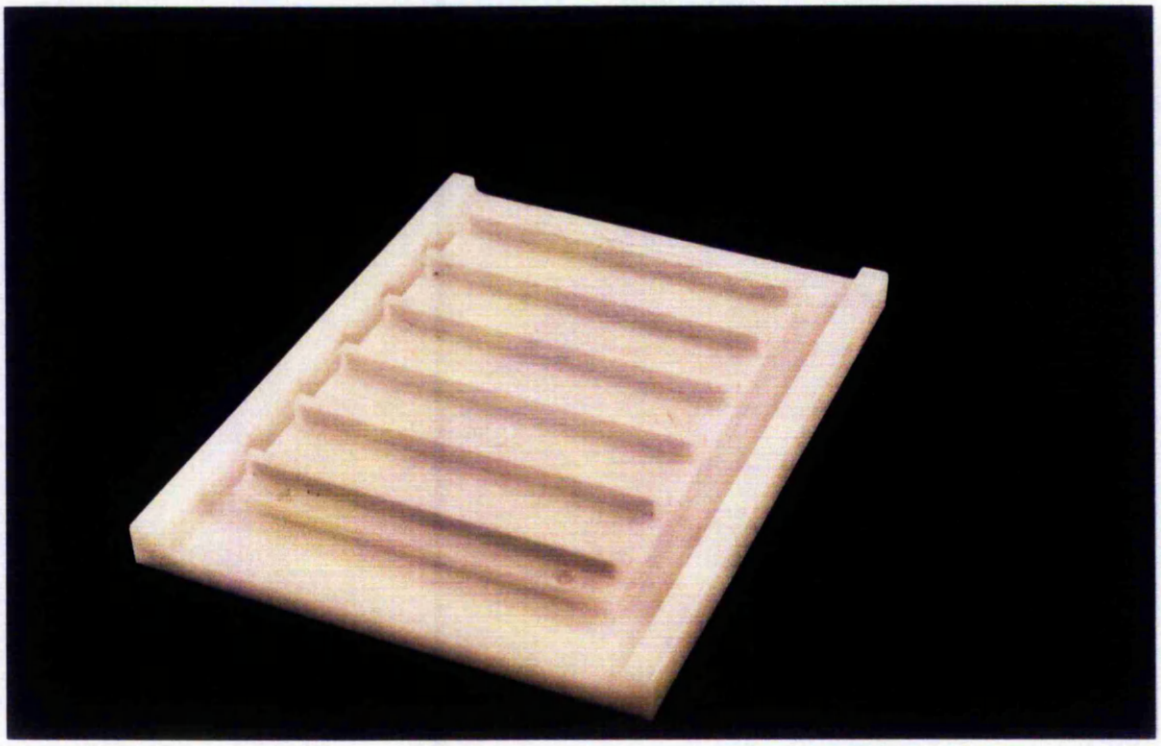


Figure 1.9: Examples of magnetic particle concentrators (MPC) Dynal MPC®-M (1) and Dynal MPC®-96 (2)

high concentrations of immunomagnetic particles at 2x 10^6-10^7 particles ml^{-1} (Safarík *et al.*, 1995).

The length of incubation with the magnetic particles affects the recovery of target cells. Incubation times of 10-60min have been reported (Safarík *et al.*, 1995). Although increased incubation times substantially increases the recovery of the target cells, prolonged incubation time can lead to the non-specific binding of the non-target microorganisms (Skjerve *et al.*, 1990; Fratamico *et al.*, 1992; Vermunt *et al.*, 1992).

1.7 DYNABEADS® ANTI-SALMONELLA

Anti-salmonella beads have been described which have coupled Dynabeads® and duPont magnetic particles with *Salmonella* spp. specific antibodies (Luk and Lindberg 1991; Vermunt *et al.*, 1992; Widjojoatmodjo *et al.*, 1993).

A number of end detection systems for salmonella have been used in conjunction with IMS (Olsvik *et al.*, 1994; Safarík *et al.*, 1995). These include microscopy (Lund *et al.*, 1988), impedance technology (Parmar *et al.*, 1992), electro-chemiluminescence (Yu and Bruno 1996), bioluminescence (Blackburn *et al.*, 1991), enzyme labelled *Salmonella* spp. specific antibodies in an ELISA based system (Holt *et al.*, 1995; Cudjoe *et al.*, 1996) and PCR techniques (Widjojoatmodjo *et al.*, 1991; 1992; Fluit *et al.*, 1993; Safarík *et al.*, 1995). However the most widely used method in food testing laboratories is that of standard plating. An example with respect to salmonella is the commercially available Dynabeads® anti-Salmonella IMS technique.

Dynabeads® anti-Salmonella are designed to replace the traditional selective enrichment stage (RV,SC and Tetrathionate broths, 24h) by concentrating and separating salmonellas directly from pre-enrichment broths by IMS. The Dynabeads® anti-Salmonella are incubated with an aliquot of a pre-enrichment broth. Any *Salmonella* spp. present in the sample will be bound to the bead by specific antibodies (Figure 1.10). The beads and any bound salmonella are subsequently separated from the pre-enrichment broth by a MCP. This form of selective enrichment takes less than 30min and presumptive results, based on plating the separated bacteria on selective agar plates (Figure 1.11), are obtained within 24h of traditional methods. The technique has been evaluated by a number of studies (Mansfield and Forsythe 1993; 1996; Cudjoe *et al.*, 1994a; 1994b; Dziadkowiec *et al.*, 1995; Ripabelli 1997).



Figure 1.10: Electron micrograph of *S.typhimurium* bound to magnetic Dynabeads® (from Dynal literature)

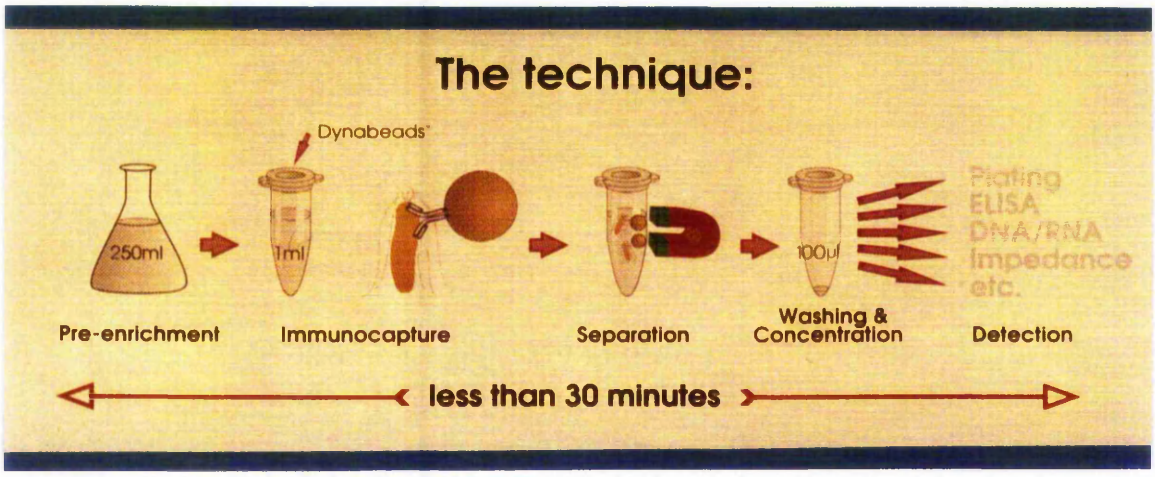


Figure 1.11: Dynabeads® anti-Salmonella immunomagnetic separation (IMS) technique (from Dynal literature)

The reactivity of Dynabeads® anti-Salmonella has been evaluated by The Centers for Disease Control (Atlanta, USA; Dynal A/S Oslo, personal communication). A total of 1482 *Salmonella* strains representing 218 serotypes were tested by slide agglutination. The strains selected represented 99.7% of the serotypes associated with human salmonella infection in the USA (prior to 1992). All gave positive slide agglutination however strains from subspecies II reacted in some case slower.

A food trial investigation was carried out by Mansfield and Forsythe (1993) which tested a range of uninoculated and artificially contaminated foodstuffs. One hundred and twenty samples were tested which included six food groups: raw chicken, prawns, skimmed milk powder (SMP), herbs and spices, cocoa powder and animal feed. Half the samples were artificially contaminated with one of a range of *Salmonella* serotypes at a level of 5-110 organisms per 25g sample. Salmonella was isolated and detected by traditional selective enrichment methods (SC, RV and Müller-Kauffmann's Tet broths) and Dynabeads® anti-Salmonella (IMS). The results are shown in Table 1.12. No salmonellas were isolated from uninoculated cocoa powder, SMP, herbs and spices and animal feed. The IMS technique isolated salmonellas from one sample of prawns which was not detected by traditional methods. Three raw chicken samples were positive for *Salmonella* spp. as detected by SC broth after 24h and RV broth after 48h. However Tet broth and the IMS technique failed to detect salmonellas in one of the three samples. Salmonellas were isolated from all artificially inoculated samples of cocoa powder, SMP, prawns, animal feed and raw chicken by all four methods. The IMS technique detected *Salmonella* spp. in all herbs and spices samples whereas SC, Tet and RV broths failed to detect salmonellas in artificially inoculated garlic granules; thus demonstrating the possible recovery of sublethally injured salmonellas. The researchers concluded that the IMS technique was as good as traditional methods and therefore could replace the time consuming selective enrichment stage with a 10min concentration step thus saving 24h.

A study was carried out by Cudjoe *et al.* (1994a) evaluated the performance of Dynabeads® anti-Salmonella compared to traditional selective enrichment and MSRV agar. One hundred and eighty naturally contaminated poultry samples consisting of cloacal/faecal swabs, raw chicken breast, egg shell samples, raw chicken liver samples and chicken neck skins were tested. Results are shown in Table 1.13. One hundred and fifty samples were confirmed salmonella positive. Of these the IMS technique detected 135 (90%), traditional methods detected 98 (65%) and MSRV detected 33 (22%). Researchers concluded that

Table 1.12: Isolation of confirmed *Salmonella* strains from six different food types (from Mansfield and Forsythe 1993)

	Confirmed salmonella isolates						
	Enrichment method						
	Selenite cystine		Tetrathionate		Rappaport- Vassiliadis		Immunomagnetic separation
Food sample	24h	48h	24h	48h	24h	48h	(10min)
Unspiked	0	0	0	0	0	0	0
Cocoa powder	0	0	0	0	0	0	0
Skimmed milk powder	0	0	0	0	0	0	0
Herbs and Spices	0	0	0	0	0	0	0
Prawns	0	0	0	0	0	0	1
Animal feed	0	0	0	0	0	0	0
Raw chicken	3	3	2	2	2	2	2
Spiked							
Cocoa powder	10	10	10	10	10	10	10
Skimmed milk powder	10	10	10	10	10	10	10
Herbs and spices	9	9	8	9	9	9	10
Prawns	10	10	10	10	10	10	10
Animal feed	10	10	10	10	10	10	10
Raw chicken	10	10	10	10	10	10	10

Dynabeads® anti-Salmonella followed by plating was superior to traditional culture techniques and the MSRV method.

Table 1.13: The detection of *Salmonella* strains from poultry samples using traditional, Dynabeads® anti-Salmonella immunomagnetic separation (IMS) and modified semi-solid Rappaport-Vassiliadis (MSRV) detection methods (from Cudjoe *et al.*, 1994b)

Samples	Enrichment method			
	Traditional	**IMS**	**MSRV**	**All methods**
Faecal/cloacal	32/40	27/40	3/40	38/40
Meat	4/10	5/10	0/10	5/10
Egg shells	0/10	1/10	0/10	1/10
Liver	38/60	59/60	29/60	60/60
Neck skin	24/60	43/60	1/60	46/60
Number of positive	98/180	135/180	33/180	150/180

Dziadkowiec *et al.* (1995) carried out a study which compared the sensitivity of Dynabeads® anti-Salmonella with traditional selective enrichment methods (SC, Müller-Kauffmann's Tet, RV). Skimmed milk powder (SMP) samples were artificially inoculated with low levels of *S.enteritidis*, *S.typhimurium* and *S.virchow* to imitate the low contamination levels often found in foods. Samples were then incubated in BPW for 24h. Table 1.14 presents the data for the three *Salmonella* serotypes according to predicted inoculum size and enrichment procedure. Broths inoculated with 20 or more cells were positive for *Salmonella* spp., while those inoculated with less than 0.2 cells were all negative. Replicate broths inoculated with 20 *S.enteritidis* cells were positive as detected by the IMS technique, Tet and RV broths. However SC broth failed to isolate salmonellas from one of the replicates. The IMS technique, Tet and RV broths detected *Salmonella* spp. in three out of five replicate broths inoculated with 2 cells, while SC broth could only detect *Salmonella* spp. in two. Only one of the five replicates inoculated with 0.2 cells was positive for *Salmonella* spp. as confirmed by the IMS technique, Tet and RV broths. SC failed to detect *Salmonella* spp. in any of the replicates. Pre-enrichment broths inoculated with 20 *S.typhimurium* cells were positive by all four techniques. The IMS technique, SC and Tet broths confirmed that three out of the five replicates inoculated with

Table 1.14: The isolation of *Salmonella* strains by Dynabeads® anti-Salmonella immunomagnetic separation (IMS) and traditional enrichment broths from skimmed milk powder-buffered peptone water pre-enrichment broths (from Dziakowiec *et al.*, 1995)

		S.enteritidis				*S.typhimurium*				*S.virchow*			
		Enrichment method				Enrichment method				Enrichment method			
Predicted inoculum size (cfu/ml)*	Replicate	IMS	SC	Tet	RV	IMS	SC	Tet	RV	IMS	SC	Tet	RV
20	1	+	+	+	+	+	+	+	+	+	+	+	+
	2	+	+	+	+	+	+	+	+	+	+	+	+
	3	+	ND	+	+	+	+	+	+	+	+	+	+
	4	+	+	+	+	+	+	+	+	+	ND	+	+
	5	+	+	+	+	+	+	+	+	+	+	ND	+
2	1	ND	ND	ND	ND	ND	ND	ND	ND	+	+	+	+
	2	+	ND	+	+	+	+	+	+	+	+	+	+
	3	+	ND	+	+	ND	ND	ND	ND	+	+	+	+
	4	+	ND	+	+	+	+	+	+	+	+	+	+
	5	ND	ND	ND	ND	+	+	+	ND	+	ND	+	ND
0.2	1	ND	ND	ND	ND	ND	ND	ND	ND	+	+	+	+
	2	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	4	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	5	+	ND	+	+	ND	ND	ND	ND	ND	ND	ND	ND

*, Colony forming units of salmonella cells that were inoculated into 250ml of skimmed milk powder-buffered peptone water (25g in 225ml BPW); IMS, immunomagnetic separation; SC, selenite cystine; Tet, Müller-Kauffmann tetrathionate; RV, Rappaport-Vassiliadis; +, positive salmonella culture isolated on selective agar; ND, not detected, no salmonella isolated on selective agar

2 salmonella cells were positive. However *Salmonella* spp. were not detected by RV broth. The IMS technique and RV broth detected *Salmonella* sp. in all replicates inoculated with 20 *S.virchow* cells. However both SC and Tet broths failed to detect *Salmonella* spp. in one (but not the same) replicate. All replicates inoculated with 2 cells were positive by IMS and Tet. SC and RV broths did not detect *Salmonella* sp. in one of the replicates. Only one of the replicates inoculated with 0.2 cells was positive and was detected by all techniques. These results confirmed that the IMS technique was as sensitive as traditional selective enrichment methods.

The Dynabeads® anti-Salmonella IMS technique was further evaluated in a collaborative ring trial study (Mansfield and Forsythe 1996). Eight laboratories participated in a salmonella detection ring trial study which compared selective enrichment by traditional broths (SC, RV, Müller-Kauffmann's Tet) with IMS using Dynabeads® anti-Salmonella from six types of herbs and spices. These included white pepper, paprika powder, garlic powder, onion powder, oregano and compound flavouring. Each herb and spice was artificially inoculated with one of six freeze-dried *Salmonella* serotypes. Each herb and spice analysis comprised of 12 samples including one uninoculated control, five spiked at a 'low' level (10 cells/25g), five spiked at a 'high' level (1000 cells/25g) and one referred to as 'very high' (10,000 cells/25g). The recovery of *Salmonella* spp. from each herb and spice is shown in Table 1.15. Where the laboratory cross-contamination of the negative control occurred, all results were removed leaving a total of 468 samples. Both the IMS technique and traditional enrichment recovered salmonellas from 195/468 (41.7%) samples. Salmonellas were recovered by the IMS technique and not traditional enrichment in 18 samples. Whereas traditional enrichment recovered *Salmonella* spp. from 19 samples which were negative by the IMS technique. Researchers concluded that artificially inoculated *Salmonella* strains were recovered from herb and spice samples almost as frequently with the IMS technique as with the traditional enrichment method (213 and 214 samples, respectively). It should be mentioned that laboratories taking part in the food trial were not familiar with the IMS technique and limited training was given. During the course of the trial it became obvious that familiarity of the technique resulted in a higher number of true positive IMS results.

A study by Ripabelli *et al.* (1997) compared the Dynabeads® anti-Salmonella and traditional SC and RV selective enrichment for the isolation and detection of *Salmonella* spp. from naturally contaminated pork sausages and chicken meat samples. *Salmonella* spp. were

Table 1.15: Comparison of the recoveries of six *Salmonella* strains from herbs and spices by Dynabeads® anti-Salmonella immunomagnetic separation (IMS) and traditional enrichment techniques (from Mansfield and Forsythe 1996)

Herbs and spice[a]	*Salmonella* strain	Number of salmonella samples detected by immunomagnetic separation and traditional enrichment techniques				Total number of samples
		Positive-positive[b]	Positive-negative[c]	Negative-positive[d]	Negative-negative[e]	
White pepper	*S.typhimurium*	58	0	5	21	84
Paprika	*S.enteritidis*	24	5	4	27	60
Onion powder	*S.ealing*	21	4	6	53	84
Garlic	*S.virchow*	3	5	0	88	96
Oregano	*S.give*	35	4	3	42	84
Compound flavouring	*S.arizonae*	54	0	1	5	60
Total number	-	195	18	19	236	468
Percentage (%)	-	41.7	3.8	4.1	50.4	100

[a], herbs and spice are presented in order of analysis; [b], number of samples positive by both techniques;
[c], number of false negative results by traditional enrichment techniques; [d], number of false neagtive results by immunomagnetic separation technique;
[e], number of samples from which *Salmonella* spp. were not recovered by either technique

detected in 18 out of the 167 samples tested. SC broth enrichment detected 15 (83.3%) of these, RV broth yielded only 7 (38.9%) positive isolations, while the IMS technique detected 12 (66.7%). Researchers concluded that the IMS technique could become a reliable alternative to traditional methods for routine salmonella enrichment.

1.8 RAPID METHODS FOR THE IDENTIFICATION AND CONFIRMATION OF *SALMONELLA* SPP.

Results from traditional and alternative methods of salmonella detection give only presumptive identification of isolates that need to be confirmed by further testing. Traditional methods of identification include colony and cell morphology and biochemical, enzymological and serological tests. Rapid test kits have been developed for the identification of bacterial isolates. They are based on culture methods, enzyme activity and latex agglutination techniques. Commercially available rapid identification and confirmation methods for *Salmonella* spp. are listed in Table 1.16.

1.8.1 *CULTURE METHOD*

Rapid test kits based on culture techniques are miniaturised, ready-to-use tests with dehydrated substances and indicators contained in disposable wells or tubes. Since they are based on culture methods, results are not obtained more rapidly. However these kits do save time in media preparation and many samples can often be tested at one time. For example the API 20E kit consists of a strip of 20 microtubes containing dehydrated substances. Each tube is inoculated with a bacterial suspension of the test organism. The strip is then incubated at 37°C for 18-24h. During this time the metabolising organism produces colour changes in the media which are either spontaneous or revealed by other reagents. The reactions are read according to an interpretation table which results in an identification number. Identification of the organism is obtained reference manual. The AutoMicrobic system for the identification of *Enterobacteriaceae* is fully automated. It uses plastic cards with wells containing substrates that are inoculated automatically and scanned hourly.

1.8.2 *ENZYME ACTIVITY*

These kits are based on the presence of performed bacterial enzymes that can be detected within a few hours by biochemical tests. For example the Micro-ID enteric identification system consists of impregnated filter papers and can identify *Enterobacteriaceae* within 4h. The MUCAP is a test reagent that can be used to identify salmonellas on selective agar.

Table 1.16 Commercially available rapid identification and confirmation methods for *Salmonella* spp.

Principle	Product name	Company	Comments and references
Culture method	API20E	bioMérieux	For *Enterobacteriaceae*
	Minitex	BBL Beckton Dickenson	For *Enterobacteriaceae*
	Enterotube 11	Roche Diagnostics	For *Enterobacteriaceae*
	ATB automated bacterial identification system	bioMérieux	Semi-automated system
	AutoMicrobic system	Vitex Systems Inc.	Fully automated system for the identification of *Enterobacteriaceae*
Enzyme activity	Micro-ID	Organon Teknica	For *Enterobacteriaceae*
	MUCAP	Immuno Ltd.	For *Enterobacteriaceae*; Manafi and Sommer 1992
Serology	Oxoid Salmonella	Unipath	Latex agglutination
	Microscreen Salmonella	Mercia Diagnostics	Latex agglutination; D'Aoust *et al.*, 1991; Manafi and Sommer 1992
	ANA Salmonella	ANI Biotech OY	Latex agglutination
	Wellcolex Colour Salmonella	Murex	Coloured latex
	Spectate Salmonella	Rhône Diagnostics	Coloured latex; D'Aoust *et al.*, Cheesbrough and Donnelly 1996

A drop of reagent is added to a colony and examined under ultra violet light (λ 366nm) after 3-5min. In a salmonella positive colony non-fluorescent methylumberliferyl caprylate is cleaved enzymatically by C_8 esterase to release fluorescent umberliferone.

1.8.3 *LATEX AGGLUTINATION TECHNIQUE*

Rapid tests based on latex agglutination techniques identify bacterial isolates within a few minutes. Latex particles are sensitised with antibodies specific to particular antigens on the cell wall of the target organism or group of organisms. A drop of pure culture is applied to test areas (usually cards) and mixed with a sensitised latex solution. The formation of visable granular clumps indicates agglutination and the presence of the target organism. Several kits are available (Table 1.16). Some use coloured latex to distinguish between *Salmonella* serogroups (Wellcolex Colour Salmonella and Spectate Salmonella).

1.9 ADVANTAGES AND DRAWBACKS OF ALTERNATIVE SALMONELLA ISOLATION AND DETECTION PROCEDURES

As previously mentioned perhaps the major disadvantage of the traditional culture method of salmonella isolation and detection with respect to the food industry is time. Food testing laboratories are required to process large numbers of samples. The faster they can do this the more cost efficient the process.

Alternative methods have attempted to fulfill this specification by reducing media preparation time. The Salmosyst Enrichment technique combines the culturing stages. Whereas the Tecra™ Unique Salmonella test provides a kit in a ready-to-use form.

The total test time of negative samples has also been reduced. A presumptive positive salmonella sample can be identified within 24-56h (Table 1.9). This means that produce (containing no *Salmonella* spp.) can be distributed 1-3 days earlier than traditional methods can allow. Alternative methods of salmonella isolation and detection have achieved this by shortening or replacing one or more of the traditional enrichment steps. Commercial ELISAs use a shortened enrichment broth stage and replace the time consuming selective agars with a shortened post enrichment broth step and a 2h assay (Table 1.9). Moreover by using microtitre plates up to 94 samples can be tested at any one time including one positive and negative control. This mutiple testing of samples ultimately result in increased sample throughput.

Traditional culture methods are also labour intensive and require trained personnel to isolate and distinguish between salmonella and non-salmonella colonies. Semi-automated systems such as the EiaFoss and VIDAS analysers means that procedures are less labour intensive. Many of the alternative methods have simplified detection procedures reducing the need for the skilled personnel by introducing colorimetric tests. The majority of which are spectrophotometric methods, a positive sample is represented by an absorbance reading above a specified threshold value as with the ELISA systems. An alternative simpler method is the immunochromatographic Path-Stik Rapid Salmonella dipstick method of Celsis Lumac. In this case a positive result is indicated by the presence of a pink/purple line.

Although alternative techniques have advantages over the traditional culture method, they do suffer from disadvantages of their own. In many cases the enrichment procedures have been complicated. Where traditional methods use a single enrichment protocol to cater for different food types. Some alternative procedures require separate enrichment protocols to detect salmonella in foods with high and low levels of background flora (Table 1.9). For example the Salmonella-Tek™ procedure for samples with low level background flora includes an 18-24h pre-enrichment, a 6-8h selective enrichment, a 14-18h post-enrichment, a 20min treatment step and a 2h assay. Samples with a high level of background flora should be incubated in pre-enrichment medium for 22-24h, selectively enriched for 18-24h, post enriched for 4-6h, before treatment and analysis by ELISA.

Some alternative methods such as conductance microbiology still require skilled personnel to interpret data and in addition require specialist tubes, incubator modules and monitors. Moreover most alternative methods still require non-traditional equipment. ELISA methods, for example require microtitre plates, multichannel pipettes and ELISA plate readers.

The hazards associated with some techniques mean that they have not been widely accepted in the food testing laboratory. For example although RIAs have a high sensitivity and are able to detect a few picograms (1 picogram = 10^{-12} g), the potential hazards associated with radioactivity restricts its useage to those laboratories equiped to handle radioisotopes.

Immunoassays are only as good as the antibodies they use. Most antibodies are not entirely *Salmonella* sp. specific. Cross-reactions with closely related *Enterobacteriaceae*

are not uncommon. Tecra™ VIA cross-reacts with *Citrobacter* spp (Jay and Comar 1988). Whereas the VIDAS Salmonella Assay cross-reacts with *Enterobacter cloacae* as well as *C.freundii* (Blackburn *et al.*, 1994). Mastazyme™-Salmonella has been reported to cross-react with *E.coli*, *Enterobacter* spp., *Proteus* spp., *Serratia* spp., *Hafnia* spp. and *Citrobacter* spp. (Bolton and Franklin 1996). Some salmonellas are not detected by ceratin techniques. For example the EiaFoss uses an antibody that detects salmonella flagella antigen. Hence it can not detect non-motile salmonellas such as *S.gallinarum* (Jones and Betts 1994).

Traditional methods are used as the yardstick to which alternative techniques are compared with respect to sensitivity and specificity. Due to the innate conservatism of the food testing industry and its apparent resistance to change; shared enrichment steps ensure a wider acceptance of alternative techniques. Most commercially available alternative methods include traditional pre- and selective enrichment (Figure 1.12). However some alternative techniques have introduced novel enrichment media (Figure 1.13). The EiaFoss system for the detection of *Salmonella* spp. replaces traditional pre- and selective enrichment with novel salmonella enrichment broths. Moreover it, somewhat unconventionally, uses BPW as a post enrichment instead of GN- or M-broths. While these novel broths may prove to be effective at increasing salmonella cell numbers and reducing competing bacteria, they do not have a long history of development. As such they may require extensive evaluation studies before they are widely accepted as a viable alternative to traditional culture media.

Many alternative salmonella isolation and detection methods have been developed. However as yet none produce results within a day. This is primarily due to the fact that the majority of techniques require relatively high numbers of target organisms to produce a positive result. Salmonellas usually contaminate processed foods at extremely low levels. However the detection limit of most ELISAs range from 10^5-10^7 cfu/ml (Table 1.9) depending on serotype. This means that all commercially available ELISAs require some form of time consuming culture enrichment to increase salmonella cell numbers to a detectable level. Hence the effectiveness of these salmonella detection methods are dependent upon selective enrichment; and as previously mentioned traditional forms of selective enrichment have limitations in their ability to isolate all contaminating salmonella.

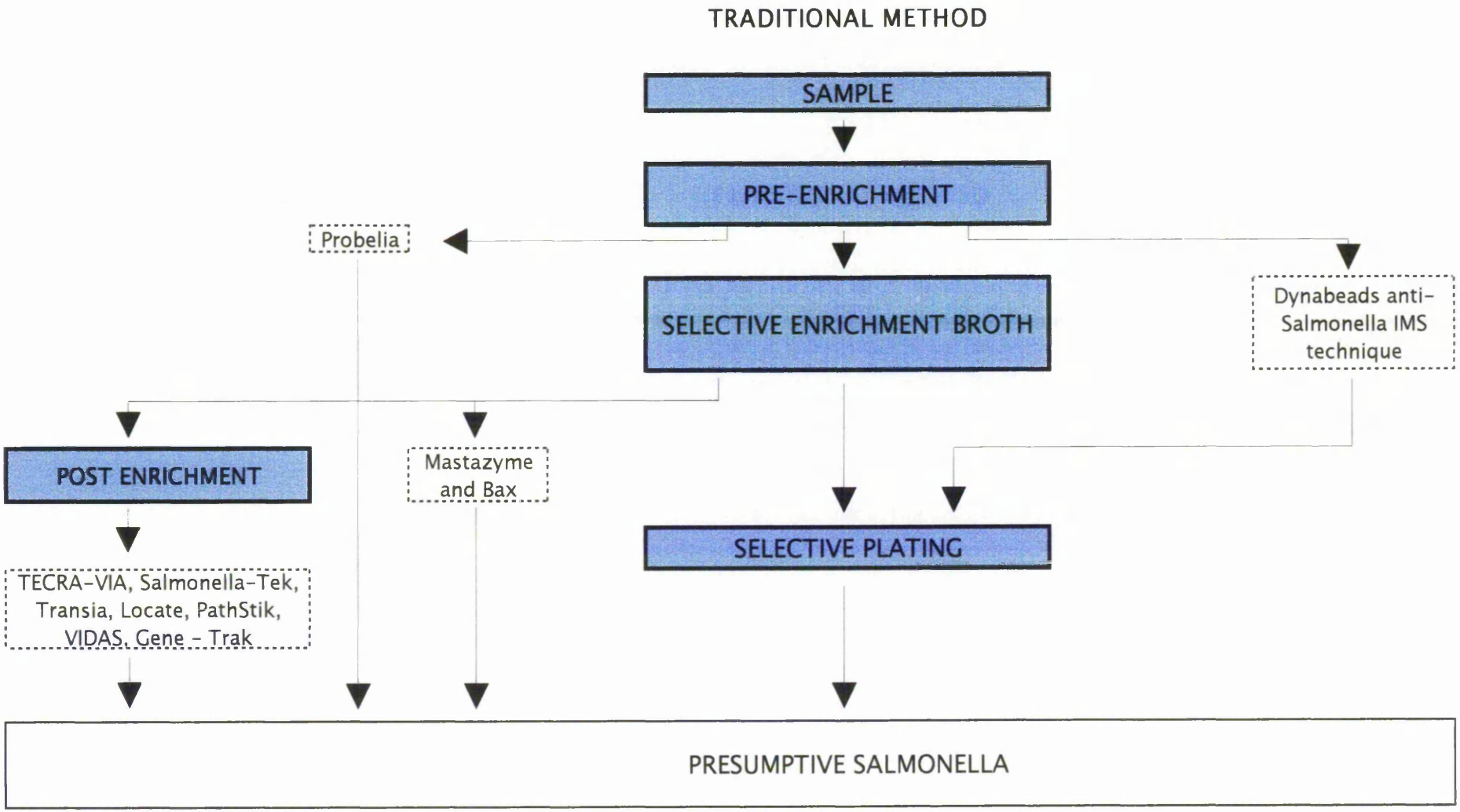


Figure 1.12: Commercially available salmonella isolation and detection methods based on traditional enrichment

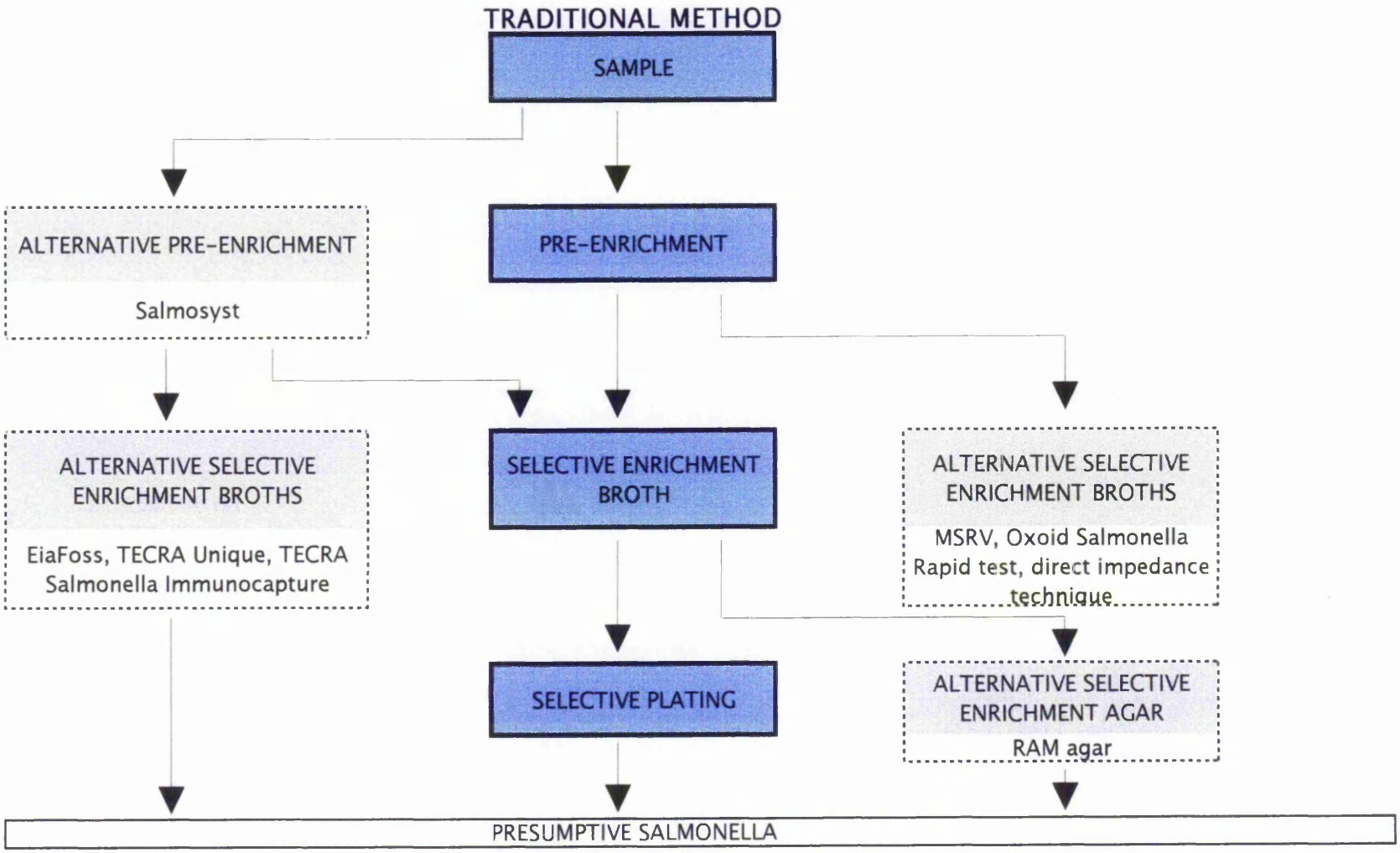


Figure 1.13: Commercially available salmonella isolation and detection methods based on alternative enrichment media

PCR technology can utilise minute quantities of target material to produce billions of copies of specific DNA (RNA) sequences. This property means that PCR technology does not suffer from the sensivity and specificity drawbacks of antibody related systems. However there are problems associated with the application of the PCR technique in food microbiology. The DNA used in the PCR process could originate from dead cells as well as viable ones and so cannot be used directly on the food sample. Moreover some substances in food and traditional selective and non-selective media are reported to inhibit the PCR process (Rossen *et al.*, 1992; Lantz *et al.*, 1994; D'Aoust *et al.*, 1995). Thus inclusion of a pre- and post enrichment step dilutes PCR inhibitors and in addition ensures that detection is based on the presence of viable cells only. This however leads to an extended protocol. Hence commercial PCR techniques such as Probelia™ and Bax™ require a minimum of 24h before a result is available (Table 1.11).

In addition there are other problems associated with PCR technology. The presence or absence of amplified product must be verified by other techniques such as gel electrophoresis and DNA-H. PCR products have generally been analysed by ethidium bromide-stained agarose gels. Although this method is rapid, it is hazardous because it uses a mutagenic compound. While contamination of the laboratory with trace amounts of amplican can wreak havoc on subsequent amplification results (Rasmussen *et al.*, 1994). Moreover PCR, gel electrophoresis and DNA-H require specialist equipment and trained personnel.

1.10 SALMONELLA CORE SPECIFIC ANTIBODIES

PCR technology has the potential to produce perhaps the most sensitive, rapid salmonella detection technique. However it requires specialist equipment and trained personnel not routinely found in the food testing laboratories. Hence at this present time its use is therefore restricted. A more 'user friendly' and well proven technique is that of the ELISA method. To date antibodies used within the ELISA systems have expressed limitations with respect to specificity and sensitivity. Two monoclonal antibodies, T6 and M105, have been described which are reported to react with an epitope common to all salmonellas. Moreover these antibodies exhibit a limited cross-reaction with non-salmonella organisms. The positive attributes of the T6 and M105 antibodies are described in the following section.

1.10.1 *THE MONOCLONAL ANTIBODY T6*

Tsang *et al.* (1987) described a murine monoclonal antibody which was directed against the N-acetylglucosamine→glucose region of the outer core structure of salmonella LPS (Figure 1.14).

A study by Tsang *et al.* (1991b) investigated the conservation of the T6 epitope in a large selection of bacteria. A total of 176 *Salmonella* strains representing 116 serotypes and 42 serogroups including representatives if the sub-species I, II, IIIa, IIIb, IV, V and VI were tested. All 20 serotypes (70 strains) belonging to serogroup A-E were positive for the T6 epitope. Additionally 12 serotypes (12 strains) of subspecies IIIa *Salmonella* reacted with the T6 antibody. Moreover 71% of the 96 serotypes (106 strains) belonging to the serogroups F-67 were positive.

While Choi *et al.* (1992) reported that the T6 antibody reacted with all 30 *Salmonella* strains tested and did not cross-react with any of the 21 non-*Salmonella* strains.

Ng *et al.* (1996) tested a total of 232 strains (136 serovars) of *Salmonella*. Ninety-nine percent of the serogroup A-E strains and 71% of the serogroup F-67 strains were T6 reactive. There was no cross-reactions with the 65 non-*Salmonella* strains assayed.

1.10.2 *THE MONOCLONAL ANTIBODY M105*

The M105 antibody was produced and characterised by Tsang *et al.* (1991a). The epitope of this IgG_{2b} antibody was determined by reacting the M105 antibody with purified LPS from different rough mutant *Salmonella* strains (Ra-Re) and related *Enterobacteriaceae* (*E.coli* and *Shigella*) by dot blot RIA. Results revealed that the M105 antibody reacted strongly with Ra LPS and in addition reacted with Rb_1 and Rb_2 LPS but with decreasing reactivity. Therefore investigators concluded that the M105 antibody binding site extends over at least three sugar residues of N-acetylglucosamine→glucose→galactose of the outer core region of salmonella LPS. This corresponds to a slightly deeper epitope than T6 (Figure 1.14).

Studies have shown that the M105 antibody reacts specifically with *Salmonella* spp. (Todd *et al.*, 1991; Tsang *et al.*, 1991; Nielsen and Tsang 1992; Nielsen *et al.*, 1993; Tsang *et al.*, 1997). The M105 antibody reactive strains included representatives of serogroups A, B, C_1, C_2, D_1, E_1, E_4, F, G, H, I, J, K, L, M, N, O, P, Q, R, S, T, U, V, W, X, Y, Z,

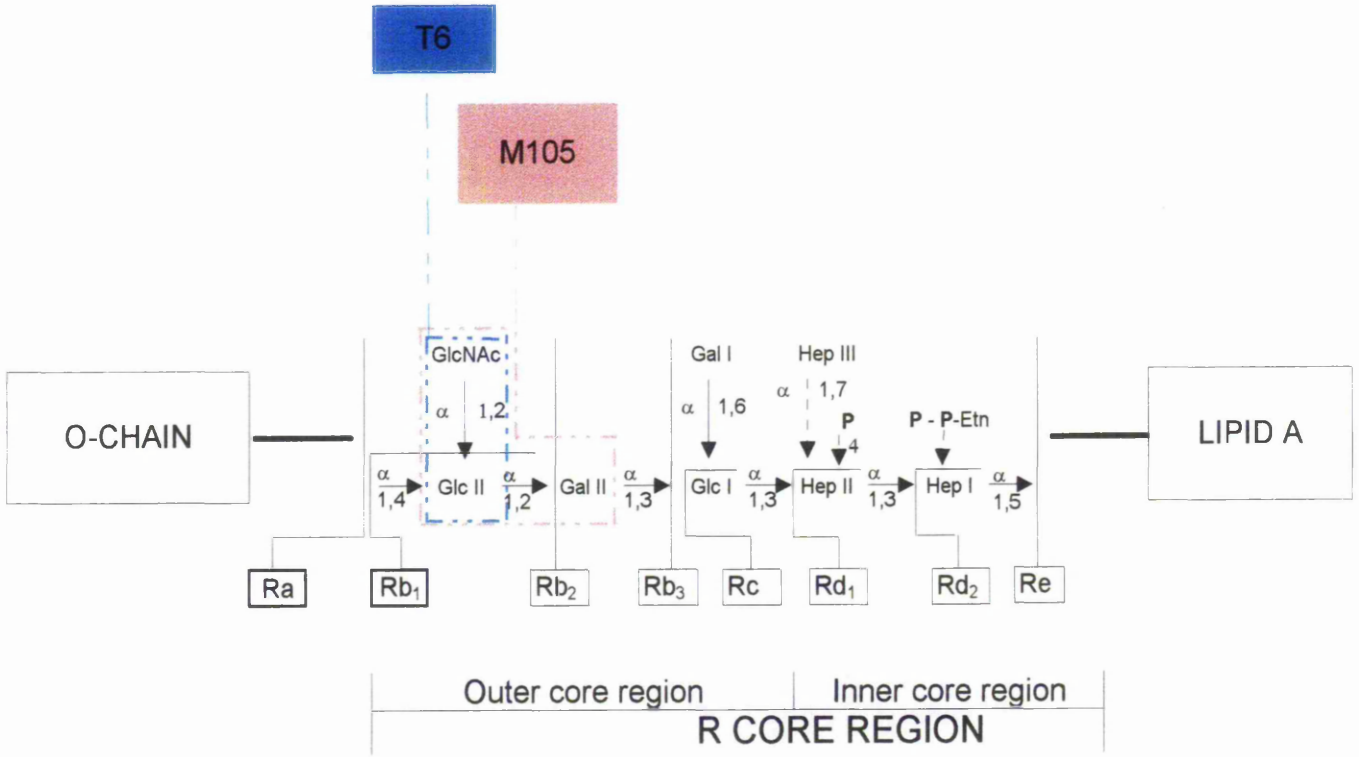


Figure 1.14: The binding sites of the *Salmonella* genus specific monoclonal antibodies T6[a] and M105[b]
([a], Tsang *et al*., 1991b; [b], Tsang *et al*., 1987) (Dotted lines indicate incomplete substitution)

51, 52, 53, 55, 56, 57, 58, 59, 60, 61, 62, 63, 65, 66 and 67. In total 43 out of 46 serogroups tested have been shown to react with the M105 antibody.

The specificity of the M105 antibody has been demonstrated by its lack of reactivity with over 300 non-*Salmonella* strains including a range of closely related Enterobacteriaceae: *Citrobacter freundii*, *C.diversus*, *Enterobacter aerogenes*, *E.cloacae*, *Edwardsiella tarda*, *Klebsiella pneumoniae*, *K.oxytoca*, *Proteus mirabilis*, *P.vulgaris*, *Providencia stuarti*, *Morganella morgannii*, *Serratia marcescens*, *Shigella sonnei*, *S.flexneri* and *Yersinia enterocolitica*. Two strains of *Enterobacter agglomerans* and a rough mutant strain of *Shigella flexneri* were shown to cross-react with M105.

1.11 AIMS

In view of the increase in human salmonella infections in many countries, the World Health Organisation (WHO) organised a Consultation on the Control of Salmonella Infections in Animals, held in Jena, Germany, on 21-26 November 1993. Among the recommendations of the participants was further research on the rapid isolation and identification procedures for salmonella (Anon. 1994).

Conventional methods of salmonella isolation and detection require a minimum of three days for a presumptive result. Dynabeads® anti-Salmonella IMS technique currently reduce this time to two days. However, both methods rely to different extents, on culture techniques which is time consuming. The proposed project aimed to reduce the time to one day. This would be achieved by combining the Dynabeads® anti-Salmonella IMS technique with an antibody detection method, within an ELISA based system (IMS-ELISA; Figure 1.15). Thereby, omitting the time dependent culture stage. The monoclonal antibody M105 has been reported to be specific for a salmonella core antigen (Tsang *et al.*, 1991a) and was provided by Dynal A/S laboratories (Oslo, Norway) as the detection antibody. It is generally believed that this 'Ra core' is common to all *Salmonella* serotypes (Kato 1993). Hence M105 was assumed to be genus specific and therefore an ideal candidate for the ELISA system.

A subsequent literature search revealed that another salmonella Ra core specific antibody T6 lacked reactivity with a number of *Salmonella* strains (Tsang and Schlecht 1990; Tsang *et al.*, 1991b). This lack of reactivity indicated that the Ra core structure was absent. Hence, the aims of the project became twofold. The primary concern of Dynal A/S was

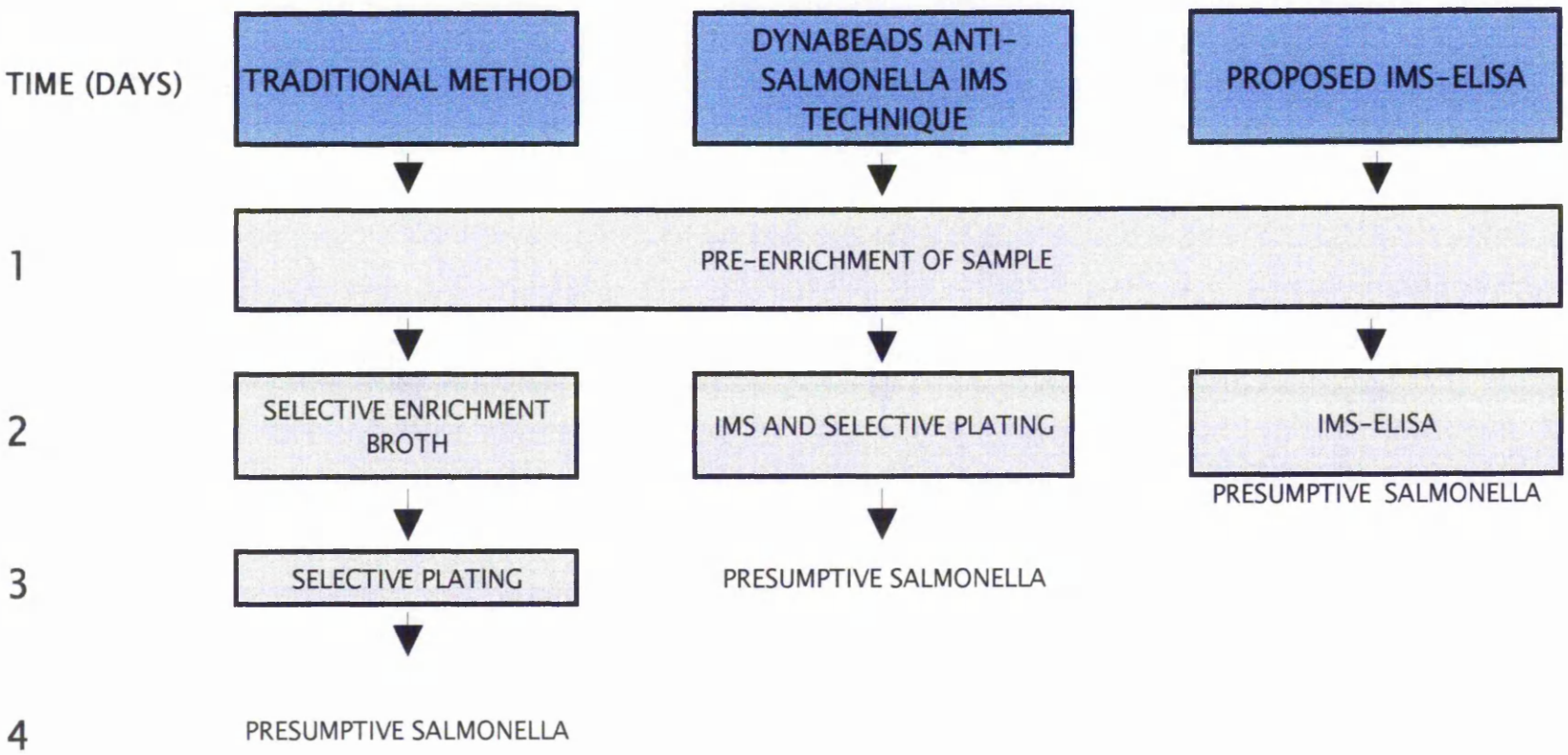


Figure 1.15: Comparison of traditional, Dynabeads anti-Salmonella IMS-selective plating and the proposed IMS-ELISA techniques for the isolation and detection of salmonella

the development and subsequent testing of an IMS-ELISA system. Additionally, if a non-Ra type or atypical core structures exist, the IMS-ELISA (based solely on M105 antibody detection) would be unable to detect all 2,400 *Salmonella* serotypes as hoped. Therefore, in addition and of equal importance to the project was to determine whether non-Ra type core structures exist. In order to achieve the aims of this project six stages were proposed (Figure 1.16).

The first stage of the project involved the confirmation of the M105 epitope site by use of rough mutant LPS by SDS-PAGE and immunoblot analysis. The second stage used dot blot analysis techniques to establish the presence of the M105 epitope in a range of *Salmonella* serotypes and serogroups. In addition a number of closely related *Enterobacteriaceae* were analysed to check for possible cross-reaction. Amplification of the antibody-antigen interaction was achieved by increasing epitope accessibility by heat and chemical treatment which was visualised by dot blot assay in stage three. Stage four involved the development and evaluation of an IMS-ELISA protocol for the isolation and detection of salmonella from foods. During this stage the monoclonal antibody M105 was purified and labelled with an enzyme. Antibody reactivity and specificity was monitored by dot blot assay, non denaturing PAGE and immunoblot assay. The next step was to develop and evaluate a detection method based on IMS and the monoclonal antibody M105 (IMS-ELISA). The final step involved a preliminary food trial which compared the IMS-ELISA salmonella isolation and detection protocol with traditional methods and was based on experience attained from previous food trials (Mansfield and Forsythe 1993; Mansfield and Forsythe 1996).

The possible presence of non-Ra type core structures was determined in stage five by observing the immunoblot assay reaction of the M105 antibody with SDS-PAGE differentiated LPS. Finally stage six involved the chemical analysis of LPS and the core of possible non-Ra type cores.

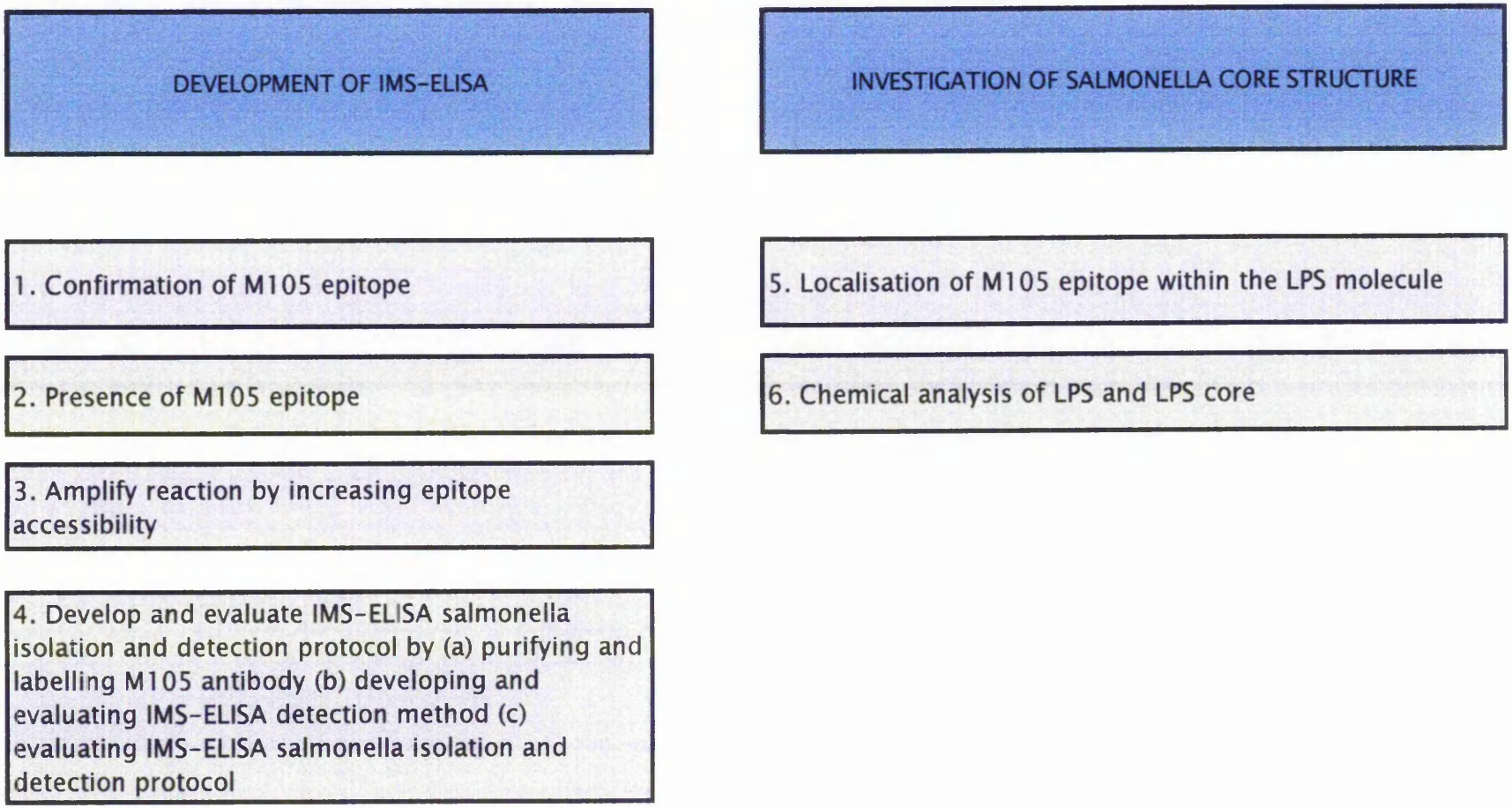


Figure 1.16: Stages of research project

CHAPTER TWO

MATERIALS AND METHODS

2 MATERIALS AND METHODS

2.1 BACTERIAL STRAINS

Organisms were obtained from the Nottingham Trent University culture collection, Dynal Research and Development laboratory (Norway), Dr Ian Poxton (The University of Edinburgh, UK), Dr Klaus Nielsen (Animal Diseases Research Institute of Agriculture Canada, Canada), British Analytical Control Ltd. (UK) and Campden Food and Research Ltd. (UK). The *Salmonella* and non-*Salmonella* strains studied are listed in Table 2.1a and 2.1b respectively.

2.2 PURE LPS PREPARATIONS FROM COMMERCIAL AND OTHER SOURCES

Pure lipopolysaccharide (LPS) was obtained from Sigma Chemicals (UK) or kindly donated by Dr Otto Holst, the Forschungs Zentrum (Germany). A list of the pure LPS preparations used in this study is shown in Table 2.2.

2.3 ANTIBODIES

Antibodies were obtained from Dr Klaus Nielsen (Animal Diseases Research Institute of Agriculture Canada, Canada), Zymed Laboratories Inc. (USA), Jackson Research Laboratories (USA), AmDex Laboratories (Denmark), Murex Diagnostics Ltd. (UK), Dako A/S. (Denmark), Sigma Chemicals (UK) and Dynal A/S (Norway). A list of the antibodies used is shown in Table 2.3.

2.4 PREPARATION OF MEDIA, REAGENTS AND AGAR PLATES

Media and reagents were in general sterilised by autoclaving at 121°C for 15min. The exceptions were Rappaport-Vassiliadis broth which was sterilised at 115°C for 15min and Xylose Lysine Desoxycholate agar which was heated to boiling and was not autoclaved. In addition the reagents used in the electrophoresis and staining of LPS and protein were not sterilised.

2.4.1 *BUFFERED PEPTONE WATER*

20g of buffered peptone water (BPW; Unipath, UK) was dissolved in 1000ml distilled water, dispensed into 9ml volumes and sterilised by autoclaving (15min, 121°C). The medium was stored at room temperature.

Table 2.1a *Salmonella* strains

Serogroup	Serotype	LPS type smooth (S)/ rough(R)	Source
B	*S.agona*	S	b
	S.bredeney	S	b
	S.gallinarum	S	c
	S.saint paul	S	b
	S.typhimurium	S	a
C_1	*S.infantis*	S	b
	S.livingstone	S	c
	S.virchow	S	a
C_2	*S.hadar*	S	b
	S.manchester	S	c
	S.newport	S	b
C_3	*S.kentucky*	S	c
D_1	*S.dublin*	S	d
	S.enteritidis	S	a
	S.panama	S	b
E_1	*S.give*	S	a
E_4	*S.senftenberg*	S	b
L	*S.minnesota*	S	c
O	*S.adelaide*	S	b
	S.ealing (NTU)	S	a
	S.ealing (Canada)	S	d
51[f]	*S.arizonae* (NTU)	S	a
	S.minnesota Ra mutant	Ra	e
	S.minnesota Rc mutant	Rc	e
	S.minnesota Rd_1 mutant	Rd_1	e
	S.minnesota Rd_2 mutant	Rd_2	e
	S.minnesota Re mutant	Re	e

a, Nottingham Trent University (NTU) culture collection strain;
b, provided by Dynal Research and Development laboratory (Norway);
c, provided by Campden Food and Research Ltd. (UK);
d, provided by Dr Klaus Nielsen (Animal Diseases Research Institute of Agriculture Canada, Canada);
e, provided by Dr Ian Poxton (The University of Edinburgh, UK);
[f], typed at the Public Health Laboratory Service, Colindale, London (51:z_4, z_{23} :-)

Table 2.1b Non-*Salmonella* strains

Enterobacteriaceae	Reference code	Source
Citrobacter freundii	NTU	a
	4/840b[d]	b
	4/841[d]	b
	4/1155[d]	c
	4001[d]	c
	4002[d]	c
	5/308[d]	b
	5/785[d]	b
Enterobacter agglomerans		a
Escherichia coli		a
Shigella flexneri	386	c

a, Nottingham Trent University (NTU) culture collection strain;
b, provided by British Analytical Control Ltd. (UK);
c, provided by Campden Food and Research Ltd. (UK);
[d], natural strains isolated from food sources

Table 2.2: Purified LPS type and source

Serogroup	Serotype	LPS type	Reference number	Source
B	*S.abortis equi*	S	L-5886	a
	S.typhimurium	S	L-6511	a
	S.typhimurium	S	1135	b
	S.typhimurium	S	C5	b
D	*S.enteritidis*	S	L-6001	a
	S.typhosa	S	L-6386	a
F	*S.arizonae* IIIa	S	1823	b
	S.arizonae IIIb	S	10455	b
G	*S.arizonae* IIIa	S	10466	b
K	*S.cerro*	S	10390	b
L	*S.minnesota*	S	L-6261	a
N	*S.godesberg*	S	1140	b
	S.godesberg	S	1431	b
O	*S.adelaide*	S	1464	b
U	*S.milwaukee*	S	1139	b
V	*S.nirembe*	S	1660	b
X	*S.bere*	S	10392	b
	S.bere	S	10427	b
Y	*S.djakarta*	S	10403	b
51	*S.arizonae* IIIa	S	1803	b
63	*S.arizonae* IIIa	S	10463	b
Rough	*S.typhimurium*	Ra	L-6016	a
	S.typhimurium	Rb_1	1780	b
	S.minnesota	Rb_2	1127	b
	S.typhimurium	Rc	L-5891	a
	S.typhimurium	Re	L-9511	a
011:B8	*E.coli*	S	L-2630	a

S, smooth (wild) type;
R, rough mutant type;
a, obtained from Sigma, UK;
b, donated by Dr Otto Holst, the Forschungs Zentrum, Germany

Table 2.3: Antibody specificity and source

Antibody (Reference code)	Specificity	Source[a]
M105[b]	Salmonella core region	Dr K.Nielsen
HRP labelled M105[b]	Salmonella core region	Zymed
	Salmonella core region	Jackson
	Salmonella core region	Amdex
Anti A-S antisera	Salmonella O-antigens serogroup A-S	Murex
Anti O antisera	Salmonella O-antigen serogroup O	Murex
AP labelled rabbit	Mouse IgG and IgM	Dako
HRP labelled goat	Mouse IgG and IgM	Dako
AP labelled goat	Rabbit IgG whole molecule	Sigma
HRP labelled sheep (MIC 13/43)	Mouse IgG	Dynal
HRP labelled donkey (MIC 13/133)	Mouse IgG	Dynal

[a], See Section 2.3 for address; [b], IgG_{2b} antibody; AP, alkaline phosphatase; HRP, horseradish peroxidase; M105 antibody was used in conjunction with AP and HRP labelled rabbit anti-mouse secondary antibodies; anti A-S and anti O antisera was used in conjunction with HRP labelled goat anti-rabbit secondary antibody

2.4.2 *RAPPAPORT-VASSILIADIS BROTH*

30g of dehydrated Rappaport-Vassiliadis broth (RV; Unipath, UK) was added to 1000ml distilled water, heated gently to dissolve, dispensed into 10ml volumes and sterilised by autoclaving (15min, 115°C). The medium was stored at room temperature.

2.4.3 *GLUCOSE NUTRIENT BROTH WITH NOVOBIOCIN*

39g of glucose nutrient broth (GN; Difco Laboratories, UK) was dissolved in 900ml distilled water, dispensed into 9ml volumes and sterilised by autoclaving (15min, 121°C). The medium was stored at room temperature.

Novobiocin (sodium salt; Sigma, UK) was dissolved in GN broth (100μg/ml) and filter sterilised (0.45μm Dynaguard filter) . The novobiocin solution was added to GN broth (1:9) to produce a 10μg/ml solution of novobiocin in GN (nGN) broth. An uninoculated control of nGN broth was incubated with test samples to check the sterility of the medium.

2.4.4 *XYLOSE LYSINE DESOXYCHOLATE*

Xylose lysine desoxycholate agar (XLD; Unipath, UK) was prepared by adding 53g of dehydrated media to 1000ml distilled water. This was heated with frequent agitation until the medium boiled (care was taken not to over heat the medium). The XLD agar was cooled to 50°C, mixed by gently swirling, before pouring approximately 25ml into 9cm plastic Petri dishes (Sterilin). The agar was left to set, after which the plates were dried in a plate drier for approximately 20min. Once dried the plates were stored in the dark at room temperature.

2.4.5 *TRYPTONE SOYA AGAR*

This was prepared by autoclaving 20g tryptone soya agar dehydrated media (TSA; Unipath, UK) in 500ml distilled water (121°C for 15min). The plates were poured, dried and stored in the same manner as the XLD plates.

2.5 MICROBIOLOGY

2.5.1 *CHEMICALS AND REAGENTS*

Buffered peptone water (BPW; Unipath, UK)

Hydrochloric acid - concentrated (HCl; Fisher Scientific, UK)

Sodium chloride (NaCl; Sigma, Germany; Fisher Scientific, UK)

Trizma base® - reagent grade (tris[hydroxymethyl]aminomethane, tris; Sigma, UK)

Tryptone (Difco, Germany)

Tryptone soya agar (TSA; Unipath, UK)

Yeast extract (Difco, Germany)

LB-medium

LB-medium was prepared by dissolving 5g yeast extract, 19g tryptone and 5g NaCl in 1000ml distilled water.

Tris buffered saline

Tris buffered saline (TBS) was prepared by dissolving tris (6.055g) and NaCl (11.688g) in 1000ml distilled water. The pH of the solution was adjusted to 7.4 with HCl.

2.5.2 *STORAGE AND MAINTAINANCE OF BACTERIAL CULTURES*

Bacterial strains were identified using API 20E biochemical tests (bioMérieux, UK). In addition *Salmonella* strains were differentiated into serogroups by serological tests (Wellcolex colour Salmonella, Murex Diagnostics Ltd., UK; Salmonella somatic agglutinating serum, polyvalent O A-S and 35-O; Murex Diagnostics Ltd., UK). Twice a year all strains were re-tested.

Master cultures of bacterial strains were stored on Protect Bacterial Preserver beads (STC Technical Service Consultants Ltd., UK). From solid cultures the colonies to be preserved were picked off using a sterile loop and mixed in the cryopreservation fluid to produce a thick suspension. The tube was capped, inverted six times and left to stand for 30 sec. Using a sterile pipette, as much fluid as possible was withdrawn and discarded. The cap was replaced and the vial was stored at -20°C.

Once every three months a single preserver bead from each master culture was streaked over a TSA plate and incubated at 37°C for 18-24h. The purity of the culture was checked by sub-culturing a single, well isolated colony onto a second TSA plate. From

this plate a single, well isolated colony was streaked onto a minimum of three TSA slopes and incubated at 37°C for 18-24h. TSA slopes were stored at 4°C. Bacterial cultures were sub-cultured onto TSA agar plates and incubated at 37°C for 18-24h when required.

2.5.3 *PROCEDURE FOR GROWING OVERNIGHT CULTURES*

All cultures with the exception of those used for LPS extraction and subsequent chemical analysis were prepared by the following method. A single well isolated colony was transferred from TSA plates (Section 2.5.2) into 10ml BPW. Cultures were incubated at 37°C for 18h under static conditions with the exception of those used for food trial experiments which were incubated for 48h. The bacteria were harvested in a bench centrifuge (MSE Centaur 1) at 2000xg for 10min. Harvested bacteria were washed once in 15ml TBS, pelleted by centrifugation as previous and resuspended in diluent to the required concentration.

For LPS extraction and subsequent chemical analysis cultures were grown in a fermentor according to the 'in-house' procedure of the Forschungs Zentrum, Borstel, Germany. Simply *S.arizonae* (NTU) and *S.ealing* (NTU) cultures were grown in LB-medium at 37°C under aerobic conditions for 16-18h.

2.5.4 *PROCEDURE FOR ADJUSTING CELL NUMBERS*

Washed cells were adjusted to an optical density reading at 600nm of 3.1 (equivalent to 10^9 cfu/ml) and resuspended in diluent (TBS, BPW or chemical treatment diluent). All adjusted cell levels were confirmed by plate count on TSA by the Miles and Misra technique (37°C, 24h).

2.6 CELL PRE-TREATMENT

2.6.1 *CHEMICALS AND REAGENTS*

Ethylene diamine tetra acetic acid (EDTA; BDH, UK)

Hydrochloric acid - concentrated (HCl; Fisher Scientific, UK)

Lauryl sulphate - reagent grade (Sodium dodecyl sulphate, SDS; Sigma, UK)

Phosphate buffered saline (PBS; Sigma, UK)

Sodium chloride (NaCl; Fisher Scientific, UK)

Trizma base® - reagent grade (tris[hydroxymethyl]aminomethane, tris; Sigma, UK)

Triton X-100 (t-octophenylpolyethoxyethanol; Sigma, UK)

Tris-EDTA solution

10mM tris (adjusted to pH 8.0 with HCl), with 10mM EDTA.

Tris-Triton solution

1% Triton X-100 in 10mM tris (adjusted to pH 8.0 with HCl).

Tris-EDTA-Triton solution

1% Triton X-100 in 10mM tris (adjusted to pH 8.0 with HCl), with 10mM EDTA.

TBS-SDS solution

4% SDS in TBS (prepared as described in Section 2.5.1).

TBS-EDTA solution

TBS (prepared as described in Section 2.5.1) was mixed with an equal volume of 0.1M EDTA solution (in PBS at pH 7.2). PBS was prepared thus: 1 PBS tablet was dissolved in 500ml distilled water according to the manufacturer's instructions.

SDS-EDTA solution

4% SDS in EDTA solution (prepared as above).

2.6.2 *PROTOCOL FOR CELL PRE-TREATMENT*

Cultures were grown in BPW as described in Section 2.5.2. Cell numbers were adjusted to 10^9 cfu/ml as described in Section 2.5.3. Cells (1ml) that were pre-treated by heat were resuspended in 100μl of TBS and incubated according to the conditions shown in Table 2.4. Cells (1ml) that were pre-treated by chemical means were resuspended in 100μl volumes of chemical treatment diluent (Section 2.6.1) and incubated according to the conditions shown in Table 2.5.

Table 2.4: Heat treatment methods for the pre-treatment of salmonella cells

Temperature (°C)	Time (min)
4	30
4	60
55	10
55	30
100	10
100	30

Table 2.5: Chemical treatment methods for the pre-treatment of salmonella cells

Resuspension solution[a]	Physical treatment	Temperature (°C)	Time (min)
Tris-EDTA-Triton[b]	S	37	5
Tris-EDTA	S	37	5
Tris-Triton	S	37	5
Tris-EDTA-Triton	S	55	5
Tris-EDTA	S	55	5
Tris-Triton	S	55	5
TBS	S	100	10
TBS-SDS	S	100	10
TBS-EDTA	S	100	10
SDS-EDTA	S	RT	10
TBS	S	RT	30
TBS-SDS	VS	RT	30
TBS-EDTA	VS	RT	30
SDS-EDTA	VS	RT	30
Distilled water	VS	RT	30

[a], See Section 2.6.1 for solution details;
[b], method used by Tsang *et al.*, 1987;
S, static;
VS, vigorous shaking using a Stuart Flask Shaker, Analytical Supplies Ltd., UK;
RT, room temperature

2.7 LPS EXTRACTION TECHNIQUE

2.7.1 *CHEMICALS AND REAGENTS*

Acetone (Sigma, Germany)

Diethylether (Sigma, Germany)

Ethanol (Sigma, Germany)

2.7.2 *PROTOCOL FOR THE EXTRACTION OF LPS FROM WHOLE CELLS*

S.arizonae (NTU) and *S.ealing* (NTU) cultures were grown in LB-medium according to the method described in Section 2.5.3. Bacteria were harvested by centrifugation (5000xg, 15min; 21g and 23g wet weight of *S.arizonae* and *S.ealing* respectively) and killed with ethanol (50ml; 18h at 4°C, stirring). Cells were decanted into centrifuge tubes and the ethanol removed following centrifugation (Hettich Rotixa/KS; 2,5000xg, 0°C, 10min). Acetone (25ml) was added and mixed for 20min to resuspend the cells. The acetone was removed as per ethanol. This step was repeated with a second quantity of acetone. Cells were then resuspended in a minimum volume of diethylether, mixed and centrifuged (2,500xg, -10°C, 5min). Following centrifugation the supernatant was discarded and the cells were allowed to air dry.

LPS was isolated by the hot phenol/water method of Galanos *et al.* (1969). Dried bacteria were resuspended in 100ml of 45% phenol in deionised distilled water (v:v) and heated to 68°C (with constant stirring). The temperature was maintained for 10min. After which the suspension was cooled to 4°C in an ice bath and the two layers were separated by centrifugation in a bench centrifuge (Hettich Rotixa/KS; 5000-10,000xg, 0°C, 30min). The water phase was removed, while the phenol layer was washed twice with deionised distilled water (50ml per wash). The three aqueous phases were combined and placed in dialysis tubing (16,000kDa). The water phase was dialysed against deionised distilled water at 4°C for 72h (with constant stirring and frequent changes of water). Following dialysis, the sample was rotary evaporated to reduce the volume by 50%. Finally the crude LPS was dried by lyophilisation.

The freeze dried crude LPS sample was resuspended in deionised distilled water to a final concentration of 50mg/ml and centrifuged in a Beckman L7-55 ultracentrifuge for 4h at 150,000 xg (4°C). The LPS sedimented as a characteristic clear, gelatinous pellet. Supernatant was removed leaving approximately 1ml which was sonicated for 10-20min. The LPS sediment was then washed twice with deionised distilled water (5 ml per wash).

Following a second ultracentrifugation (Beckman L7-55 ultracentrifuge for 4h at 150,000 xg and 4°C) LPS samples were lyophilised and stored at room temperature.

Purified LPS samples were dissolved in deionised distilled water to a final concentration of 5mg/ml and the purity of the preparation was assessed by detecting the UV spectra (200-300nm). LPS absorbs at 214nm, where as contaminating DNA and RNA would show a peak predominantly at 260nm.

2.8 DOT BLOT ASSAY

2.8.1 *CHEMICALS AND REAGENTS*

Albumin, bovine (BSA; Sigma, UK)

Bromo chloro indolyl phosphate (BCIP; Sigma, UK)

4-Chloro-1-naphthol (4-CN; Sigma, UK)

Dimethyl sulphoxide (DMSO; Sigma, UK)

Hydrochloric acid - concentrated (HCl; Fisher Scientific, UK)

Hydrogen peroxide - 30% (H_2O_2; Sigma, UK)

Methanol (BDH, UK)

Nitroblue tetrazolium (NBT; Sigma, UK)

Skimmed milk powder - dried (SMP; Tesco, UK)

Sodium chloride (NaCl; Fisher Scientific, UK)

Trizma base® - reagent grade (tris[hydroxymethyl]aminomethane, tris; Sigma, UK)

Tween 20 (polyoxyethylenesorbitan monolaurate; Sigma, UK)

Tris buffered saline

The tris buffered saline (TBS) solution was prepared as described in Section 2.5.1.

Tris buffered saline-Tween 20

Tris buffered saline-Tween 20 (TBS-T) solution was prepared by mixing Tween 20 (0.1%) with TBS (v/v).

Antibody diluent

Antibody diluent was prepared by dissolving 1% BSA in TBS or TBS-T (w/v)

Blocking solution

Blocking solution was prepared by dissolving 3% SMP in TBS-T.

BCIP/NBT substrate solution

A 33μl BCIP stock solution (50mg BCIP dissolved in 1ml DMSO; stored at 4°C) and 44μl NBT stock solution (75mg NBT dissolved in 1ml aqueous 70% DMSO (v/v); stored at 4°C) were added to 20ml substrate buffer (9.0825g tris in 100ml distilled water; pH adjusted to 9.5 with HCl), mixed and used immediately.

4-Chloronaphthol (4-CN) substrate solution

A 60μl aliquot of ICE COLD 30% H_2O_2 was added to 100ml TBS. This solution was mixed at room temperature with 60mg 4-CN pre-dissolved in 20ml methanol. The substrate solution was used immediately.

2.8.2 *PROTOCOL FOR THE DOT BLOT ASSAY*

The original protocol was from a procedure used by the Nottingham Trent University for protein analysis and was further developed as appropriate for the analysis of LPS.

The dot blot procedure was carried out in large polystyrene weigh boats (14x14cm size; Fisher Scientific, UK). However the weigh boats were not large enough to accomodate the dot blot. Therefore the nitrocellulose membrane was cut into two. Hence the volumes stated in the protocol below represent the volume of solution added to each weigh boat. The incubations were performed at room temperature unless otherwise stated. The washing and colour development steps were performed using a Titertek ELISA plate shaker (Level 4; Flow Laboratories, UK).

The dot blot procedure was performed using a SRC 96D Minifold 1 dot blotter (Schleicher and Schull, Anderman, UK) according to the manufacturer's instructions. The filter support plate was placed onto the vacuum filtrate chamber. Gel blotting paper (Schleicher and Schull, Anderman, UK) moistened with TBS buffer was placed on the filter plate. Nitrocellulose membrane (cellulose nitrate, 0.45 μm BA85, Schleicher and Schull, Anderman, UK) wetted in TBS buffer was placed on the filter paper. Then the sample well plate was placed on top. Finally the whole manifold was clamped together. The vacuum was turned on and a small amount of TBS buffer was poured into each well to ensure that the wells were clear. Volumes (50μl) of either LPS (0.1mg/ml) or treated bacteria (10^9 cfu/ml) were introduced. The vacuum was switched off when the filtration had finished. The membrane was removed using flat ended forceps and allowed to air dry (to fix antigen). Dot blots were usually developed within one day but could be stored at

-20°C until required for up to 60h with no adverse effects (determined by preliminary investigation). Antigen was detected either by M105 antibody and AP labelled secondary antibody or by HRP labelled M105 antibody.

Detection of antigen by the M105 antibody and AP labelled secondary antibody

The nitrocellulose membrane was re-wetted with TBS and incubated in 80ml blocking solution at 37°C for 30min. After blocking the unbound sites, the nitrocellulose was incubated with 40ml M105 antibody diluted in 1% BSA in TBS (a 1/1000 and 1/100 dilution for ascites and purified antibody respectively) at 37°C for 3h. Unbound M105 antibody was removed by washing thrice in 40ml TBS-T for 5min each wash. The nitrocellulose was then incubated with 40ml of a 1/1000 dilution of AP labelled rabbit anti-mouse antibody (Dako; diluted in 1% BSA in TBS-T) at 37°C for 2h. Unbound secondary antibody was removed by washing as previously described. In addition the nitrocellulose was rinsed with 80ml distilled water and equilibrated in 40ml substrate buffer. Colour was developed by incubating the nitrocellulose in the dark in 40ml BCIP-NBT substrate buffer solution for 8min. The reaction was stopped by extensive washing in distilled water. Once the dot blot had dried the relative colour intensity readings were taken using the red diode of a Nyco Card Reader (Nycomed Pharma, A/S, Norway).

Detection of antigen by HRP labelled M105 antibody

The nitrocellulose membrane was re-wetted with TBS and incubated in 80ml 3% SMP in TBS-T blocking solution at 37°C for 30min. After blocking the unbound sites, the nitrocellulose was incubated with 40ml of HRP labelled M105 (diluted in 1% BSA in TBS; the antibody concentration used was determined by titration) and incubated at 37°C for 3h. Unbound antibody was removed by washing thrice with 40ml TBS-T for 5min each wash and rinsing once in 40 ml distilled water. Colour was developed by incubating the nitrocellulose in 4-CN substrate solution for 30min. The reaction was stopped by immersing the membrane in 80ml distilled water for 10min with gentle agitation (with at least one change of water). Once the dot blot had dried the relative colour intensity readings were taken using the red diode of a Nyco Card Reader (Nycomed Pharma, A/S, Norway).

The reader should note that the relative colour intensity readings were expressed as a K/S value. This value was not defined in the Nyco Card Reader user manual. Moreover the manufacturer did not respond to requests by this researcher to clarify the issue.

2.9 IMMUNOBLOT ASSAY

2.9.1 *CHEMICALS AND REAGENTS*

The chemicals and reagents used were as for the dot blot assay (Section 2.8).

2.9.2 *PROTOCOL FOR THE IMMUNOBLOT ASSAY OF ELECTROPHORETICALLY TRANSFERRED LPS*

The protocol was adapted from the dot blot assay (Section 2.8.2). The volumes stated in the protocol below represent the volume of solution added to each weigh boat. The incubations were carried out at room temperature unless otherwise stated. The washing and colour development steps were performed using a Titertek ELISA plate shaker (Level 4; Flow Laboratories, UK).

LPS was electrophoretically transferred onto nitrocellulose membrane (cellulose nitrate, 0.45 μm BA85, Schleicher and Schull, Anderman, UK) by methods described in Section 2.14.2. Unbound sites on the nitrocellulose were blocked with 80ml 3% SMP in TBS-T at 37°C for 30min. The membrane was then incubated with 40ml primary antibody (M105 or serogroup O specific antisera diluted in 1% BSA in TBS and 1% BSA diluted in TBS-T respectively) at 4°C for 18h. The unbound primary antibody was removed by washing thrice in 40ml TBS-T for 5min each wash. The nitrocellulose was then incubated with 40ml of a 1/1000 dilution of AP labelled species specific antibody (diluted in 1% BSA in TBS-T) at 37°C for 2h. Unbound secondary antibody was removed by washing thrice as above, rinsing once with 80ml distilled water and equilibrated with 40ml substrate buffer. Colour was developed by incubating the nitrocellulose in 40ml BCIP-NBT substrate buffer solution for 8min (in the dark). The reaction was stopped by extensive washing in distilled water. The immunoblot was recorded on thermal printer paper (Sony, Type II, Upp-110 HD; high density printer paper; Ultraviolet Products, UK) by an Image analyser (Image Store 5000 Annotator 5.0; Ultra Violet Products, UK).

2.9.3 *PROTOCOL FOR THE IMMUNOBLOT ASSAY OF ELECTROPHORETICALLY TRANSFERRED HRP LABELLED M105 ANTIBODY*

The protocol was adapted from the dot blot assay as described in Section 2.9.2. Protein was electrophoretically transferred onto nitrocellulose membrane (cellulose nitrate, 0.45 μm BA85, Schleicher and Schull, Anderman, UK) by methods described in Section 2.14.3. The nitrocellulose was blocked with 80ml 3% SMP in TBS-T at 37°C for 30min. Excess blocking solution was removed by washing thrice with 40ml TBS-T for 5min each

wash and rinsing once in 80ml distilled water. Colour was developed by incubating the membrane in 4-CN substrate solution for 30min. The reaction was stopped by immersing the membrane in 80ml distilled water for 10min with gentle agitation (with at least one change of water).

2.10 IMMUNOMAGNETIC SEPARATION (IMS) TECHNIQUE

2.10.1 *CHEMICALS AND REAGENTS*

Phosphate buffered saline (PBS; Sigma, UK)

Tween 20 (polyoxyethylenesorbitan monolaurate; Sigma, UK)

Xylose lysine desoxycholate agar (XLD; Unipath, UK)

Phosphate buffered saline (PBS) washing buffer, pH 7.2

One PBS tablet was dissolved in 500ml distilled water according to the manufacturer's instructions. PBS washing buffer was prepared by mixing 0.05% Tween 20 in PBS (v/v).

2.10.2 *THE DYNABEADS® ANTI-SALMONELLA IMS PROTOCOL*

Salmonella cells were immunomagnetically separated with Dynabeads® anti-Salmonella (Dynal UK Ltd., UK) in accordance with the manufacturer's instructions. All stages were carried out at room temperature. Aliquots (1ml) of BPW pre-enriched food sample were mixed with 20μl of Dynabeads® anti-Salmonella in a 1.5ml plastic Eppendorf tube and incubated for 10min. Beads were separated using a magnetic particle concentrator (MPC®-M; Dynal UK Ltd., UK) for 3min. The beads were resuspended in 1ml PBS washing buffer, separated and resuspended in 50μl washing buffer. Aliquots (25μl) were loop streaked on duplicate XLD plates for overnight incubation at 37°C. This standard IMS technique is referred to as the 'IMS-XLD method'.

2.11 IMMUNOMAGNETIC SEPARATION ENZYME LINKED IMMUNOSORBENT ASSAY (IMS-ELISA)

2.11.1 *CHEMICALS AND REAGENTS*

Acetic acid (Fisons, UK)

Dimethyl sulphoxide (DMSO; Sigma, UK)

Hydrochloric acid - concentrated (HCl; Fisher Scientific, UK)

Hydrogen peroxide - 30% (H_2O_2; Sigma, UK)

Methanol (BDH, UK)

p-nitrophenylphosphate (pNPP; Sigma, UK)

Sodium acetate (BDH, UK)

Sulphuric acid (H_2SO_4; Fisher Scientific, UK)

3',3',5',5' tetramethylbenzidine (TMB; Sigma, UK)

Trizma base® - reagent grade (tris[hydroxymethyl]aminomethane, tris; Sigma, UK)

Tween 20 (polyoxyethylenesorbitan monolaurate; Sigma, UK)

Antibody diluent, blocking and washing solutions

As described in Section 2.8.1.

p-Nitrophenylphosphate (pNPP) substrate solution

The p-NPP substrate solution was prepared by dissolving 5mg of p-NPP in 5ml tris buffer (0.2M tris, pH 9.3). The substrate solution was used immmediately.

Tetramethylbenzidine substrate (TMB) solution

The TMB substrate solution was prepared fresh by mixing 20ml of sodium acetate solution (0.82g of sodium acetate dissolved in 100ml distilled water and adjusted to pH 6.0 with 0.1M acetic acid; stored at 4°C), 150μl TMB solution (10mg of TMB was dissolved in 1ml DMSO; stored at 4°C) and 8μl 30% H_2O_2 (4°C).

2.11.2 *PROTOCOL FOR THE IMS-ELISA*

The IMS-ELISA was carried out in flexible microtitre plates (96 well, flat bottomed; Falcon, Becton Dickinson, UK) to allow the application and removal of the magnetic particle concentrator (MPC®-96 Dynal UK Ltd., UK; Figure 1.9). The supernatant was removed after each incubation (with the exception of the substrate) and washing step. This was achieved by concentrating the Dynabeads® to the side of the well by application of the MPC®-96. All stages of the IMS-ELISA protocol were carried out at room

temperature. All incubation steps with the exception of the washing stages were carried out under shaking conditions (Level 4; Titertek ELISA shaker, Flow Laboratories, UK). All solutions were added using an Eppendorf Multipette® 4780 pipetter and Eppendorf Combitips®. Solutions were removed by using an eight channel multichannel pipetter (Titertek, Flow Laboratories, UK).

To eliminate binding of non-*Salmonella* strains to the ELISA tray wells 200μl/ well of 3% BSA in TBS blocking solution was added (30min). A 10μl aliquot of Prototype Enhanced Group E Dynabeads® (Prototype Dynabeads®, Dynal Olso, Norway) and 100μl of antigen were added to each well and incubated for 10min. Antigen comprised of either heat treated cells and heat released antigen (IMS-ELISA development tests and double bead IMS-ELISA protocol) or IMS captured heat released antigen (single bead IMS-ELISA preliminary food trial protocol). Unbound cells were removed by washing once with TBS-T (100μl/well). The bead-antigen complex was detected by a 1/100 dilution of 1mg/ml pure M105 antibody diluted in 1% BSA in TBS-T (30min; 100μl/ well). Non-bound M105 antibody was removed by washing thrice with TBS-T (100μl/well). The Prototype Dynabeads® were then incubated either with AP or HRP enzyme labelled antibody directed against the M105 antibody. AP labelled secondary antibody (option A) was used for the IMS-ELISA development and food trial tests. HRP labelled secondary antibody (option B) was used for investigations into the high background readings produced by the IMS-ELISA.

Option A - detection by AP labelled secondary antibody

The Prototype Dynabeads® were incubated with a 1/1000 dilution of AP labelled visualisation antibody (0.0018g/ml, diluted in 1% BSA in TBS-T) for 30min (100μl/well). Unbound antibody was removed by washing thrice with TBS-T (100μl/well) and rinsing once with tris buffer (100μl/well). Colour was developed by adding p-NPP substrate solution for 21min (175μl/well). The Prototype Dynabeads® were concentrated to the sides of the wells and the OD was read at 405nm immediately using Titertek Multiscan Plus reader (Flow Laboratories, UK).

Option B - detection by HRP labelled antibody

The Prototype Dynabeads® were incubated with a 1/1000 dilution of HRP labelled visualisation antibody (0.001g/ml, diluted in 1% BSA in TBS-T) for 30min (100μl/well). Unbound antibody was removed by washing thrice with TBS-T (100μl/well) and rinsing

once in distilled water (100μl/well). Colour was developed by adding TMB substrate solution (100μl/well) for 10min. The reaction was stopped by adding 2.5M H_2SO_4 (50μl/well). The Prototype Dynabeads® were concentrated to the sides of the wells and the OD was read at 450nm using Titertek Multiscan Plus reader (Flow Laboratories, UK).

2.12 POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

The routine protocol for the analysis of LPS by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was obtained from Dr Henrik Chart of the Public Health Laboratories (PHL), Colindale, London. Whereas the protocols of protein analysis by non-denaturing PAGE were taken from Current Protocols in Molecular Biology Volume 2 (Ausubel *et al.*, 1990).

2.12.1 *THE SOLUBILISATION TECHNIQUE*

2.12.1.1 *Chemicals and reagents*

Glycerol[1] (Sigma; UK)

Hydrochloric acid - concentrated (HCl; Fisher Scientific, UK)

2-Mercaptoethanol (Sigma, UK)

Proteinase K from tritirachium album (Sigma, UK)

Lauryl sulphate[1] (sodium dodecyl sulphate, SDS; Sigma, UK)

Bromophenol blue[1] (Sigma, UK)

Trizma base®[1] (tris[hydroxymethyl]aminomethane, tris; Sigma, UK)

[1], Electrophoresis grade

Solubilisation buffer for the analysis of pure LPS

The LPS solubilisation buffer was prepared by mixing 50ml of tris solution (0.757g tris dissolved in 80ml deionised distilled water; pH adjusted to 6.8 with HCl; final volume made up to 100ml with deionised distilled water), 10ml glycerol, 3g SDS, 5ml 2-mercaptoethanol and 0.01g bromophenol blue. The final volume of the solution was made up to 100ml with tris solution. The solubilisation buffer was distributed into 10ml volumes and stored at -20°C.

LPS sample buffer for the analysis of whole cells

A 2x concentrated LPS sample buffer was prepared by mixing 4ml glycerol, 5ml tris buffer (0.5M tris, pH 6.8), 8ml 10% SDS solution (w/v) and 2.6ml deionised distilled water. To this solution 10mg bromophenol blue was dissolved. The solution was

dispensed into 2.5ml aliquots and stored at -20°C. Prior to use 50μl 2-mercaptoethanol was mixed with a 2.5ml aliquot of 2x concentrated LPS sample buffer. Once the 2-mercaptoethanol was added the solution could be stored at 4°C but had to be used within a week.

LPS proteinase K solution for the analysis of whole cells

The LPS proteinase K solution was prepared by mixing 2.5mg proteinase K with 1ml 1x concentrated sample buffer (2x concentrated sample buffer mixed with an equal volume of deionised distilled water).

Protein sample buffer for the analysis of protein

A 2x concentrated protein sample buffer was prepared by mixing 25ml 4x concentrated tris buffer (6.05g tris in 40 ml deionised distilled water; pH adjusted to 6.8 with 1M HCl; total volume made up to 100ml with deionised distilled water; stored at 4°C), 20ml glycerol and 1mg bromophenol blue. The total volume was made up to 100ml with deionised distilled water. The 2x concentrated protein sample buffer was aliquoted into 1ml volumes and stored at -20°C.

2.12.1.2 ***Solubilisation procedure of pure LPS***

Samples of purified LPS were dissolved in deionised distilled water to a 1mg/ml concentration, mixed with an equal volume of LPS solubilisation buffer and boiled for 5min. Treated samples were cooled to room temperature, aliquoted into 10μl volumes and analysed or stored at -20°C until required. Frozen samples were warmed to room temperature and mixed using a whirlimixer and a hypodermic syringe before analysis. The volume of solubilised LPS which produced a clear banding profile was determined by titration.

2.12.1.3 ***Solubilisation procedure of LPS from whole cells***

Lipopolysaccharide extracts from bacteria were prepared by proteinase K digestion of whole cell lysates (Hitchcock and Brown 1983). A 3ml volume of overnight culture grown in BPW (Section 2.5.3) was centrifuged in a MSE Centaur 1 (2000 xg, 15min), resuspended in 100μl of 2x concentrated LPS sample buffer and boiled at 100°C for 10min. After which, 100μl of deionised distilled water was added, together with 40μl of proteinase K in 1x concentrated LPS sample buffer (2x concentrated sample buffer mixed with an equal volume of deionised distilled water). The resulting solution was mixed

thoroughly before incubating at 60°C for a minimum of 2h. Samples were then cooled to room temperature, mixed and analysed or aliquoted into 10μl volumes and stored at -20°C. Frozen samples were warmed to room temperature and mixed using a whirlimixer and a hypodermic syringe before analysis.

2.12.1.4 *Solubilisation procedure of protein*

Samples analysed by the non-denaturing method were diluted with 2x concentrated protein sample buffer for the analysis of proteins and analysed immediately.

2.12.2 *THE SDS-PAGE TECHNIQUE*

2.12.2.1 *Chemicals and reagents*

30% Acrylamide:bisacrylamide[1] (Sigma, UK)

Ammonium persulphate (Sigma, UK)

Hydrochloric acid - concentrated (HCl; Fisher Scientific, UK)

Glycine[1] (Sigma, UK)

Lauryl sulphate[1] (sodium dodecyl sulphate, SDS; Sigma, UK)

Temed[1] (Sigma, UK)

Trizma base®[1] (tris[hydroxymethyl]aminomethane, tris; Sigma, UK)

[1], Electrophoresis grade

Lower gel buffer for the SDS-PAGE of LPS

Tris (90.87g) was dissolved in approximately 400ml deionised distilled water and the pH was adjusted to 8.8 with concentrated HCl. The total volume was made up to 500ml with deionised distilled water. The solution was stored at 4°C.

Upper gel buffer for the SDS-PAGE of LPS

Tris (30.29g) was dissolved in approximately 400ml deionised distilled water. The pH was adjusted to 6.8. The final volume was made up to 500ml with deionised distilled water. The solution was stored at 4°C.

Running buffer number one for the SDS-PAGE of LPS

Tris (6.06g), glycine (28.80g) and SDS (2.00g) were dissolved in 1800ml of deionised distilled water. The pH was adjusted to 8.3 with HCl. The total volume was made up to 2000ml with deionised distilled water.

2.12.2.2 *Protocol for the SDS-PAGE of LPS*

Solubilised LPS was applied in 5-10μl volumes (determined by titration) onto gels comprising a 4.5% acrylamide stacking gel and a 12.5% acrylamide separation gel.

SDS was omitted from both the stacking and resolving gels since SDS in the gels' buffers reduced the transfer efficiency of LPS from gel to nitrocellulose membrane (Pyle and Schill 1985).

The gel mould apparatus used (Mini-Protean® II Electrophoresis Cell, Bio-Rad, UK) was assembled according to the manufacturer's instructions after the glass plates were cleaned successively with detergent, tap water, distilled water and ethanol. A 12.5% resolving gel was prepared by mixing 5ml lower gel buffer, 8.3ml 30% acrylamide:bisacrylamide solution and 6.7ml deionised distilled water. This solution was then degased for 5min. Temed (10μl) and 60μl of freshly prepared 10% ammonium persulphate solution (w/v) was added and mixed using a 10ml glass pipette. Care was taken to avoid the introduction of air. The solution was immediately poured into the gel mould to within 1cm of the bottom of the well comb. The resolving gel was overlayed with water saturated butanol to exclude air and produce a flat top to the gel. Polymerisation took up to 40min. Before applying the stacking gel, the saturated butanol was removed and the surface of the gel washed with deionised distilled water. Any excess water was absorbed with filter paper (Whatman Number 1). A 4.5% stacking gel was prepared by mixing 2.5ml of upper gel buffer, 1.5ml 30% acrylamide: bisacrylamide solution and 6.0ml deionised distilled water. This solution was then degased for 5min. Temed (10μl) and 30μl of freshly prepared 10% ammonium persulphate solution (w/v) was added and mixed using a 10ml glass pipette. Care was taken to avoid the introduction of air. The solution was immediately poured onto the resolving gel. A ten well comb was inserted into the stacking gel. Polymerisation took up to 20min, after which the comb was removed and gels were placed onto the inner cooling core. A 20μl aliquot of running buffer number one was pipetted into each well using a Hamilton syringe with a flat ended needle (Hamilton Microtitre 700 series syringe; Aldrich, UK). After which 5-10μl of solubilised LPS sample was pipetted into eight of the ten wells (the two outside well were not used). To do this the syringe was inserted to approximately 1-2mm from the well bottom. The inner cooling core was carefully lowered into the lower buffer chamber. Approximately 115ml of running buffer number one was added to the upper buffer chamber. The remainder was poured into the lower buffer chamber so that at least the bottom 1cm of gel was covered.

The gel was electrophoresed at 50mA using a PowerPac 300 power pack (Bio-Rad Laboratories, UK) until the tracking dye ran to within 1cm of the bottom of the gel (approximately 35min).

Following electrophoresis LPS gels were either fixed for subsequent carbohydrate silver staining, coomassie blue stained for contaminating proteins or used for electrotransfer. Electrotransferred gels were also carbohydrate silver stained to check the effectiveness of transfer.

2.12.3 *THE NON-DENATURING PAGE TECHNIQUE FOR THE ANALYSIS OF PROTEINS*

2.12.3.1 *Chemicals and reagents*

The chemicals used were as per LPS (Section 2.12.2).

Lower gel buffer for the non-denaturing PAGE of proteins

Tris (91g) was dissolved in 300ml deionised distilled water. The pH was adjusted to 8.8 with 1M HCl. The total volume was made up to 500ml with deionised distilled water. The solution was stored at 4°C.

Upper gel buffer for the non-denaturing PAGE of proteins

Tris (6.05g) was dissolved in 40 ml deionised distilled water. The pH was adjusted to 6.8 with 1M HCl. The total volume was made up to 100ml with deionised distilled water. The solution was stored at 4°C.

Running buffer number two for the non-denaturing PAGE of proteins

A 5x concentrated running buffer number two was prepared by dissolving tris (15.1g) and glycine (72.0g) in 700ml deionised distilled water. The final volume was made up to 1000ml. The working solution (pH 8.3) was prepared by diluting one volume of 5x concentrate with four volumes of deionised distilled water. The solution was stored at 4°C.

2.12.3.2 *Protocol for the non-denaturing PAGE of protein*

Gels were poured as per LPS protocol Section 2.12.2.2 with the exception different resolving and stacking gels were used. These were as follows: a 4.5% acrylamide stacking solution was prepared by mixing 1.25ml upper gel buffer for non denaturing

3.05ml of deionised distilled water
PAGE and 0.65ml 30% acrylamide:bisacrylamide. This solution was then degased for 5min. Temed (5μl) and 25μl of 10% ammonium persulphate solution (w/v) was added and mixed using a 10ml glass pipette. Care was taken to avoid introducing air. The solution was used immediately. A 7.5% acrylamide resolving gel was prepared by mixing 3.75ml lower gel buffer for non-denaturing PAGE, 3.75ml 30% acrylamide:bisacrylamide and 7.50ml deionised distilled water. This solution was then degased for 5min. Temed (10μl) and 50μl of 10% ammonium persulphate solution (w/v) was added and mixed using a 10ml glass pipette. Care was taken to avoid introducing air. The solution was used immediately.

A 10μl volume of non-denatured protein sample was pipetted into each well. Running buffer number two was used for the electrophoresis of protein samples. The gel was electrophoresed at 100V until the tracking dye ran to within 1cm of the bottom of the gel (approximately 1h 25min). Protein gels were either stained by coomassie blue protein stain or protein silver stain.

2.13 STAINING

2.13.1 *CARBOHYDRATE SILVER STAINING TECHNIQUE*

2.13.1.1 *Chemicals and reagents*

Acetic acid - glacial (Fisons, UK)
Ammonia (Fisons, UK)
Citric acid (trisodium, dihydrate; Sigma, UK)
Formaldehyde - 40% (BDH, UK)
Methanol - HPLC grade (BDH, UK)
Periodic acid - electrophoresis grade (Sigma, UK)
Silver nitrate (Sigma, UK)
Sodium hydroxide (NaOH; BDH, UK)

Silver stain fixing solution

Fixing solution was prepared by mixing 200ml of methanol with 25ml of glacial acetic acid. The final volume was made up to 500ml with deionised distilled water. The fixing solution was stored at room temperature and used within 2-3 days of preparation.

Silver stain oxidising solution

Periodic acid (0.75g) was dissolved in 100ml silver stain fixing solution. The oxidising

solution was made up fresh.

Silver staining solution

Immediately prior to use the silver staining solution was prepared as follows: 28ml of 0.1M NaOH (w/v) was placed into a dark reagent bottle with a magnetic stirrer. The solution was stirred continuously. A 2ml volume of ammonia was added. To this solution silver nitrate solution (1g silver nitrate in 5ml deionised distilled water) was added dropwise using a pasteur pipette. The final volume was made up to 150ml with deionised distilled water. The resulting silver staining solution was used within 5min of preparation. If solutions were too warm (i.e. ambient temperature above 22-25°C) a dark precipitate formed and the solution was discarded. Under these conditions all solutions were stored at 4°C prior to use.

Silver stain developer solution

Citric acid (50mg) was dissolved in 1000ml deionised distilled water. To this solution 0.5ml formaldehyde was added. The silver stain developer solution was made up fresh.

2.13.1.2 ***Protocol for the carbohydrate silver stain***

LPS profiles were detected by a modified Tsai and Frasch (1982) carbohydrate silver stain procedure (PHL, Colindale, London, UK). All stages of the protocol were carried out in large polystyrene weigh boats (14x14cm; Fisher Scientific, UK), at room temperature with gentle agitation using a Titertek ELISA shaker (Flow Laboratories, UK). The volumes stated represent the volume added to each weigh boat. The solutions were discarded after each incubation and washing step. A clean weigh boat was used after each last washing step to ensure minimum carry over of the oxidising, silver staining and developer solutions.

The gel was fixed in 100ml fixing solution for 18h, then incubated with 50ml oxidising solution for 5min. Oxidising solution was removed by washing thrice in 100ml deionised distilled water for 15min each wash. During the last washing step the silver staining solution was prepared. The gel was then silver stained (50ml) for 10min. Silver staining solution was removed by washing thrice in deionised distilled water for 15min each wash. During the last washing step silver stain developer solution was prepared. The LPS bands were visualised by incubating the gels in 100ml developing solution over a light source (light box, Whatman) for 2-5min. The reaction was stopped with copious amounts of

deionised distilled water. LPS profiles were recorded on thermal printer paper (Sony, Type II, Upp-110 HD; high density printer paper; Ultraviolet Products, UK) by Image analyser (Image Store 5000 Annotator 5.0; Ultra Violet Products, UK), by photograph or HP deskscan II (Hewlett Packard, Scanjet 3c). Scanned gels were further analysed by QuantiScan (BioSoft, UK).

2.13.2 *COOMASSIE BLUE PROTEIN STAIN*

2.13.2.1 *Chemicals and reagents*

Acetic acid - glacial (Fisons, UK)

Coomassie brilliant blue (Sigma, UK)

Methanol - HPLC grade (BDH, UK)

Coomassie blue fixing and staining solution

Coomassie brilliant blue (0.25g) was dissolved in 500ml methanol. To this solution 25ml glacial acetic acid was added and the final volume was made up to 1000ml with deionised distilled water. The stain was stored at room temperature.

Coomassie blue destain

A 10% acetic acid destain solution was prepared by mixing 100ml glacial acetic acid with 900ml deionised distilled water. The solution was stored at room temperature.

2.13.2.2 *Protocol for the Coomassie blue protein stain*

All stages of the protocol were carried out in large polystyrene weigh boats (14x14cm; Fisher Scientific, UK), at room temperature with gentle agitation (IKA Labortechnik, K250 basic shaker). The electrophoresed gels were incubated in 100ml coomassie blue stain for 1h until bands became visible. The stain was discarded and the gel was destained in 100ml 10% acetic acid (v/v) for 18h (with two solution changes). The gel was recorded on thermal printer paper (Sony, Type II, Upp-110 HD; high density printer paper; Ultraviolet Products, UK) by Image analyser (Image Store 5000 Annotator 5.0; Ultra Violet Products, UK).

2.13.3 *PROTEIN SILVER STAINING TECHNIQUE*

2.13.3.1 *Chemicals and reagents*

Acetic acid - glacial (Fisons, UK)

Ethanol - reagent grade (Hayman, UK)

Silver staining kit for acrylamide gels (AG-25 and AG-5; Sigma Chemicals, UK) consisted of silver concentrate (silver nitrate), developer 1 concentrate (sodium carbonate), developer 2 concentrate (formaldehyde), reducer A concentrate (potassium ferricyanide), reducer B concentrate (sodium thiosulphate) and reducer C concentrate (sodium carbonate).

Fixing solution

Fixing solution was prepared for the fixing process by adding 90ml of glacial acetic acid to 270ml of ethanol. The final concentration was made up to 900ml with deionised distilled water.

Silver equilibration solution

Silver concentrate (1.5ml) was diluted to 300ml with deionised distilled water.

Developer solution

Developer 1 concentrate (30ml) was diluted to 300ml with deionised distilled water. To this solution 0.17ml of developer 2 concentrate was added.

Stop solution

Glacial acetic acid (3ml) was diluted with 297ml deionised distilled water.

Reducer solution

Reducer concentrate A (2ml) was mixed together with 4ml of reducer concentrate B. To this solution 0.7ml of reducer concentrate C was added. The final volume was made up to 300ml with deionised distilled water.

2.13.3.2 *Protocol for the protein silver stain*

All steps were performed in large polystyrene weigh boats (14x14cm; Fisher Scientific, UK) at room temperature with gentle agitation using a IKA Labortechnik, KS 250 basic shaker. After removal from the gel electrophoresis chamber the gel was placed in 100ml fixing solution for 60min (during which the fixing solution was changed twice). The gel

was washed thrice with deionised distilled water for 10min each wash and equilibrated in silver stain equilibration solution for 30min. The equilibration solution was removed and rinsed for 10-20s with deionised distilled water. Half the 300ml volume of developer solution was placed over the gel. After 5-8min this solution was removed and the remaining developer solution was added. When the protein bands were dark enough the reaction was stopped by pouring off the developer solution and incubating the gel for 5min in stopping solution. Stopping solution was removed and the gel was washed thrice in deionised distilled water for 10min each wash. The gel was placed in reducer solution for 10-30s and rinsed immediately under tap water for 1min. Finally the gel was washed thrice with deionised distilled water for 5min each wash. The protein profiles were recorded immediately on thermal printer paper (Sony, Type II, Upp-110 HD; high density printer paper; Ultraviolet Products, UK) by Image analyser (Image Store 5000 Annotator 5.0; Ultra Violet Products, UK).

2.14 ELECTROTRANSFER

2.14.1 *CHEMICALS AND REAGENTS*

Glycine[1] (Sigma, UK)

Methanol - HPLC grade (BDH, UK)

Lauryl sulphate (sodium dodecyl sulphate, SDS; Sima, UK)

Sodium hydroxide (NaOH; BDH, UK)

Trizma base®[1] (tris[hydroxymethyl]aminomethane, tris; Sigma, UK)

[1], Electrophoresis grade

LPS transfer buffer

The electrotransfer buffer used was from Hancock and Poxton (1988). Tris (3g) was dissolved in a mixture of methanol (250ml) and deionised distilled water (1000ml). To this solution 14.42g of glycine was added. When necessary the pH was adjusted to 8.3 with 1M NaOH.

Protein electroblotting buffer

Tris (5.8g) was dissolved in a mixture of methanol (200ml) and deionised distilled water (800ml). To this solution 2.9g of glycine was dissolved followed by 0.37g SDS.

2.14.2 *THE ELECTROBLOTTING PROTOCOL FOR LPS*

Disposable gloves were worn throughout the electroblotting procedure. Electroblotting performed using a Trans-Blot® SD semi-dry transfer cell (Bio-Rad, UK) with a Mode 200/2.0 Power Supply power pack (Bio-Rad, UK). The graphite electrode of the transfer unit was soaked in deionised distilled water and the excess was removed with tissue paper. A total of 18 sheets of filter paper (3MM Chr chromatography paper, Whatman International Ltd., UK), cut to a size slightly larger than the gel, were required for the transfer unit. All filter paper sheets were soaked in transfer buffer. Nine pre-soaked filter papers were placed onto the anode electrode one at a time. Air bubbles were excluded by gently rolling a glass rod over the surface. A sheet of nitrocellulose membrane (cellulose nitrate, 0.45μm BA85, Schleicher and Schull, Anderman, UK) cut to the size of the filter papers was soaked in transfer buffer and placed on top of the filter papers. Any air bubbles were removed and the electrophoresed gel (pre-soaked in transfer buffer for 30min) placed onto the membrane. To complete the transfer unit a further nine pre-soaked filter papers were placed on the gel and the air excluded. The components were transblotted at 100mA constant current for 2h.

2.14.3 *THE ELECTROBLOTTING PROTOCOL FOR PROTEIN*

Protein samples from non-denaturing PAGE were transblotted onto nitrocellulose membrane as per LPS samples with the exception that protein electroblotting buffer was used and protein samples were run at 50mA constant current for 1h.

2.15 COMPOSITIONAL ANALYSIS TECHNIQUES

The compositional analysis of *S.arizonae* (NTU) and *S.ealing* (NTU) LPS and isolated polysaccharide was carried out under the supervision of Dr Otto Holst and Dr Simon Haseley at the Forschungs Zentrum, Borstel, Germany. *S.minnesota* rough mutant R345 LPS of known composition was used as a standard to monitor the compositional analysis techniques. All LPS samples were dissolved in deionised distilled water to a concentration of 5mg/ml prior to analysis.

2.15.1 *THE IDENTIFICATION AND QUANTITATIVE DETERMINATION OF URONIC ACID AND NEUTRAL SUGARS*

2.15.1.1 *Chemicals and reagents*

Acetic acid (Sigma, Germany)

Acetic anhydride (Sigma, Germany)

Chloroform ($CHCl_3$; Sigma, Germany)

Hydrochloric acid - concentrated (HCl; Merck, Germany)

Methanol (Merck, Germany)

Pyridine (Merck, Germany)

Sodium borohydride ($NaBH_4$; Merck, Germany)

Sodium hydroxide (NaOH; Sigma, Germany)

2.15.1.2 *Protocol for the identification and quantitative determination of uronic acid and neutral sugars*

The presence of uronic acid and neutral sugars (galactose, glucose, heptose, ribose) was determined as alditol acetate derivatives by the following method.

Step 1 - Methanolysis

A 100μl aliquot of 5mg/ml LPS (*S.minnesota* R345 LPS and test LPS from either *S.arizonae* or *S.ealing*) was dried in a desiccator for 1h, resuspended in 500μl 0.5M HCl (in methanol) and incubated at 85°C for 45min. After which the sample was evaporated, washed once with 20% methanol (v/v in deionised distilled water), once with methanol and then evaporated to dryness.

Step 2 - Carboxyl-reduction

A 500μl volume of $NaBD_4$ (3mg in 20% methanol) was added and the sample was incubated for 18h at 4°C. After warming to room temperature 1-2 drops 2M HCl was added. The sample was then evaporated, washed thrice with 5% acetic acid (v/v in methanol) and evaporated to dryness.

Step 3 - Hydrolysis

A 1ml volume of 0.1M HCl was added to either carboxyl-reduced sample or 100μl of a 5mg/ml *S.minnesota* R345 LPS or test LPS (pre-dried in a desiccator; for neutral sugar analysis only), mixed and incubated at 100°C for 48h. The LPS sample was cooled to room temperature and evaporated to dryness. At this stage a 30μl volume of an internal xylose standard was added (200mM final concentration).

Step 4 - Reduction

Deionised distilled water (500μl) was added to the LPS sample, followed by 1-2 drops of 1M NaOH. To this 2mg $NaBH_4$ in 0.3ml deionised distilled water was added and

incubated at room temperature for 4h. The sample was neutralised by adding 2M HCl dropwise until a pH of 6.0 was reached. The LPS sample was evaporated, washed thrice with 5% acetic acid (v/v in methanol) and evaporated to dryness. Acetic anhydride (200μl) was then added and the solution incubated at 85°C for 15min. After which the solution was dried with stream of nitrogen. Then 200μl pyridine and 100μl acetic anhydride was added and incubated at 85°C for 25min. The solution was dried for a second time under a stream of nitrogen. The sample was dissolved in 50μl chloroform and read on a Varian 3600 Gas Chromatograph with flame-ionisation detector and fused-silica capilary column (25m x 0.32 mm) with chemically bonded SE-54 (0.25μm, Weeke,Mühleim). The temperature programm was as follows: 140°C for 3min, a gradient of 3°C/min to 280°C and 280°C for 20min.

The GLC standards included rhamnose, ribose, arabinose, xylose, allose, mannose, glucose, galactose.

2.15.2 *THE IDENTIFICATION AND QUANTITATIVE DETERMINATION OF 3-DEOXY-D-MANNO-OCTO-2-ULOPYRANOSONIC ACID (KDO)*

Kdo was determined by the periodate-thiobarbituric acid assay described by Brade *et al.* (1983).

2.15.2.1 *Chemicals and reagents*

Dimethyl sulfoxide (DMSO; Sigma, Germany)
Hydrochloric acid - concentrated (HCl; Merck, Germany)
Sulphuric acid (H_2SO_4; Merck, Germany)
Periodic acid (H_5IO_6; Sigma, Germany)
Sodium acetate (Sigma, Germany)
Sodium arsenide ($NaAsO_2$; Sigma, Germany)
Thiobarbiturate (Sigma, Germany)

2.15.2.2 *Protocol for the identification and quantitative determination of total Kdo (Kdo-HCl)*

All Kdo linkages were cleaved by using strong acid hydrolysis by the following method. A 50μl volume of 2M HCl was added to 50μl of sample (10mM Kdo standard, 5mg/ml R345 LPS, or 5mg/ml test LPS). The resulting mixture was incubated at 100°C for 2h. The sample was cooled to room tempertature and tubes were set up as follows:

Blank (in triplicate)

Standard (in duplicate)..... 1,2,3,4 and 5μl

Sample..... 5,10 and 15μl

Deionised distilled water (250μl) was added to each tube and mixed. The periodate-thiobarbituric acid assay was carried out as follows: the hydrolysed LPS sample was oxidised by addition of 125μl of 40mM H_5IO_6 in 0.125N H_2SO_4 (room temperature for 30min). The reaction was stopped by adding 125μl of 0.2M $NaAsO_2$ in 0.5M HCl. Periodate was reduced by mixing (indicated by the disappearance of the yellow/brown colour). Freshly prepared 0.6% thiobarbituric acid solution (250μl; w/v) was added and incubated at 100°C for 15min. DMSO (500μl) was added to each tube while hot. After mixing and cooling to room temperature the pink colour was measured at 549nm using a Uvikon spectrophotometer against the reagent blanks.

2.15.2.3 *Protocol for the identification and quantitative determination of branching Kdo (Kdo-Ace)*

Mild acid hydrolysis was used to cleave the glucosidic linkages between Kdo residues by the following procedure. Using a 2mM Kdo standard, 5mg/ml R345 LPS or 5mg/ml test LPS tubes were set up tubes as follows:

Blank (in triplicate)

Standard (in duplicate)..... 2,4,6,8 and 10μl

Sample..... 2,4 and 8μl

A 250μl volume of 0.1M sodium acetate buffer at pH 4.4 was added to each tube and incubated at 100°C for 60min. Samples were cooled to room temperature and the periodate-thiobarbituric acid assay was carried out as previous.

2.15.3 *THE IDENTIFICATION AND QUANTITATIVE DETERMINATION OF AMINO SUGARS*

2.15.3.1 *Chemicals and reagents*

Acetic anhydride (Sigma, Germany)

Hydrochloric acid - concentrated (HCl; Merck, Germany)

p-dimethylaminobenzaldehyde (Sigma, Germany)

Potassium borate ($K_2B_4O_7$; Sigma, Germany)

Sodium bicarbonate ($NaHCO_3$; Merck, Germany)

Reagent A

Reagent A was prepared by mixing 10μl of $NaHCO_3$, 40μl of deionised distilled water and 10μl 5% acetic anhydride.

Morgan-Elson reagent (MER)

Morgan-Elson reagent was prepared by mixing two volumes of Morgan-Elson stock solution (16g of p-dimethylaminobenzaldehyde in 5ml of concentrated HCl; stored at 4°C) with five volumes of acetic acid. The solution was used immediately.

2.15.3.2 *Protocol for the identification and quantitative determination of glucosamine (HCl)*

Glucosamine was quantified using a modified Morgan-Elson reaction after hydrolysis (Reissig *et al.*, 1955). A 50μl volume of 8M HCl was added to 50μl of sample (5mg/ml R345 LPS or 5mg/ml test LPS) and incubated for 18h at 100°C. The samples were then cooled to room temperature and centrifuged to remove the solid fraction. The supernatant was used to set up the following tubes:

Blank (in triplicate)

Standard (5mM glucosamine, in duplicate)..... 2,4,6,8 and 10μl

Sample..... 5,10 and 15μl

Deionised distilled water (50μl) was added to each sample which was then dried in a vacuum desiccator for 1h. The step was then repeated.

The Morgan-Elson assay was performed as follows. The dried sample was reacted with 60μl of Reagent A for 10min at room temperature, mixed and incubated at 100°C for 3min. After cooling to room temperature 50μl of 5% $K_2B_4O_7$ was added and the sample was incubated at 100°C for 7min. The sample was cooled to room temperature, 700μl Morgan-Elson reagent was added and the sample incubated at 37°C for 20min. The solution was then mixed and the absorbance read at 585nm against the reagent blanks.

2.15.3.3 *Protocol for the identification and quantitative determination of reducing glucosamine*

Reducing glucosamine was determined by the Morgan-Elson assay (Reissig *et al.*, 1955) performed without hydrolysis. Using 5mM glucosamine standard, 5mg/ml R345 LPS or 5mg/ml test LPS tubes were set up as follows:

Blank (in triplicate)

Standard (in duplicate)..... 2,4,6,8 and 10μl

Sample..... 10,15 and 20μl

Samples were dried in a vacuum desiccator for 1h. The Morgan-Elson reaction was performed on the dried sample as previous.

2.15.4 *THE IDENTIFICATION AND QUANTITATIVE DETERMINATION OF FATTY ACIDS*

2.15.4.1 *Chemicals and reagents*

Methanol (Merck, Germany)

Hydrochloric acid - concentrated (HCl; Merck, Germany)

Chloroform ($CHCl_3$; Sigma, Germany)

Sodium sulphate (Na_2SO_4; Sigma, Germany)

Heptadeconoic acid (17:0; Merck, Germany)

Sodium methylate ($NaOCH_3$; prepared at the Forschungs Zentrum, Borstel, Germany)

Cyanamide (CH_2N_2; prepared at the Forschungs Zentrum, Borstel, Germany)

2.15.4.2 *Protocol for the identification and quantitative determination of total fatty acids*

Step 1 - Hydrolysis

A standard of heptadecanoic acid (17:0) solution (10μl of a 10mg/ml diluted in methanol) was mixed with $CHCl_3$ (100μl) and dried under a stream of nitrogen. A LPS sample was then added (40μl of 5mg/ml *S.minnesota* R345 or test LPS) and dried in a vacuum desiccator for 1h. Fatty acids were liberated by incubating the sample in 500μl 2M methanolic HCl at 120°C for 24h.

Step 2 - Extraction

The fatty acid derivatives (methyl esters) were extracted with $CHCl_3$ in the following procedure. The hydrolysed LPS was cooled to room temperature and further cooled under running tap water until cold. $CHCl_3$ (1ml) and deionised distilled water (4ml) were added, the solution was mixed and the $CHCl_3$ layer removed. Another 1ml of $CHCl_3$ was added, mixed and removed as previous. This step was repeated once more. The three $CHCl_3$ layers were combined and 200mg Na_2SO_4 was added to remove any water contamination. The combined organic phase was concentrated to 100μl under a stream of nitrogen. The fatty acid derivatives were analysed by GLC and GLC-MS (GLC using a Varian 3600 Gas Chromatograph with flame-ionisation detector and fused-silica capilary column [25m x 0.32 mm, Duraband, DB 1]; GLC-MS using a Hewlett-Parkard 5985

instrument, equiped with SE-54 capillary column [as above] and a HP-1000 data system). The temperature programme for the GLC was: 130°C for 5min, a gradient of 5°C/min to 250°C and 250°C for 10min.

2.15.4.3 *Protocol for the identification and quantitative determination of ester and amide bound fatty acids*

For selective release and transesterification of ester-bound fatty acids the procedure of Wollenweber and Riestchel (1990) was followed. Heptadecanoic acid (17:0) was used as a standard.

Step 1 - Hydrolysis

A 10μl aliquot of heptadecanoic acid (10mg/ml diluted in methanol) was mixed with $CHCl_3$ (100μl) and dried under a stream of nitrogen. Eighty μl of LPS sample (5mg/ml *S.minnesota* R345 or test LPS) was added and dried in a vacuum desiccator for 18h. The dried sample was then treated with 500μl 0.25M $NaOCH_3$ (w/v in absolute methanol) for 16h at 37°C.

Step 2 - Extraction

After addition of 5ml deionised distilled water the solution was acidified with 1M HCl (1ml). The methyl esters were extracted thrice with $CHCl_3$ (1ml) as per total fatty acids and the $CHCl_3$ layers were combined. The combined extracts were dried by the addition of Na_2SO_4 (200mg; 5min) and concentrated to 100μl under a stream of nitrogen. The ester bound fatty acids derivatives were analysed by GLC-MS as for total fatty acids.

Amide bound fatty acids

Hydrolysis and extraction steps were carried out as for ester bound fatty acids. Amide bound fatty acids were liberated by the addition of CH_2N_2 (2 drops) at room temperature for 5-10min. A stream of nitrogen was passed over the yellow solution until it turned clear and was then analysed by GLC-MS as for total fatty acids.

2.15.5 *THE IDENTIFICATION AND QUANTITAIVE DETERMINATION OF PHOSPHATE*

2.15.5.1 *Chemicals and reagents*

Ammonium molybdate (Sigma, germany)

Ascorbic acid (Sigma, Germany)

Sodium acetate (Sigma, Germany)

Sodium phosphate, dibasic (Na_2HPO_4; Merck, Germany)

Sulphuric acid - concentrated (H_2SO_4; Merck, Germany)

Perchloric acid ($HClO_4$; Sigma, Germany)

Hydrolysis reagent

Hydrolysis reagent was prepared by mixing deionised distilled water (62.7ml), H_2SO_4 (30.6ml) and 70% $HClO_4$ (6.7ml). The solution was stored at 4°C.

Ammonium molybdate reagent

Ammonium molybdate reagent was prepared by mixing 1M sodium acetate (1ml), 2.5% ammonium molybdate (1ml), deionised distilled water (7ml) and 10% ascorbic acid (1ml). The solution was made up fresh.

Reagent for inorganic phosphate analysis

Reagent for inorganic phosphate analysis was prepared at 4°C by mixing cooled 0.65M H_2SO_4 (5.4ml), 2.5% ammonium molybdate (0.6ml) and ascorbic acid (3.0ml). The solution was made up fresh.

2.15.5.2 *Protocol for the identification and quantitative determination of organic phosphate*

Using a phosphate standard of 5mM Na_2HPO_4 and 5mg/ml R345 LPS or 5mg/ml test LPS, tubes were set up as follows:

Blank (in triplicate)

Standard (in duplicate)..... 2,4,6,8 and 10μl

Sample..... 2,4 and 8μl

Samples were dried in a vacuum desiccator for 18h. To the dried sample 100μl of hydrolysis reagent was added. The solution was incubated at 100°C for 1h, mixed and incubated at 165°C for 2h. Samples were cooled to room temperature and the liberated inorganic phosphorus was reacted with ammonium molybdate reagent (1ml) at 37°C for 90min. The solution was re-mixed and the intensity of the resulting blue coloured complex was measured at 820nm against the reagent blanks.

2.15.5.3 *Protocol for the identification and quantitative determination of inorganic phosphate*

Inorganic phosphate was determined by the method of Lowry *et al.* (1954). Using a phosphate standard of 5mM Na_2HPO_4 standard and 5mg/ml R345 LPS and test LPS tubes were set up as follows:

Blank (in triplicate)

Standard (in duplicate)..... 2,4,6,8 and 10μl

Sample..... 5,10 and 15μl

The total volume of each sample was made up to 100μl with deionised distilled water. A 900μl volume of inorganic phosphate reagent was added and solution was incubated at 37°C for 30min. The solution was re-mixed and the intensity of the resulting blue coloured complex was measured at 820nm against the reagent blanks.

2.16 GEL PERMEATION CHROMATOGRAPHY

2.16.1 *CHEMICALS AND REAGENTS*

Acetic acid (Sigma, Germany)

Sodium chloride (NaCl; Sigma, Germany)

Desoxycholate (Sigma, Germany)

Ethylene diamine tetra acetic acid (EDTA; Sigma, Germany)

Sodium nitrite (NaN_3; Merck, Germany)

Pyridine (Merck, Germany)

Trizma base® (tris[hydroxymethyl]aminomethane, tris; Sigma, Germany)

Pyridine-acetic acid buffer

Pyridine-acetic acid buffer (pH 5.4) was prepared by mixing 4ml pyridine, 10ml acetic acid and 986ml deionised distilled water.

EDTA-desoxycholate buffer

Sodium chloride (11.688g), EDTA (1.211g), NaN_3 (0.2g) and tris (1.211g) were dissolved in 500ml deionised distilled water. Desoxycholate (2.5g) was added to 250ml deionised distilled water and heated to dissolve. The two solutions were mixed and the pH adjusted to 8.0 with 10M HCl. The final volume was made up to 1000ml with deionised distilled water.

2.16.2 *PROTOCOL FOR THE ISOLATION OF CORE OLIGOSACCHARIDE*

Cleavage of polysaccharide and lipid A was achieved by mild hydrolysis (10mg LPS in 1ml 1% acetic acid [1ml concentrated acetic acid: 99ml deionised distilled water] at 100°C, 1.5-2h). The solution was centrifuged to remove insoluble lipid A. The resulting supernatant was evaporated to dryness and re-dissolved in pyridine-acetic acid buffer. Samples were then fractionated on a Sephadex G-50 column (40cm by 2.5cm) at room temperature, in pyridine-acetic acid buffer, and eluted with the same buffer. Five ml volumes were collected. Absorption at 214nm was monitored over time. Fractions corresponding to the two main absorption peaks were pooled. Buffer was removed by dialysing (molecular weight: 12-14,000 kDal) against deionised distilled water (4°C for 3 days). Dialysed samples were rotary evaporated to concentrate, freeze dried and stored at room temperature.

2.16.3 *PROTOCOL FOR THE ISOLATION OF R-TYPE LPS*

The separation of long chain LPS and core was achieved using a modified Peterson and McGroarty (1985) gel permeation method. The separation was carried out on a Sephadex G50 column (120cm by 1.5cm) at room temperature in EDTA-desoxycholate buffer. LPS (10mg) was pre-dissolved in 1ml EDTA-desoxycholate buffer by heating to 60°C for 30min. This LPS solution was applied to the column and 5ml fractions were collected. Fraction samples corresponding to the peaks were combined, placed in dialysis tubing (molecular weight: 12-14,000 kDal) and dialysed against deionised distilled water at 4°C for 72h (stirring) to remove detergent and buffer. The deionised distilled water was changed twice daily. Following dialysis the fractionated LPS samples were concentrated by rotary evaporation, freeze dried and stored at room temperature.

2.17 THE ANTHRONE-SULPHURIC ACID TEST FOR NEUTRAL SUGARS

2.17.1 *CHEMICALS AND REAGENTS*

Anthrone (Sigma, Germany)

Sulphuric acid - concentrated (H_2SO_4; Merck, Geramny)

Anthrone-sulphuric acid reagent

Anthrone-sulphuric acid reagent was prepared by dissolving 200mg anthrone in 100ml concentrated H_2SO_4. To this solution 34.5ml deionised distilled water was carefully added.

2.17.2 *PROTOCOL FOR THE ANTHRONE-SULPHURIC ACID TEST*

A 100μl aliquot of fractionated LPS eluate sample was incubated with 900μl anthrone-sulphuric acid reagent at 100°C for 10min. The sample was cooled to room temperature, mixed and the absorbance read at 610nm.

2.18 ANTIBODY PURIFICATION

2.18.1 *CHEMICALS AND REAGENTS*

BioRad protein assay kit (BioRad, USA)

Bovine serum albumin (BSA; Sigma, USA)

Citric acid monohydrate (Merck, Germany)

Glycine (BioRad, USA)

Sodium azide (NaN_3; Merck, Germany)

Sodium bicarbonate ($NaHCO_3$; Merck, Germany)

Sodium chloride (NaCl; Merck, Germany)

Sodium hydroxide (NaOH; Prolabo, France)

Glycine buffer

Buffer was prepared by dissolving glycine (75g), NaCl (8.8g), NaN_3 (0.001ml) in 800ml deionised distilled water. The pH was adjusted to 8.6 with 5M NaOH. The total volume was made up to 1000ml with deionised distilled water.

Citrate buffer

Citrate buffers pH 3.0, 4.5 and 6.0 were prepared by dissolving 21g of citric acid in 800ml deionised distilled water. The pH of the buffers was achieved with 5M NaOH. The final volume was made up to 1000ml with deionised distilled water.

Dialysing solution

Dialysing solution was prepared by dissolving $NaHCO_3$ (8.4g) in 800ml deionised distilled water. The pH was adjusted to 9.5 with 2M NaOH. The final volume was made up to 1000ml with deionised distilled water.

2.18.2 *SAMPLE PREPARATION*

The ascities sample was prepared by a three step protocol. Firstly particulate matter was removed by centrifugation in a IEC Micromax® digiral microcentrifuge (Heigar Laboratories, Norway) at 9,300xg for 5min. Secondly fat and fibrigen was removed by

chemical pre-treatment (in-house procedure, Dynal A/S, Oslo, Norway). Finally the sample was diluted 50:50 (v/v) with glycine buffer.

2.18.3 *ISOLATION OF IgG_{2b} MONOCLONAL ANTIBODY*

M105 IgG_{2b} antibody was purified from ascites preparations on a protein A affinity chromatography column packed with Prosept™-A affinity absorbent (BioProcessing, UK). The column was equilibrated for 18h with glycine buffer. Sample was applied to the column and washed through with ten column volumes of glycine buffer. The column was washed successively with ten column volumes of citrate buffer at pH 6.0, 4.5 and 3.0. The IgG_{2b} antibody M105 was eluted with pH 3.0 citrate buffer. The column was re-equilibrated to pH 8.6 with ten column volumes of glycine buffer. Eluate fractions representing the IgG_{2b} antibody were combined and placed into dialysis tubing (Spectra/ Por molecular porous membrane tubing; molecular weight : 12-14,000 kDal). The antibody was dialysed at 4°C for 18h in dialysing solution with a minimum of two solution changes.

2.19 THE PROTEIN ASSAY TECHNIQUE

The concentration of the M105 antibody was determined by protein assay using the Bio-Rad protein assay kit. A standard curve was prepared using known concentrations of BSA (1.48mg/ml stock solution). The Bio-Rad solution was diluted with distilled water (20%, v/v). Before use the diluted reagent was filtered using Whatman Number 1 filter. To 5ml of diluted Bio-Rad reagent was added 100μl of each standard BSA concentration and incubated at room temperature according to the manufacturer's instructions. The OD at 595nm was read after 20min and a calibration curve plotted of the OD against protein concentration. Purified dialysed M105 antibody was similarly assayed using the Bio-Rad protein assay kit and the OD measured. From the standard curve the concentration of protein present in each sample was calculated.

2.20 THE HRP ENZYME ANTIBODY LABELLING TECHNIQUE

M105 antibody was labelled with HRP by a modified periodate activation process using the Zymed Actizyme-peroxidase kit (Zymed Laboratories Inc., USA). Additional samples were labelled by Jackson Research Laboratories (USA) and AmDex (Denmark) by undisclosed methods.

2.20.1 *CHEMICALS AND REAGENTS*

Zymed actizyme-peroxidase kit (Zymed Laboratories Inc., USA) consisting of three vials of activated enzyme and one vial of conjugate stabiliser.

Lysine (Sigma, USA)

Potassium phosphate, dibasic (KH_2PO_4; Merck, Germany)

Sodium bicarbonate ($NaHCO_3$; Merck, Germany)

Sodium chloride (NaCl; Merck, Germany)

Sodium hydroxide (NaOH; Merck, Germany)

Disodium hydrogen phosphate ($Na_2PO_4.7H_2O$; Merck, Germany)

Sodium bicarbonate ($NaHCO_3$) buffer

Buffer was prepared by dissolving 8.4g $NaHCO_3$ in 1000ml distilled water. The pH was adjusted to 9.5 with 2M NaOH.

Lysine solution

A 1M lysine solution was prepared by dissolving 18.27g lysine in 100ml distilled water.

Phosphate buffered saline (PBS), pH7.4

A 10mM PBS solution at pH 7.4 was prepared by dissolving KH_2PO_4 (0.26g), $Na_2PO_4.7H_2O$ (2.17g) and NaCl (8.71g) in 1000ml distilled water.

2.20.2 *THE ZYMED ACTIZYME-PEROXIDASE PROTOCOL*

The M105 antibody was dialysed extensively in sodium bicarbonate buffer (8h with 2 changes of buffer). The concentration of antibody was adjusted to 2mg/ml by using a Centricon-100, Amicon concentrator (Amicon division, W.R. Grace and Co, USA) according to the manufacturer's instructions (1000xg, 10min). A 1mg sample of M105 antibody was added to a vial of activated enzyme, mixed and incubated at room temperature for 3h (or overnight at 4°C). To block the remaining sites 200μl of lysine solution was added to the reaction mixture and incubated for 1h at room temperature. The conjugate was dialysed extensively with PBS (with at least 2 solution changes). Two parts stabiliser was added to one part conjugate. The conjugate was stored at 4°C.

2.21 PRELIMINARY FOOD TRIAL PROCEDURES

2.21.1 *CHEMICALS, MEDIA, REAGENTS AND FOOD TEST SAMPLES*

Skimmed milk powder (SMP; purchased at the local supermarket)

Chicken breast samples (fresh; purchased at the local supermarket)

Buffered peptone water (BPW; Unipath, UK)

Rappaport-Vassiliadis broth (RV; Unipath, UK)

Xylose lysine desoxycholate agar (XLD; Unipath, UK)

GN broth (Difco Laboraotories Ltd., UK)

Novobiocin - sodium salt (Sigma, UK)

Phosphate buffered saline (PBS; Sigma, UK)

Hydrochloric acid - concentrated (HCl; Fisher Scientific, UK)

Trizma base® - reagent grade (tris[hydroxymethyl]aminomethane, tris; Sigma, UK)

Sodium chloride (NaCl; Fisher Scientific, UK)

Tris buffered saline - Tween 20

A tris buffered saline - Tween 20 (TBS-T) solution was prepared as described in Section 2.8.1.

2.21.2 *THE SINGLE BEAD PROTOCOL FOR THE ISOLATION AND DETECTION OF SALMONELLA STRAINS FROM FOOD USING THE IMS-ELISA DETECTION TECHNIQUE*

Twenty-five gram samples of raw chicken breast (including skin) were weighed out into stomacher bags (4" x 6" Stomacher® Lab-Blender Mode 80 Bags; Seward Medical, UK). A 50ml volume of sterile BPW was added to each sample of chicken, homogenised in a stomacher (Seward Laboratory Blender, Stomacher 400; Fisher Scientific, UK) at high speed for 30s, added to 175ml BPW and mixed by swirling. Samples of SMP (25g) were added to 225ml BPW directly and mixed by swirling. Cultures were prepared as per Section 2.5.3. Cell numbers were adjusted by methods described in Section 2.5.4 and resuspended in BPW. Eighteen samples each of the chicken breast and SMP BPW pre-enrichment were artificially inoculated with approximately 1, 10 and 100 cells of *S.typhimurium*, *S.virchow*, *S.give*, *S.ealing* (NTU) and *S.arizonae* (NTU). Cell numbers were confirmed by plate count. Two samples of each food type were left as uninoculated controls. All forty food samples were then incubated at 37°C for 18h in accordance with traditional methods.

Following pre-enrichment aliquots of the supernatant were removed for further testing as follows:

(i) Traditional selective enrichment (RV-XLD) method

Aliquots (0.1ml) of BPW pre-enriched chicken and SMP samples were removed into RV broth (9.9ml) for traditional selective enrichment at 42°C. After 24 and 48h, a loopful was streaked onto XLD agar plates and incubated at 37°C for 18-24h.

(ii) Standard IMS-XLD method

Aliquots (1ml) of BPW pre-enriched chicken and SMP samples were removed for IMS as described in Section 2.10.

(iii) Single bead IMS protocol

Aliquots (1ml) of BPW pre-enriched chicken and SMP samples were transferred into pierced 1.5ml Eppendorf tubes and boiled at 100°C for 30min. The boiled samples were cooled to room temperature then incubated with 20μl Prototype Dynabeads® (Prototype Enhanced Group E Dynabeads®; Dynal A/S Olso, Norway) for 10min. The subsequent bead-antigen complex was washed in TBS-T (1ml), resuspended in 100μl TBS-T, divided into two and transferred to pre-blocked microtitre plate wells. The routine IMS-ELISA procedure was followed thereafter using AP-labelled secondary antibody (Section 2.11).

Where appropriate, presumptive salmonella colonies were confirmed by serology tests for O antigens using Wellcolex colour Salmonella (Murex Diagnostics Ltd., UK) and serogroup specific antisera (Salmonella somatic agglutinating serum, polyvalent O A-S and 35-O; Murex Diagnostics Ltd., UK) and biochemical tests (API 20E; bioMérieux Ltd., UK).

2.21.3 *THE DOUBLE BEAD (WITH ENRICHMENT) PROTOCOL FOR THE ISOLATION AND DETECTION OF SALMONELLA STRAINS FROM FOOD USING THE IMS-ELISA DETECTION TECHNIQUE*

The preliminary food trial using a double bead protocol was carried out whereby 25g samples of raw chicken breast (including skin) was analysed as per single bead protocol. BPW-raw chicken samples were artificially inoculated with approximately 1, 10 and 100 cells of *S.typhimurium*, *S.virchow*, *S.give*, *S.ealing* (NTU) and *S.arizonae* (NTU) and incubated in accordance with the traditional protocol in at 37°C for 18h. Aliquots of pre-enriched chicken samples were removed for further testing as follows:

(i) Traditional selective enrichment (RV-XLD) method

A 0.1ml aliquot was removed for traditional enrichment as described for the single bead protocol.

(ii) Standard IMS-XLD method

A 1ml aliquot was removed for IMS. The standard IMS protocol (Section 2.10) was followed with the exception that only 25μl was streaked onto XLD plates. The other 25μl was used for the double bead protocol described below.

(iii) Double bead (with enrichment) protocol with IMS-ELISA detection

A 25μl aliquot of IMS concentrated salmonella was inoculated into 1ml nGN broth and incubated at 42°C for 6h. Following this post-enrichment step, 100μl aliquots were removed and boiled for 30min in pierced Eppendorf tubes. Salmonella antigen was detected by IMS-ELISA as per Section 2.11. In addition a loopful of non-boiled nGN broth was streaked onto XLD agar plates and incubated for 18-24h at 37°C.

2.22 DATA ANALYSIS

Results were analysed using ANOVA (Microsoft Excel. Version 5.0).

The photographs contained within this thesis represent the best examples from the period of study. The photographs may have been taken on different dates with different film (colour or black and white). Therefore the SDS-PAGE profiles and immunoblots, in any one figure, may not be of equivalent size or colour.

CHAPTER THREE

RESULTS

3 RESULTS

A number of methods have been routinely used throughout the present study. In order to avoid repetition these are stated only once and are as follows: buffered peptone water (BPW) is recommended by the British Standards Institution (BSI) as the pre-enrichment step for the isolation of salmonella from foods (Figure 1.8). Hence all cells were grown in BPW as standard, unless otherwise stated, as per Section 2.5.3. Cell numbers were adjusted by the optical density (OD) method described in Section 2.5.4 in tris buffered saline (TBS), BPW or glucose nutrient broth with novobiocin (nGN broth) as specified. Confirmation of cell numbers was by the Miles and Misra plate count method on tryptone soya agar (TSA).

3.1 METHOD DEVELOPMENT

3.1.1 *DOT BLOT ASSAY*

A dot blot assay method was developed to optimise the reactivity between the M105 antibody and purified lipopolysaccharide (LPS) from *S.typhimurium* Ra mutant (L-6016). A procedure was devised which used circles of nitrocellulose membrane (Gelman Sciences BioTrace™ NT) held in the wells of flat bottomed microtitre plates (Sterilin). TBS wetted nitrocellulose membrane was cut into circles with a hole punch. One circle of nitrocellulose was placed at the bottom of each well. Antigen was applied with an automatic pipetter. Later a dot blot apparatus was purchased.

The original dot blot assay protocol was from Immutec (The Nottingham Trent University) and was as follows: after application of the antigen, the nitrocellulose membrane was blocked with 3% skimmed milk powder (SMP) in TBS-Tween 20 (TBS-T) for 30min at 37°C. The blocking solution was discarded and the antigen was detected by primary antibody diluted in 1% bovine serum albumin (BSA) in TBS-T (1-2h, room temperature). Unbound primary antibody was removed by washing in TBS-T (3x 20min). The nitrocellulose membrane was then incubated with enzyme labelled secondary antibody diluted in 1% BSA in TBS-T (1-2h, room temperature). Unbound secondary antibody was removed as per primary antibody. In addition the membrane was rinsed with distilled water and equilibrated in substrate buffer before incubation with enzyme substrate. This basic protocol was further developed as appropriate. A summary of the experimental parameters that were altered are shown in Table 3.1.

Table 3.1: Dot blot assay development conditions

Parameters		
Nitrocellulose	Type	Cellulose nitrate[a], BioTrace™ NT[b]
LPS	Volume applied per dot (μl/ml)	1, 2, 3, 4, 5, 50
Method of application		5x 1μl, 1x 5μl
Blocking solution		3% BSA in TBS-T, 3% SMP in TBS-T, 3% gelatin in TBS[c]
Antibody diluent		1% BSA in TBS-T, 1% BSA in TBS
Primary M105 antibody (Ascities)	Dilution	1/1000, 1/5000, 1/10,000, 1/20,000
	Incubation temperature (°C)	4, 30, 37
	Incubation time (min)	15, 30, 45, 60, 90, 120, 180, overnight
Secondary AP labelled antibody	Dilution	1/1000, 1/2,500, 1/5000, 1/10,000, 1/20,000
	Incubation temperature (°C)	4, 22-25, 37
	Incubation time (min)	15, 30, 45, 60, 90, 120, overnight
Washing solution		0.5% SDS in TBS-T, 1% BSA in TBS-T, 1% gelatin in TBS-T, TBS-T, PBS-T, TBS, PBS
Washing time (3x min)		2, 5, 10, 15, 20
Enzyme substrate		BCIP, BCIP/NBT, pNPP

[a], Schleicher and Schull; [b], Gelman Sciences; [c], Pyle and Schill 1985; LPS, lipopolysaccharide; AP, alkaline phosphatase; BSA, bovine serum albumin; TBS, tris buffered saline; TBS-T, TBS-Tween; SDS, sodium dodecyl sulphate; PBS, phosphate buffered saline; PBS-T, PBS-Tween; BCIP, bromo chloro indolyl phosphate; BCIP/NBT, BCIP/ nitroblue tetrazolium; pNPP, p-nitrophenylphosphate

A considerable length of time was spent at this stage which produced a large amount of data. Since the results were determined by a semi-quatitative method, where '+' indicated that a very feint dot was produced and '+++++' indicated that a very intense dot was produced, it is difficult to summarise the data as an 'average colour intensity'. Hence only the pertinent results are presented here as text.

LPS was dissolved in TBS to a concentration of 1mg/ml. This was the same concentration used by Tsang *et al.* (1991) in a dot blot radioimmunoassay with M105 antibody detection. A series of experiments were performed to establish the minimum volume of antigen required to generate reproducible intense dots. There was little difference in the intensity of the dots produced by 3-5μl volumes of antigen. However a 5μl volume was used in subsequent experiments so that the antigen was not limiting.

Two methods of application were compared: 1x 5μl and 5x 1μl. By concentrating the LPS onto a smaller area of membrane, using the 5x 1μl method, a more intense dot was produced. Since whole bacterial cells may have a limited amount of antigen exposed this method of enhancing the reaction was considered to be beneficial. Prior to antigen binding the nitrocellulose membrane was incubated with one of three blocking solutions (3% SMP in TBS-T, 3% BSA in TBS-T, 3% gelatin in TBS) to test their ability to block sites on the membrane. No dots were produced when 3% SMP in TBS-T and 3% BSA in TBS-T were used. SMP was chosen in preference to BSA simply because it was cheaper.

Different antibody diluents, dilutions, incubation time and temperatures were assessed (Table 3.1). The titration of the primary M105 antibody demonstrated that the 1/1000 dilution was the minimum dilution required to give intense, reproducible dots with relatively low non-specific binding. Previous researchers who have used the M105 antibody diluted it in 1% BSA in TBS with Tween (Tsang *et al.*, 1991a). However the results of the present study showed that addition of Tween 20 to the M105 antibody diluent markedly reduced the intensity of the dots. It was also noted that when 1% gelatin in TBS or TBS-T was used as the diluent no dots were produced. In summary the most intense dots were exhibited when blots were incubated with a 1/1000 dilution of the primary M105 antibody for 3h at 37°C or overnight at 4°C (static). In order to reduce the dot blot assay protocol time, blots were incubated at the higher temperature. The optimal working conditions for the secondary antibody against a 1/1000 dilution of M105 antibody was 1/1000 diluted in 1% BSA in TBS-T incubated at 37°C for 2h.

Seven different washing solutions were tested (Table 3.1). Washing blots with TBS-T produced the most intense dots with the lowest background colour. Addition of 0.05% Tween 20 to PBS or TBS resulted in more intense dots. However blots which were washed in 0.5% SDS in TBS-T resulted in very feint dots. This suggested that antigen was removed from the membrane by SDS.

The washing stages during preliminary experiments were 60 minutes in duration (3x 20min). Further tests were performed to check whether these prolonged washing times were necessary. Four additional washing times were examined (2, 5, 10, 15min) using TBS-T washing solution. A 15min wash (3x 5min) was the maximum time required. Longer washing periods produced dots that were notably feinter. Shorter washing times resulted in blots with increased background colour.

Three enzyme substrates were assessed (BCIP, BCIP/NBT, pNPP). Addition of NBT to BCIP enzyme substrate dramatically increased the intensity of the dots. Moreover substrate development time was reduced from 20min (BCIP) to 8min (BCIP/NBT). A series of experiments were performed using pNPP enzyme substrate. This substrate produces a soluble product which can be read quantitatively and is often used in enzyme linked immunosorbent assays (ELISAs). However this method of reading AP activity of dot blots resulted in unacceptably diverse absorbance readings between replicates and duplicate experiments. Hence BCIP/NBT substrate was used in all future dot blot assay experiments.

Further work was undertaken to ascertain the number of bacteria required to produce intense reproducible dots. *S.typhimurium* (serogroup B), *S.virchow* (serogroup C), *S.enteritidis* (serogroup D), *S.give* (serogroup E), *S.ealing* (NTU; serogroup O) and *S.arizonae* (NTU; serogroup 51) were grown and adjusted to 10^8, 10^9 and 10^{10} colony forming units (cfu)/ml in TBS. The bacterial suspensions were applied to the nitrocellulose membranes in 5x 1μl volumes. The 10^9 and 10^{10} cfu/ml volumes produced the most intense dots. The higher cell suspension was used throughout the dot blot assay experiments to ensure that antigen was not limiting.

A Schleicher and Schulls dot blot apparatus was purchased from Anderman, UK. Following which the performance of an alternative nitrocellulose membrane (cellulose nitrate; 0.45μl; BA85) was compared with the Gelman Sciences BioTrace™ NT

membrane. Fifty μl volumes of 0.1mg/ml *S.typhimurium* Ra mutant were applied to all 96 wells. The blots were assayed as standard (Section 2.8). Relative absorbance readings, expressed as a K/S value, were recorded using a Nyco Card Reader (see Section 2.8.2 for definition). The type and source of nitrocellulose significantly affected the intensity of the dots produced ($P<0.05$). The mean absorbance readings ($n=96$) were K/S 0.97 and 0.27 for Schleicher and Schulls cellulose nitrate and Gelman Sciences BioTrace™ NT respectively. Hence the cellulose nitrate membrane was used throughout this study. The final dot blot assay protocol for the M105 antibody is described in Section 2.8.

3.1.2 *THE ELECTROTRANSFER CONDITIONS FOR THE PARTIAL TRANSFER OF LPS FROM SDS-PAGE GEL TO NITROCELLULOSE MEMBRANE*

Electrotransferance was used to transfer LPS from polyacrylamide gel to nitrocellulose membrane for further analysis by immunoblot assay. To achieve this many researchers have adapted the protein transfer method of Towbin *et al.* (1979). These are based on wet transfer systems (Bradbury *et al.*, 1984; Vullo *et al.*, 1987; Chart 1995). In general, electrotransferance techniques result in the complete transferance of LPS from gel to nitrocellulose membrane. Therefore the comparison of LPS banding profiles of the immunoblot and gel are generally achieved by running duplicate gels. Pyle and Schill (1985) reported that misalignment of bands between duplicate gels is a frequent problem. Therefore they developed a method, based on a wet blotter, which resulted in the partial transfer of LPS profiles and effected the uniform representation of all bands. Hence the immunoblot and electrotransferred gel could be compared directly. In the present study a wet blotter was not available. Instead a semi-dry system was used. Therefore studies were carried out to assess the conditions required for partial transfer of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) differentiated LPS by a semi-dry system.

The electrotransfer procedure was adapted from a method routinely used for proteins with the exception of the transfer buffer which was replaced by that described by Hancock and Poxton (1988).

Purified LPS from wild type *S.typhimurium* (1mg/ml in deionised distilled water) was solubilised (Section 2.12.1.2) and electrophoresed (Section 2.12.2.2). Electrotransfer was performed using a semi-dry transfer system as per Section 2.14 with the exception that the electrotransfer conditions were replaced by those summarised in Table 3.2. In addition

a second sheet of nitrocellulose membrane was positioned under the first. The electrophoretically transferred LPS was detected with the M105 antibody by the immunoblot method described in Section 2.9.2.

Table 3.2: The electrotransfer conditions tested to effect the partial transfer of SDS-PAGE differentiated purified *S.typhimurium* LPS

Electrotransfer	
Current (mA)	**Time (h)**
45[a]	1[a]
100	1
100	2
200	1

[a], electrotransfer conditions for protein mini gels

The electrotransfer conditions used for the transfer of proteins from mini-gel to nitrocellulose membrane (45mA, 1h) was ineffective at transferring LPS (Lane 2, Figure 3.1). Hence the current and transfer time were extended. A silver stained SDS-PAGE differentiated LPS profile has been included for comparative purposes (Lane 1, Figure 3.1).

The electrotransfer conditions of 100mA for 2h produced the most well defined bands with the least amount of background interference (Lane 5, Figure 3.1). No LPS was present on the backup sheet of nitrocellulose. The lack of reaction at the very top of the blot may be due to poor transferance of very long chain LPS or may simply reflect the lack of M105 antibody reactivity caused by steric interference of the very long O-chains. This was tested by reacting the blots with O-chain specific antisera (anti-serogroup A-S antisera; Table 2.3). The dilutions of primary (1/1000) and secondary (1/1000; AP labelled goat anti-rabbit; Table 2.3) antibodies were determined by titration. It can be seen from Figure 3.2 that the very long chain LPS was detected. From these results it can be concluded that the lack of M105 antibody reactivity was probably due to steric hindrance of very long O-chains.

The partial transferance of LPS was assessed by silver staining the gel after it was used for electrotransferance (Section 2.13.1). Figure 3.3 shows the silver stained gel of

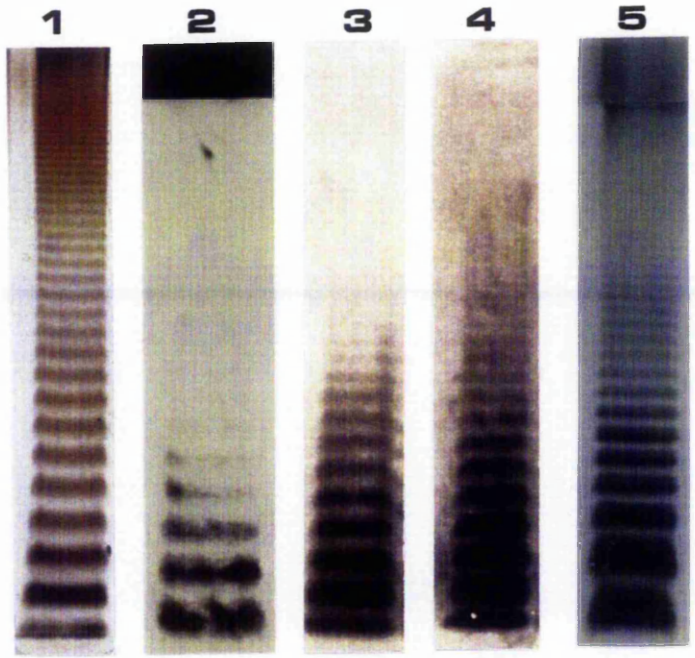


Figure 3.1: The silver stained profile of SDS-PAGE differentiated purified *S.typhimurium* LPS and immunoblots, as detected by M105 antibody, following alternative electrotransfer conditions

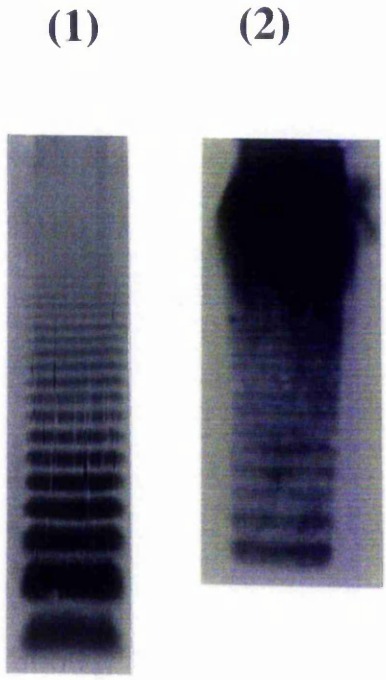


Figure 3.2: Immunoblot of *S.typhimurium* as detected by M105 antibody (Lane 1) and O-chain specific antisera (Lane 2) (from Bire 1997)

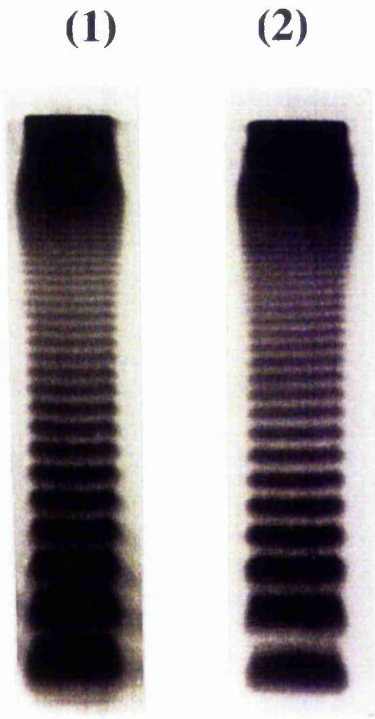


Figure 3.3: Silver stained LPS profiles of *S.typhimurium* without (Lane 1) and after (Lane 2) electrotransferance

S.typhimurium LPS (PK digest) after electrotransferance (Lane 2; 100mA, 2h) compared with a silver stained gel which was not electrotransferred (Lane 1). Clearly a significant portion of LPS remained in the gel.

3.2 DETERMINATION OF THE M105 EPITOPE SITE

The LPS from rough mutant strains of *S.typhimurium* and *S.minnesota* were solubilised and separated by SDS-PAGE as per Section 2.12, electrophoretically transferred onto nitrocellulose membrane (Section 2.14) and reacted with the M105 antibody by immunoblot assay (Section 2.9.2). The M105 antibody reacted with Ra and Rb_1 type mutant LPS only (Table 3.3). The reaction band produced by Rb_1 type LPS was feinter than that produced by Ra type LPS.

Table 3.3: Immunoblot reaction of purified LPS from rough mutant *Salmonella* strains with the M105 antibody

Serotype	LPS type	Reaction with M105
S.typhimurium	Ra	+
	Rb_1	+[a]
	Rc	-
	Re	-
S.minnesota	Ra	+
	Rb_2	-
	Rc	-
	Rd_1	-
	Rd_2	-
	Re	-

[a], feinter than Ra

3.3 PRESENCE OF THE M105 EPITOPE

The dot blot assay technique (Section 2.8) was used to determine the presence of the M105 epitope in a range of whole cell salmonellas, non-salmonellas and purified LPS. Bacteria were prepared and adjusted to a cell density of 10^9 cfu/ml in TBS as standard (*ca* $5x10^7$ cfu/dot). Purified LPS was diluted in TBS to 0.1mg/ml (0.5μg/dot). *S.typhimurium* Ra mutant LPS was run as a positive control. Relative absorbance readings were recorded

using a Nycocard Reader and were expressed as K/S values.

The M105 antibody reacted with the whole cells of all 22 *Salmonella* strains tested. A range of reactivity was expressed from K/S value 0.15 - 2.94 (Table 3.9). Ten *Salmonella* strains gave readings above K/S 1.0 including *S.agona* (K/S 2.94), *S.enteritidis* (K/S 2.55) and *S.senftenberg* (K/S 2.10). The lowest readings (K/S <0.30) were produced by *S.livingstone* (K/S 0.25), *S.adelaide* (K/S 0.17), *S.ealing* (NTU; K/S 0.15), *S.ealing* (Canada; K/S 0.17) and *S.arizonae* (NTU; K/S 0.27). Purified *S.minnesota* LPS did not react with the M105 antibody, although purified LPS from *S.abortis equi*, *S.enteritidis*, *S.typhosa* and *S.typhimurium* did (Figure 3.4).

A low level of reactivity was expressed by the whole cells of all non-*Salmonella* strains (Table 3.10). *Enterobacter agglomerans* produced the strongest reaction of K/S 0.27. The weakest was produced by *Shigella flexneri* at K/S 0.02. Although whole cell *E.coli* reacted with the M105 antibody (K/S 0.13), there was no reaction with purified *E.coli* LPS (Figure 3.4, Row 6).

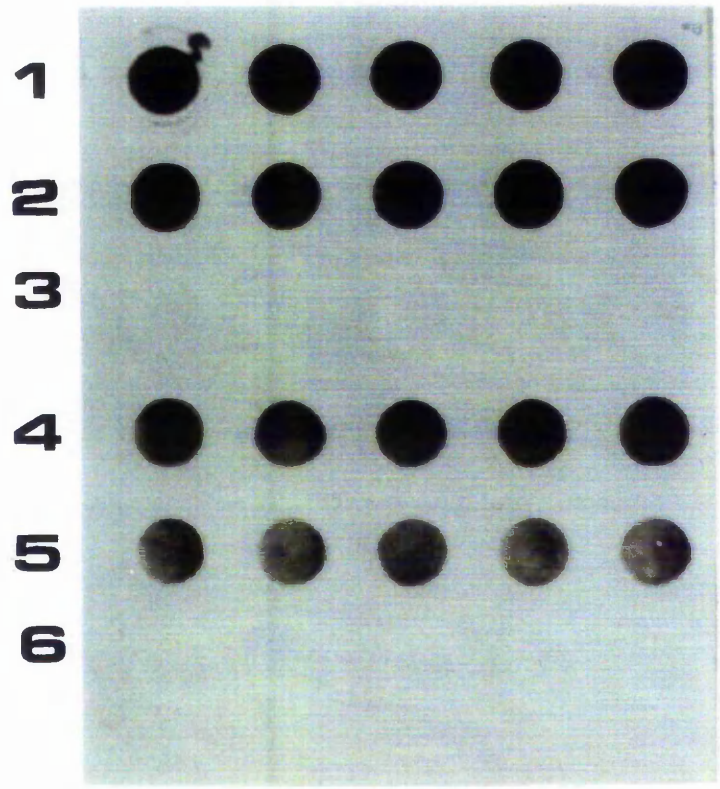


Figure 3.4: Dot blot reactions with purified LPS (0.5μg/dot) from wild type *S.abortis equi* (Row 1), *S.enteritidis* (Row 2), *S.minnesota* (Row 3), *S.typhosa* (Row 4), *S.typhimurium* (Row 5) and *E.coli* (Row 6)

3.4 AMPLIFICATION OF THE M105 REACTION

Preliminary experiments demonstrated that untreated *S.ealing* (NTU) and *S.arizonae* (NTU) cells had a weak reaction with the M105 antibody in the dot blot assay (Table 3.9). It is plausible that a large proportion of the M105 antibody reactive core structures of these weakly reactive strains were inaccessible to antibody binding. A number of reports have described that heat and chemical treatment leads to increased exposure of cryptic core epitopes (Leive *et al.*, 1968; Tsuchido *et al.*, 1985; Blais and Yamazaki 1989a). Hence *S.ealing* (NTU) and *S.arizonae* (NTU) were exposed to a variety of heat, chemical and physical treatments prior to analysis by dot blot assay in an attempt to increase the reaction with the M105 antibody. *S.typhimurium*, *S.virchow*, *S.enteritidis* and *S.give* which represent serogroups B, C, D and E respectively were also tested to observe the effects on strongly M105 antibody reactive serotypes. Cells were grown in BPW as standard, adjusted to a cell density of 10^9 cfu/ml and pre-treated by heat and chemical methods as described in Section 2.6. M105 antibody reactivity of heat and chemically pre-treated cells were analysed by dot blot assay (Section 2.8).

In order to clarify results the mean relative colour intensity readings ($\pm$standard deviation) are presented in four tables. These show the effect of combinations of tris, ethylene diamine tetra acetic acid (EDTA) and Triton X-100 solutions (Table 3.4), the effect of boiling cells in different chemical solutions (Table 3.5), the effect of shaking cells in different chemical solutions (Table 3.6) and the effect of temperature (Table 3.7) on the reactivity of six *Salmonella* strains. Although treatment significantly affected the intensity of the dots produced ($P<0.05$), the extent of the change caused by a single treatment was dependent upon strain type ($P<0.05$). Hence statistical analysis (least significant difference) was carried out on individual strains to ascertain the significance of treatments (See Tables 3.4-3.7). More general comments on the treatments are given below (Sections 3.4.1-3.4.4). A summary of the results with respect to each *Salmonella* strain in given in Section 3.4.5.

3.4.1 *THE EFFECT OF COMBINATIONS OF TRIS, EDTA AND TRITON X-100*

Any treatment of *S.typhimurium* resulted in a significant decrease in the relative colour intensity of the blots ($P<0.001$). Increasing the incubation temperature from 37°C to 55°C lead to less intense dots regardless of treatment. Addition of Triton X-100 produced dots of reduced colour intensity, which was exaggerated by increasing the incubation temperature. Treatment with tris+EDTA solution at 37°C resulted in increased reactivity

Table 3.4: Dot blot assay to show the effect of combinations of tris (T), EDTA (E) and Triton X-100 (X) on the M105 antibody reactivity with six *Salmonella* strains

		Treatment temperature (°C)					
		37			55		
		Chemical treatment			Chemical treatment		
Serotype (Source)[a]	Untreated	T+E+X[b]	T+X	T+E	T+E+X	T+X	T+E
S.typhimurium	4.40±0.66	1.07±0.72 (---)	2.19±0.88 (---)	2.13±0.68 (---)	0.97±0.47 (---)	0.75±0.42 (---)	1.06±0.23 (---)
S.virchow	5.37±1.08	3.72±0.29 (--)	5.09±0.19 (nsd)	6.37±0.65 (+)	2.14±1.25 (---)	4.16±0.73 (-)	4.62±0.48 (nsd)
S.enteritidis	2.08±0.26	2.87±0.26 (+++)	2.57±0.28 (+)	2.70±0.25 (++)	2.07±0.36 (nsd)	1.91±0.86 (nsd)	2.57±0.27 (+)
S.give	4.12±0.83	3.70±0.64 (nsd)	4.06±0.40 (nsd)	4.49±0.65 (nsd)	2.51±0.08 (---)	3.16±0.50 (-)	2.86±0.64 (--)
S.ealing (NTU)	0.08±0.06	0.02±0.01 (nsd)	0.05±0.04 (nsd)	0.29±0.10 (+++)	0.03±0.01 (nsd)	0.02±0.01 (nsd)	0.07±0.07 (nsd)
S.arizonae (NTU)	0.35±0.23	0.10±0.06 (--)	0.21±0.06 (nsd)	0.81±0.13 (+++)	0.19±0.06 (-)	0.03±0.01 (---)	0.52±0.21 (nsd)

[a], *ca* $5x10^7$ cfu/dot; [b], method used by Tsang *et al.*, 1987; mean colour intensity reading of 5 replicates ±standard deviation; least significant difference is noted in brackets where +, ++, and +++ denotes a significant increase in colour intensity compared to untreated cells at $P<0.05$, $P<0.01$ and $P<0.001$ respectively; and -, --, and --- denotes a significant decrease in colour intensity to untreated cells at $P<0.05$, $P<0.01$ and $P<0.001$ respectively; nsd, not significantly different ($P>0.05$)

Table 3.5: Dot blot assay to show the effect of boiling cells for 10min in different chemicals on the M105 antibody reactivity with six *Salmonella* strains

		Chemical treatment			
Serotype (Source)[a]	**Untreated**	**TBS**	**TBS+SDS**	**TBS+EDTA[b]**	**SDS+EDTA**
S.typhimurium	4.40±0.66	2.85±0.14 (---)	0.41±0.06 (---)	3.03±0.40 (---)	0.96±0.06 (---)
S.virchow	5.37±1.08	2.85±1.59 (---)	2.87±0.50 (---)	5.16±0.52 (nsd)	1.95±0.57 (---)
S.enteritidis	2.08±0.26	2.77±0.30 (+++)	2.35±0.23 (nsd)	2.71±0.25 (+++)	1.02±0.16 (---)
S.give	4.12±0.83	3.84±0.33 (nsd)	3.53±0.23 (nsd)	4.16±0.48 (nsd)	1.47±0.27 (---)
S.ealing (NTU)	0.08±0.06	0.40±0.09 (+++)	0.17±0.08 (nsd)	0.44±0.12 (+++)	0.03±0.01 (nsd)
S.arizonae (NTU)	0.36±0.23	0.77±0.19 (+)	0.93±0.14 (++)	0.96±0.52 (++)	0.11±0.02 (++)

[a], *ca* $5x10^7$ cfu/dot; [b], method used by Blais and Yamazaki 1989a; mean colour intensity reading of 5 replicates ±standard deviation, least significant difference is noted in brackets where +, ++, and +++ denotes a significant increase in colour intensity compared to untreated cells at $P<0.05$, $P<0.01$ and $P<0.001$ respectively; and -, --, and --- denotes a significant decrease in colour intensity to untreated cells at $P<0.05$, $P<0.01$ and $P<0.001$ respectively; nsd, not significantly different ($P>0.05$)

Table 3.6: Dot blot assay to show the effect of shaking cultures in different chemicals on M105 antibody reactivity with six *Salmonella* strains

Serotype (Source)[a]	Untreated	Chemical treatment				
		TBS	TBS+SDS	TBS+EDTA	SDS+EDTA	Distilled water
S.typhimurium	4.40±0.66	2.26±0.41 (---)	1.95±0.37 (---)	1.88±0.66 (---)	2.37±0.45 (---)	2.69±0.56 (---)
S.virchow	5.37±1.08	4.97±0.51 (nsd)	4.07±0.35 (-)	4.90±0.77 (nsd)	3.30±0.82 (---)	4.26±0.73 (-)
S.enteritidis	2.08±0.26	2.22±0.28 (nsd)	2.07±0.54 (nsd)	1.95±0.16 (nsd)	2.03±0.34 (nsd)	1.82±0.25 (nsd)
S.give	4.12±0.83	5.22±0.43 (+)	3.88±1.05 (nsd)	4.11±0.66 (nsd)	2.44±0.32 (--)	5.00±0.78 (nsd)
S.ealing (NTU)	0.08±0.06	0.36±0.06 (+++)	0.41±0.05 (+++)	0.30±0.12 (+++)	0.03±0.02 (nsd)	0.24±0.08 (++)
S.arizonae (NTU)	0.36±0.23	0.87±0.22 (+)	1.57±0.33 (+++)	0.37±0.34 (nsd)	1.57±0.33 (+++)	1.74±0.39 (+++)

[a], *ca* $5x10^7$ cfu/dot; mean colour intensity reading of 5 replicates ±standard deviation; least significant difference is noted in brackets where +, ++, and +++ denotes a significant increase in colour intensity compared to untreated cells at $P<0.05$, $P<0.01$ and $P<0.001$ respectively; and -, --, and --- denotes a significant decrease in colour intensity to untreated cells at $P<0.05$, $P<0.01$ and $P<0.001$ respectively; nsd, not significantly different ($P>0.05$)

Table 3.7: Dot blot assay to show the effect of temperature on the M105 antibody reactivity with six *Salmonella* strains

		Temperature (°C)					
		4		55		100	
		Time (min)		Time (min)		Time (min)	
Serotype (Source)[a]	**Untreated**	**30**	**60**	**10**	**30**	**10**	**30**
S.typhimurium	4.39±0.26	4.41±0.90 (nsd)	3.92±0.24 (nsd)	1.10±0.29 (---)	0.78±0.34 (---)	0.81±0.21 (---)	1.06±0.18 (---)
S.virchow	4.54±0.50	4.43±0.87 (nsd)	3.76±0.53 (nsd)	4.17±0.34 (nsd)	3.23±0.66 (--)	3.93±0.89 (nsd)	3.04±0.44 (---)
S.enteritidis	3.03±0.55	3.09±0.45 (nsd)	2.99±0.32 (nsd)	2.62±0.34 (nsd)	2.27±0.60 (--)	2.13±0.37 (--)	2.59±0.35 (nsd)
S.give	3.67±0.82	3.95±0.44 (nsd)	3.71±0.32 (nsd)	2.97±0.23 (-)	2.56±0.21 (--)	2.92±0.29 (-)	3.34±0.80 (nsd)
S.ealing (NTU)	0.10±0.03	0.12±0.02 (nsd)	0.21±0.03 (+++)	nd	nd	0.10±0.02 (nsd)	0.25±0.03 (+++)
S.arizonae (NTU)	0.19±0.07	0.52±0.10 (+++)	0.63±0.06 (+++)	0.13±0.05 (nsd)	0.33±0.03 (++)	0.36±0.07 (++)	1.16±0.14 (+++)

[a], *ca* $5x10^7$ cfu/dot; mean colour intensity reading of 5 replicates ±standard deviation; least significant difference is noted in brackets where +, ++, and +++ denotes a significant increase in colour intensity compared to untreated cells at $P<0.05$, $P<0.01$ and $P<0.001$ respectively; and -, --, and --- denotes a significant decrease in colour intensity to untreated cells at $P<0.05$, $P<0.01$ and $P<0.001$ respectively; nsd, not significantly different ($P>0.05$); nd, not done

of *S.ealing* and *S.arizonae* cells.

3.4.2 *THE EFFECT OF BOILING CELLS IN DIFFERENT CHEMICALS*

Again any treatment of *S.typhimurium* resulted in a significant decrease in the relative colour intensity of the blots ($P<0.001$). With the exception of *S.ealing* which was apparently unaffected, boiling *Salmonella* serotypes in sodium dodecyl sulphate (SDS)+EDTA solution for 10min resulted in significantly less intense dots ($P<0.01$). Boiling strains of *S.ealing* and *S.arizonae* in TBS+EDTA solution increased the intensity of the dots significantly ($P<0.01$).

3.4.3 *THE EFFECT OF SHAKING CELLS IN DIFFERENT CHEMICALS*

Any treatment of *S.typhimurium* cells resulted in a significant decrease in the relative colour intensity of the dots ($P<0.001$). Shaking *S.ealing* and *S.arizonae* cells in TBS+SDS solution, distilled water and TBS produced significantly more intense dots ($P<0.001$, $P<0.01$ and $P<0.05$ respectively).

3.4.4 *THE EFFECT OF TEMPERATURE*

The intensity of dots produced by *S.ealing* and *S.arizonae* were increased by cooling cells at 4°C in TBS for 60min ($P<0.001$) and boiling cells at 100°C in TBS for 30min ($P<0.001$).

3.4.5 *SUMMARY OF AMPLIFICATION RESULTS*

The treatment conditions required to optimally increase the M105 antibody reaction with the six *Salmonella* strains are shown in Table 3.8. No single treatment optimised the reaction of the M105 antibody with the *Salmonella* strains analysed. Moreover each strain required a different treatment to optimise the M105 antibody reactivity.

In summary any treatment of *S.typhimurium* led to a reduction of M105 reactivity. Treatment of *S.virchow* with tris and EDTA (37°C, 5min) resulted in a significant ($P<0.05$) increase in M105 reactivity from K/S 5.37 to 6.37. All other treatments resulted in a decrease in M105 antibody reactivity or had no significant effect. The optimum treatment conditions for *S.enteritidis* was tris, EDTA and Triton X-100 (37°C, 5min). Mean relative colour intensity readings were increased from K/S 2.08 for untreated cells to K/S 2.87 following treatment. The reactivity of the M105 antibody was either increased, decreased or was apparently unaffected by all other treatments to

Table 3.8: Optimum treatment conditions to expose the M105 epitope

Serotype	Optimum treatment conditions
S.typhimurium	No treatment
S.virchow	Tris+EDTA, 37°C, 5min
S.enteritidis	Tris+EDTA+Triton X-100, 37°C, 5min
S.give	TBS, shaking, 30min
S.ealing (NTU)	TBS+EDTA, 100°C, 10min
S.arizonae (NTU)	Distilled water, shaking, 30min

S.enteritidis cells. Shaking *S.give* cells in TBS solution for 30min resulted in optimal M105 reactivity. This treatment lead to a significant ($P<0.05$) increase in M105 antibody reactivity from K/S 4.12 for untreated cells to K/S 5.22 for treated cells. All other treatments resulted in a decrease in M105 reactivity or had no significant effect. Treatment of the NTU strain of *S.ealing* in TBS and EDTA (37°C), TBS (100°C, 10min), TBS and EDTA (100°C, 10min), TBS (shaking, 30min), TBS and SDS (shaking, 30min) and TBS and EDTA (shaking, 30min) resulted in a significant ($P<0.001$) increase in M105 reactivity. The optimum treatment conditions was TBS and EDTA (100°C, 10min) increasing K/S values from 0.08 to 0.44. The NTU strain of *S.arizonae* was significantly increased ($P<0.001$) by treatment with tris and EDTA (37°C,5min), TBS and SDS (shaking, 30min), SDS and EDTA (shaking, 30min), distilled water (shaking, 30min), TBS (4°C, 30 and 60min) and TBS (100°C, 30min). The optimum treatment conditions was distilled water (shaking, 30min) which increased K/S values from 0.36 to 1.74.

The increase in M105 antibody reactivity was thought to reflect an increase in eptitope accessibility. It was clear from the results that no single treatment could be used to optimise the epitope accessibility for all *Salmonella* strains. Therefore a standard treatment would have to be a compromise. In considering a standard treatment it was important to focus on the weakly reactive strains.

3.4.6 *STANDARD TREATMENT - THE EFFECT OF BOILING ON THE REACTIVITY OF THE M105 ANTIBODY*

A standard treatment was required which could easily be carried out in the industrial food testing laboratory. A simple treatment method was regarded as the most feasible. It can be seen from Table 3.7 that boiling cells at 100°C for 30min increased the reactivity of the M105 antibody weakly reactive *Salmonella* strains *S.ealing* (NTU) and *S.arizonae* (NTU). Since boiling might not optimise M105 antibody epitope exposure of all *Salmonella* serotypes, the effect of boiling on a wider range of *Salmonella* and non-*Salmonella* strains was assessed. Cells grown in BPW were adjusted to 10^9 cfu/ml in TBS and pre-treated by boiling at 100°C for 30min. The reactivity of the M105 antibody on boiled cells was assessed by dot blot assay as described in Section 2.8.

Tables 3.9 and 3.10 present the mean relative colour intensity (K/S) values of untreated and boiled cells from strains of *Salmonella* and non-*Salmonella* respectively. In addition the statistical significance (least significant difference) of boiling strains with respect to untreated cells is shown. From this analysis it can be determined that boiling salmonella cells either increased or had no apparent effect on M105 antibody reactivity. The exception was *S.virchow*, whereby significantly less intense dots were produced. Despite this reduction in M105 antibody reactivity, it should be noted that the dot intensity was still strong (K/S 1.35). Five *Salmonella* strains which expressed low M105 antibody reactivity when untreated remained the least reactive when boiled. However the readings of four of these strains were significantly increased by boiling (*S.livingstone*; *S.adelaide*; *S.ealing*, NTU; *S.arizonae*, NTU). All non-*Salmonella* strains showed a decrease in M105 antibody reactivity when boiled (Table 3.10). Hence the results clearly show that boiling can be used to successfully increase the M105 antibody reaction with certain *Salmonella* serotypes. Moreover boiling could be used to significantly reduce cross-reaction with non-*Salmonella* strains. Furthermore heat treatment at 100°C for 30min would kill the salmonellas and hence reduce a possible source of cross-contamination within the food testing laboratory.

There is a perceived discrepancy between the mean relative colour intensity readings for untreated and boiled *S.typhimurium*, *S.virchow*, *S.enteritidis*, *S.give* and *S.arizonae* cells recorded in Tables 3.7 and 3.9. This may have been due to the effect of storage on the antigenicity of salmonella master cultures and is discussed in Section 3.5.1.

Table 3.9: Dot blot assay to show the reactivity of the M105 antibody with untreated and boiled *Salmonella* strains

Serogroup	Serotype (Source)	Mean[a] K/S readings for		(Statistical analysis[c])
		Untreated cells	Boiled cells[b]	
B	*S.agona*	2.94	2.48	(nsd)
	S.bredeney	1.38	2.42	(+++)
	S.gallinarum	0.49	0.65	(+)
	S.saint paul	1.54	1.75	(+)
	S.typhimurium	0.96	1.37	(+++)
C_1	*S.infantis*	1.03	1.15	(nsd)
	S.livingstone	0.25	0.34	(+++)
	S.virchow	1.86	1.35	(---)
C_2	*S.hadar*	0.88	0.95	(nsd)
	S.manchester	0.58	0.60	(nsd)
	S.newport	1.89	2.30	(nsd)
C_3	*S.kentucky*	0.44	0.58	(nsd)
D_1	*S.dublin*	0.99	1.22	(+)
	S.enteritidis	2.55	1.40	(nsd)
	S.panama	1.94	2.84	(++)
E_1	*S.give*	1.87	2.20	(nsd)
E_4	*S.senftenberg*	2.10	2.05	(nsd)
L	*S.minnesota*	0.34	0.62	(+++)
O	*S.adelaide*	0.17	0.42	(+++)
	S.ealing (NTU)	0.15	0.18	(++)
	S.ealing (Canada)	0.17	0.19	(nsd)
51	*S.arizonae* (NTU)	0.27	0.71	(+++)

[a], mean colour intensity reading (n= 8); [b], 30min at 100°C; [c], least significant difference is noted in brackets where +, ++, and +++ denotes a significant increase in colour intensity compared to untreated cells at $P<0.05$, $P<0.01$ and $P<0.001$ respectively; and -, --, and --- denotes a significant decrease in colour intensity to untreated cells at $P<0.05$, $P<0.01$ and $P<0.001$ respectively; nsd, not significantly different ($P>0.05$)

Table 3.10: Dot blot assay to show the reactivity of the M105 antibody with untreated and boiled non-salmonella *Enterobacteriaceae*

Bacteria	Reference	Mean[a] K/S readings for		
		Untreated cells	Boiled cells[b]	(Statistical analysis[c])
C.freundii	NTU	0.06	0.02	(nsd)
	4/840	0.14	0.00	(---)
	4/841	0.09	0.01	(---)
	4/1155	0.13	0.01	(---)
	4001	0.14	0.01	(---)
	4002	0.17	0.01	(---)
	5/308	0.12	0.01	(---)
	5/785	0.08	0.02	(---)
Enterobacter agglomerans		0.27	0.02	(---)
E.coli		0.13	0.05	(--)
Shigella flexneri	386	0.02	0.00	(---)

[a], mean colour intensity reading (n=10); [b], 30min at 100°C; [c], least significant difference is noted in brackets, where -- and --- denotes a significant decrease in colour intensity to untreated cells at $P < 0.01$ and $P < 0.001$ respectively; nsd, not significantly different ($P > 0.05$)

3.4.6.1 *The effect of boiling on the release of the M105 antigen and the accessibility of cell bound antigen*

The increased reactivity of the M105 antibody with heat and chemically treated *Salmonella* strains can be explained by the release of outer membrane fragments including LPS and the disruption of barrier function, which leads to an increase in the accessibility of core epitopes (Leive *et al.*, 1968; Tsuchido *et al.*, 1985; Blais and Yamazaki 1989a). This hypothesis was investigated to determine the effect of boiling on the release of M105 antibody reactive antigen into solution and the accessibility of the cell bound antigen. *S.typhimurium* cells were grown in BPW as standard and adjusted to a cell density of 10^9 cfu/ml in TBS. Cells were either untreated or boiled at 100°C for 30min. Duplicate samples of the untreated and boiled cells were separated by centrifugation into supernatant and cell pellet. One sample was recombined while the separated pellet of the other was resuspended in TBS. The recombined, separated cell pellet and supernatant samples were

assessed for M105 antibody reactivity by dot blot assay (Section 2.8).

M105 antibody reactivity in untreated *S.typhimurium* was almost exclusively confined to the cell (Figure 3.5). The limited reactivity produced by the supernatant was either due to release of the M105 antigen by centrifugation or experimental error (some cells may have contaminated the supernatant). The increase in K/S values of boiled cells clearly shows that boiling released antigen into solution. In addition boiling increased exposure of the M105 antibody epitope on the cell. Recombined cell and supernatant produced readings lower than those from separate cell and supernatant samples. Since a maximum absorbance value was not reached, the reason for this is unclear.

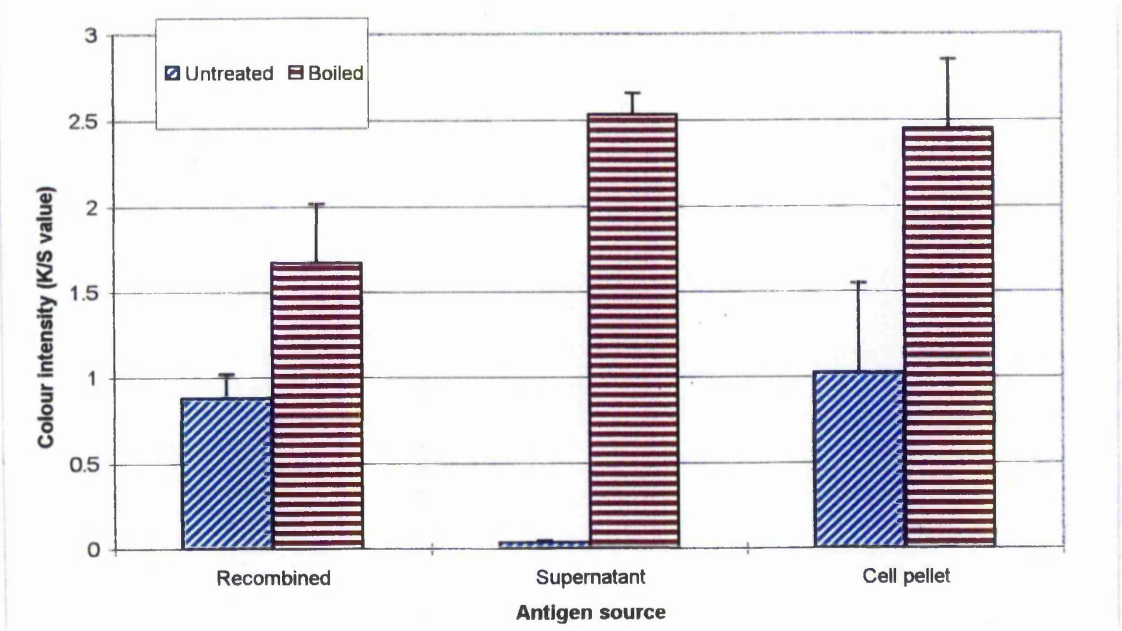


Figure 3.5: The effect of boiling (100°C, 30min) on the release of M105 antigen from *S.typhimurium* as detected by dot blot assay (vertical bars represent 95% confidence limits, where n= 4)

3.5. ADDITIONAL OBSERVATIONS

3.5.1 *THE EFFECT OF STORAGE ON THE ANTIGENICITY OF SALMONELLA SEROTYPES*

The master cultures of the bacterial strains used in this study were stored on Protect Bacterial Preserver beads at -20°C (Section 2.5.2) for up to three years. During this period some *Salmonella* strains were analysed yearly by dot blot assay (Section 2.8; cultures were analysed following subculture onto TSA and BPW). At the end

of the study data was combined and it became obvious that storage of some cultures had affected the reactivity of the strains with the M105 antibody. The reactivity of *S.agona*, *S.bredeney*, *S.saint paul*, *S.infantis*, *S.livingstone*, *S.virchow*, *S.hadar*, *S.newport*, *S.dublin*, *S.enteritidis*, *S.panama* and *S.senftenberg* with the M105 antibody had decreased. Statistical analysis of data confirmed that this reduction in reactivity was significant ($P<0.05$). Figure 3.6 presents some examples where a significant reduction in M105 antibody reactivity was noted.

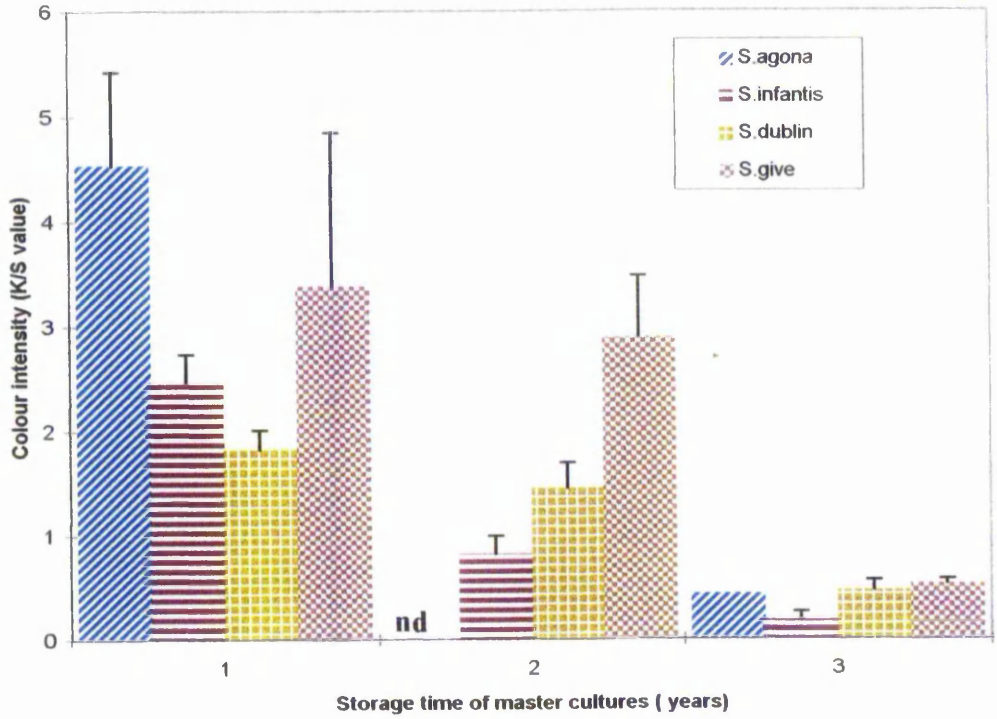


Figure 3.6: The effect of storage on the antigenicity of heat treated *Salmonella* strains as detected by dot blot assay (vertical bars represent 95% confidence limits, where n=4; nd, not done)

3.5.2 *THE EFFECT OF STORAGE ON THE ANTIGENICITY OF PURIFIED LPS*

Purified *S.typhimurium* Ra mutant LPS (dissolved in TBS) was used as a control in dot blot and immunoblot assays. Material was aliquoted and stored at -20°C. After storage aliquots were thawed to room temperature, mixed using a whirlimixer and analysed. During the course of investigations it was noted that dot blotted LPS would sometimes appear as a precipitate rather than a smooth even coloured dot (Figure 3.7). The colour intensity readings of the precipitate (K/S 0.3) were significantly lower ($P<0.05$) than those produced by the smooth dot (K/S 0.97). This discrepancy between standards was noted. It is likely that the precipitate was a characteristic of the purified LPS and was a direct

result of the storage conditions. It did not indicate a deterioration of antibody specificity or the chemicals used in the dot blot assay.

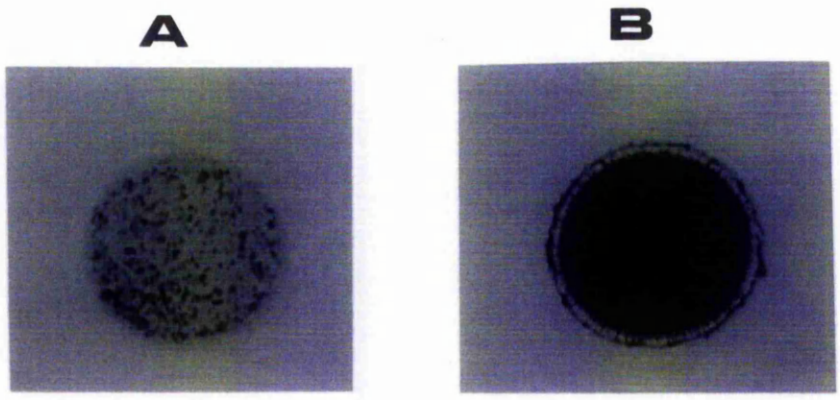


Figure 3.7: Purified *S.typhimurium* Ra mutant LPS as detected by dot blot assay appeared as a precipitate (A) or as a smooth even coloured dot (B)

3.6 DEVELOPMENT AND EVALUATION OF AN IMMUNOMAGNETIC SEPARATION ENZYME LINKED IMMUNOSORBENT ASSAY (IMS-ELISA)

The reader is reminded of the standard Dynabeads® anti-Salmonella immunomagnetic separation (IMS) - selective plating method of salmonella isolation and detection. In brief, an aliquot of traditionally pre-enriched food sample is incubated with Dynabeads® anti-Salmonella. During which salmonella cells bind specifically to the antibodies coated on the Dynabeads®. The Dynabeads®-salmonella cell complex is plated out onto selective agar plates to produce presumptive salmonella colonies. Confirmation is by traditional biochemical and serology tests.

The aim of the present study was to develop a microtitre plate based enzyme immunoassay. In addition to its use as an IMS capture and concentration technique Dynabeads® anti-Salmonella beads would be used as the solid support matrix. Since the dot blot assay results had shown that the M105 epitope was present in all *Salmonella* serotypes analysed (Table 3.9), it was concluded that the M105 antibody would be an ideal candidate for a salmonella detection antibody. Combined into a single method, the

Dynabeads® anti-Salmonella beads and the M105 antibody was referred to as the immunomagnetic separation-enzyme linked immunosorbent assay (IMS-ELISA) technique. The lack of reaction of purified LPS from *S.minnesota* (in the dot blot assay) is discussed later.

For the first stage in the development of the IMS-ELISA system a labelled M105 antibody was required. The anti-mouse detection antibody used in the dot blot and immunoblot assays could not be used in the IMS-ELISA. This was because one of the salmonella specific antibodies coated to commercially available Dynabeads® anti-Salmonella was raised in mouse. Hence attempts were made to purify and label the M105 antibody before developing an IMS-ELISA.

3.6.1 *PURIFICATION, LABELLING AND EVALUATION OF THE M105 ANTIBODY*

At the request of Dynal, the M105 antibody was labelled with horseradish peroxidase (HRP) enzyme. The M105 antibody was purified on a Protein A affinity column (Section 2.18). It was confirmed to be of isotype IgG_{2b} since it was eluted off the column with pH 3.0 buffer. The antibody concentration was established by protein assay (Section 2.19) and adjusted using an Amicon concentrator (Section 2.20) to a concentration of 1mg/ml. Purified antibody was titrated and its reactivity confirmed by dot blot assay (Section 2.8). An antibody dilution of 1/100 produced readings comparable with a 1/1000 dilution of ascities.

The antibody M105 was labelled with HRP enzyme by three separate methods. In the Zymed procedure M105 antibody was labelled with HRP by a modified periodate oxidation process (Section 2.20). The M105 antibody was labelled by Jackson Research Laboratories (USA) and AmDex (Denmark) using undisclosed methods. The reactivity of labelled M105 antibody was assessed by dot blot assay (Section 2.8) and further evaluated by non-denaturing PAGE (Section 2.12.3) and immunoblot assay (Section 2.9.3). Results of the evaluations are presented below.

3.6.1.1 *Zymed laboratory procedure*

Dilutions of the labelled M105 antibody (1/10, 1/50, 1/100, 1/500) were reacted with heat treated (100°C, 30min) cultures of *S.typhimurium*, *S.enteritidis* and *C.freundii* (10^9 cfu/ml) by dot blot assay. Antibody dilutions 1/10 and 1/50 reacted very feintly with *S.typhimurium*. This reduced reactivity could have resulted from ineffective conjugation.

The majority of the M105 antibody preparation may have remained unlabelled and was therefore not visualised by addition of the HRP substrate. Alternatively the reduced reactivity could have been due to a reduced specificity of the successfully labelled M105 antibody caused by the conjugation of HRP to or near the epitope recognition site. Both hypotheses were assessed by incubating dot blots with AP labelled secondary antibody (Section 2.8). The M105 antibody was not detected. It was therefore concluded that the Zymed labelling procedure reduced M105 antibody specificity.

3.6.1.2 *Jackson laboratory procedure*

Jackson HRP labelled M105 antibody was diluted to 1/50, 1/100 and 1/1000 and reacted with *S.typhimurium* Ra mutant LPS in a dot blot assay. A feint reaction was noted with 1/50 and 1/100 dilutions. Positive detection of the M105 antibody by AP labelled secondary antibody inferred that the lack of reactivity was due to an ineffective conjugation procedure. Therefore the HRP labelled M105 antibody was further evaluated.

Samples of HRP-labelled M105 antibody, unlabelled M105 antibody, HRP and BSA (used as a stabiliser in antibody diluent at a level of 15mg/ml) were solubilised and analysed by non-denaturing PAGE. Proteins were detected by coomassie blue and protein silver stain (Section 2.13). In addition samples were electrophoretically transferred onto nitrocellulose membrane (Section 2.14) and visualised by 4CN substrate. Multiple banding profiles were produced for BSA, HRP and the HRP-labelled M105 antibody. These probably reflected complexes (BSA:BSA, HRP:HRP) in addition to BSA, HRP and HRP conjugated M105 antibody. Many of the bands produced by the electrophoresed BSA (Figure 3.8A) and HRP (Figure 3.8B) were common to the profile produced by the HRP labelled M105 antibody. These results suggested that the Jackson labelled M105 antibody was made up of a significant portion of BSA stabiliser and in addition contained unconjugated HRP and HRP complexes. Hence the high antibody titres required for reaction could be explained by a low concentration of HRP labelled M105 antibody.

3.6.1.3 *Amdex laboratory procedure*

HRP labelled M105 antibody (1/1000 dilution) was assessed by dot blot assay against six strains of *Salmonella* serogroup B, two strains of *Salmonella* serogroup C, three strains of *Salmonella* serogroup D and two non-salmonella Enterobacteriaceae. The M105 antibody produced strong reactions with all salmonellas and no reaction with the non-salmonella *Enterobacteriaceae* (Dr Berit Grün, Dynal Research and Development

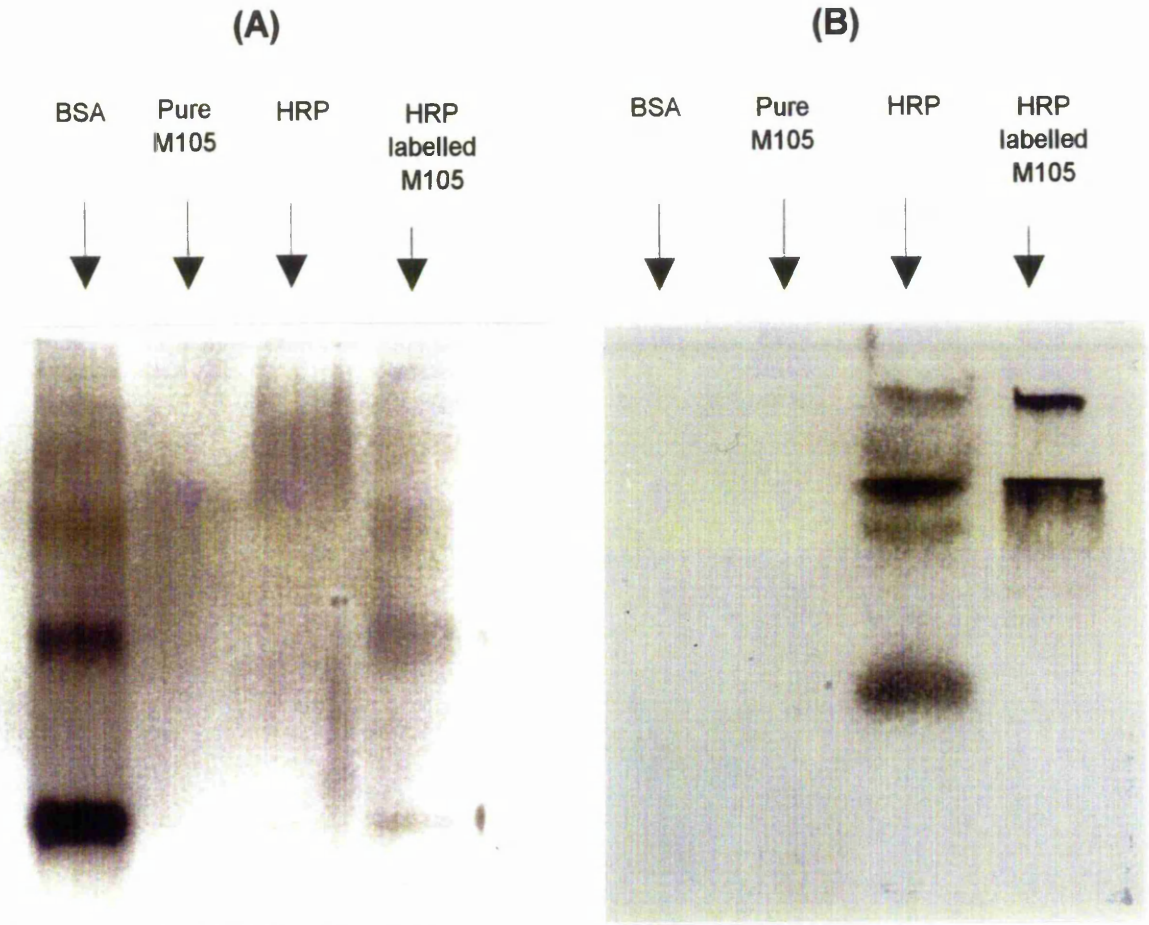


Figure 3.8: Non-denaturing PAGE analysis of HRP labelled M105 antibody - silver stained gel (A) and nitrocellulose membrane incubated with HRP substrate (B)

Laboratories Oslo, Norway; personal communication). However following two months storage at 4°C, the antibody failed to exhibit any reactivity. A 20μl aliquot of HRP labelled M105 antibody was mixed with an equal volume of enzyme substrate resulting in a blue colour. This suggested that the loss of antibody reactivity was not due to a loss of enzyme activity but could be explained by a loss of antibody specificity caused by prolonged storage at 4°C.

3.6.2 *EVALUATION OF PROTOTYPE ENHANCED GROUP E ANTI-SALMONELLA DYNABEADS®*

Attempts to label the M105 antibody with HRP failed due to a loss of specificity at 4°C. Therefore a secondary enzyme labelled anti-mouse antibody was required to visualise the reaction between the murine M105 antibody and salmonella core epitope in the IMS-ELISA. This meant that the commercially available Dynabeads® anti-Salmonella could not be used, since one of its antibodies was raised in mouse. Instead prototype enhanced group E anti-Salmonella Dynabeads® (Prototype Dynabeads®) provided by Dynal A/S (Oslo, Norway) were used. The Prototype Dynabeads® are coated in exactly the same antibodies as commercially available Dynabeads® anti-Salmonella with the exception that the murine antibody has been replaced with one which was not raised in mouse (replacement antibody was not specified by Dynal). The specificity and sensitivity of the Prototype Dynabeads® was assessed and compared with commercially available Dynabeads® anti-Salmonella.

Cultures were grown in BPW as standard. Cell densities were adjusted to 10^2-10^5 cfu/ml in BPW. The *Salmonella* strains used included *S.give*, *S.typhimurium* and *S.ealing* (NTU) which had expressed high, medium and low M105 reactivity by dot blot assay (Table 3.9). In addition two M105 cross-reactive non-*Salmonella* strains, *E.coli* and *C.freundii* (NTU) (Table 3.10) were tested to assess the cross-reactivity of the Dynabeads®. The conventional IMS-selective plating method was used for both types of beads (Section 2.10).

Table 3.11 compares the recovery of *Salmonella* strains by IMS as expressed by growth on xylose lysine desoxycholate (XLD) agar plates by a semi-quantitative streak plating method depicted in Figure 3.9. All *Salmonella* serotypes were detected at all cell levels by both bead types. *C.freundii* was isolated at all cell levels which suggested that *C.freundii* cross-reacted with both types of anti-Salmonella Dynabeads®. However the number of colonies isolated did not increase with increased cell levels. A very small number of colonies were isolated from 10^4 and 10^5 cfu/ml dilutions of *E.coli* which

Table 3.11: Recovery of *Salmonella* strains by Dynabeads® anti-Salmonella and Prototype Dynabeads®

Bacteria (Origin)	Estimated cell number (cfu/ml)	IMS bead	
		Dynabeads® anti-Salmonella	Prototype Dynabeads®
S.typhimurium	10^5	+3	+3
	10^4	+2	+2
	10^3	+2	+2
	10^2	+2	+2
	0	-	-
S.give	10^5	+2	+2
	10^4	+2	+2
	10^3	+1	+2
	10^4	+1	+1
	0	-	-
S.ealing (NTU)	10^5	+2	+2
	10^4	+2	+1
	10^3	+1	+1
	10^2	+1	+1
	0	-	-
C.freundii (NTU)	10^5	+2	+2
	10^4	+1	+2
	10^3	+1	+1
	10^4	+1	+1
	0	-	-
E.coli	10^5	+a	+a
	10^4	+b	+b
	10^3	-	-
	10^4	-	-
	0	-	-

[a], four colonies; [b], one colony;
+, signifies growth; -, signifies no growth

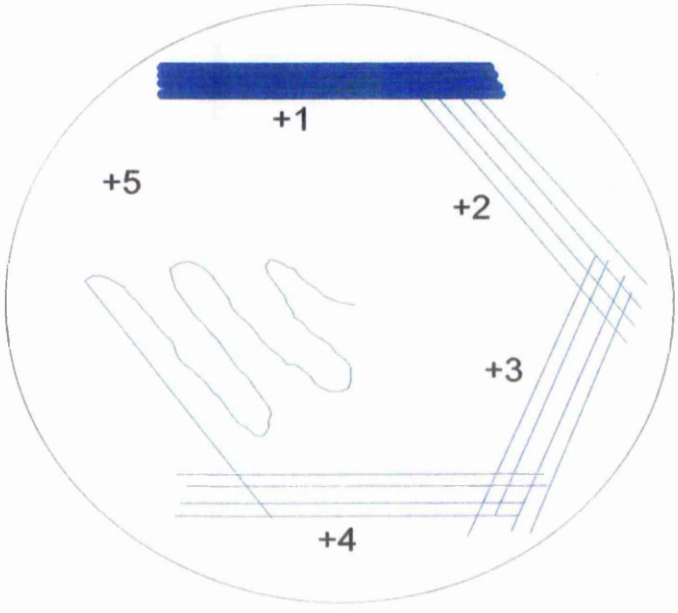


Figure 3.9: Semi-quantitative streak plating method used to allow comparison of anti-Salmonella Dynabeads® +1, growth on the first streak; +2, growth on the first and second streaks and so forth

suggested that cells were binding non-specifically to the beads themselves rather than cross-reacting with the antibodies coated onto the beads.

3.6.3 *THE DEVELOPMENT OF AN IMS-ELISA METHOD FOR THE DETECTION OF SALMONELLA SPP.*

The use of IMS in the proposed IMS-ELISA system is quite separate from the Dynabeads® anti-Salmonella IMS technique described in Section 2.10.2. The IMS-ELISA is so called because IMS is performed not in Eppendorf tubes but in microtitre plates. Detection is not by growth on an agar plate but by a *Salmonella* spp. specific double antibody detection system.

In the following sections the anti-Salmonella antibodies immobilised on the Prototype Dynabeads® (referred to as salmonella capture antibodies) will be considered as separate to those involved in detection and visualisation. Hence the M105 detection antibody will be referred to as the primary antibody and the enzyme labelled anti-mouse visualisation antibody will be referred to as the secondary antibody.

3.6.3.1 *Titration experiments*

A working IMS-ELISA protocol was established and optimised for heat treated (100°C, 30min) *S.typhimurium* cells. Cultures were grown in BPW as standard. Cell densities were adjusted in BPW. An AP labelled secondary antibody was used in preference (although not exclusively) to an HRP labelled secondary antibody. The results of the AP visualisation system produced consistently higher positive absorbance readings and hence are described below. Prototype Dynabeads® incubated with no antigen (TBS) were used to monitor background absorbance values.

Preliminary investigations demonstrated that heat treated cells non-specifically adhered to the microtitre plate wells. Mean absorbance readings of optical density $(OD)_{405nm}$ 2.05 were recorded for those wells which remained unblocked. In contrast wells which were blocked with 3% SMP in TBS-T (30min, 37°C) produced significantly lower ($P < 0.05$) absorbance values of OD_{405nm} 0.39. To ensure that reactivity was due to bead bound antigen and to reduce the possibility of non-*Salmonella* strains interfering with the reaction, a blocking stage was incorporated into the protocol. The optimal working conditions for the IMS-ELISA were determined by titrating the Prototype Dynabeads® and primary (M105) and secondary (AP labelled) antibodies. The parameters altered are listed in Table

3.12.

Ten, 15 and 20μl volumes of Prototype Dynabeads® were incubated with heat treated *S.typhimurium* cells for 10min. Antigen was detected by a 1/100 dilution of primary (purified M105) and 1/1000 dilution of secondary (AP labelled rabbit anti-mouse) antibodies incubated at room temperature for 30min. The 15μl volume of beads produced mean absorbance readings of OD_{405nm} 2.62$\pm$0.05. These were significantly ($P<0.05$) greater than the readings produced by the 10 and 20μl volumes (2.49$\pm$0.03 and 2.40$\pm$0.03). However the 10μl volume was chosen in preference. Since the ultimate aim of this study was to produce a commercially available IMS-ELISA, costs were an important factor. The 5.6% increase in absorbance by the 15μl volume of beads could not justify the 50% increase in costs.

A 10μl volume of beads was incubated with heat treated cells for 10, 20 and 30min. Antigen was detected by a 1/100 dilution of primary (purified M105) and a 1/1000 dilution of secondary (AP labelled rabbit anti-mouse) antibodies at room temperature for 30min. Similar absorbance readings were produced: 2.57$\pm$0.05, 2.54$\pm$0.04, 2.58$\pm$0.05 for the 10, 20 and 30min incubation respectively. The shortest bead incubation time of 10min was chosen. This would minimise assay time and would ensure that non-specific interactions between beads and non-*Salmonella* strains were kept to a minimum.

Ten μl of Prototype Dynabeads® were incubated with antigen for 10min. Bead captured antigen was then reacted with purified M105 antibody (1/50, 1/100, 1/500) and secondary antibody (1/500, 1/1000, 1/5000) for 30 and 60min at room temperature. The extended incubation did not increase absorbance values. Hence in order to keep the IMS-ELISA as rapid as possible, the shorter incubation period was used. The results for the titration of primary and secondary antibodies for 30min at room temperature are shown in Figure 3.10. The lowest absorbacnce readings of OD_{405nm} 1.89, 2.38 and 2.58 were produced by the following combinations of primary and secondary antibodies 1/500:1/5000, 1/100:1/5000 and 1/500:1/1000. For all other combinations of antibody dilutions absorbance readings between OD_{405nm} 2.60-2.66 were noted. The lowest concentrations of primary (1/100) and secondary (1/1000) antibodies which produced one of the highest absorbance readings (OD_{405nm} 2.66) were used in future experiments to minimise cost. A range of cell numbers were assayed (10^7-10^9 cfu/ml). The 10^9 cells produced the strongest reaction and was the level used for all IMS-ELISA specificity experiments.

Table 3.12 : IMS-ELISA development conditions

Parameters		
Prototype Dynabeads®	Volume per well (µl)	10, 15, 20
	Incubation time (min)	10, 20, 30
Primary M105 antibody (purified)	Dilution	1/50, 1/100, 1/500, 1/1000, 1/2000
	Incubation temperature (°C)	Room temperature
	Incubation time (min)	30, 60
Secondary AP labelled antibody	Dilution	1/500, 1/1000, 1/5000
	Incubation temperature (°C)	Room temperature
	Incubation time (min)	30, 60
Secondary HRP labelled antibody	Dilution	1/500, 1/1000, 1/5000
	Incubation temperature (°C)	Room temperature
	Incubation time (min)	30, 60
Enzyme substrate development time (min)	pNPP	5, 13, 21, 30, 40, 50, 60
	TMB	5, 10, 15, 20

AP, alkaline phosphatase; HRP, horseradish peroxidase; pNPP, p-nitrophenyl phosphate; TMB, tetramethylbenzidine

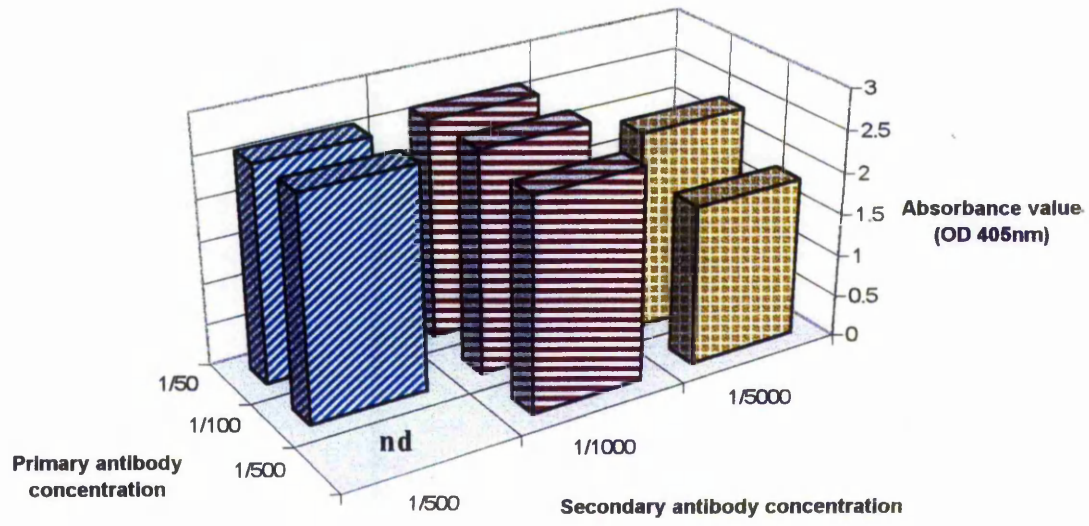


Figure 3.10: Absorbance readings for the titration of primary M105 and secondary enzyme labelled species specific antibodies incubated at room temperature for 30 minutes (where n=5; nd, not done)

3.6.3.2 *Investigations into high background absorbance values*

Background absorbance values recorded for the AP and HRP visualisation systems were unexpectedly high. The mean absorbance readings (n=39) were $0.45_{OD405nm}$ and $0.84_{OD450nm}$ for the AP and HRP systems respectively. Therefore experiments were undertaken to reduce these values. All experiments were based on HRP visualisation (Section 2.11). This was because the HRP background readings were generally higher and therefore any difference in the absorbance readings would be more easily recognised. A series of TBS negative controls were set up and the routine IMS-ELISA performed with the following adjustments: alternative antibody diluents (1% BSA in TBS and TBS-T) and blocking solutions (3% BSA in TBS and TBS-T; 3% SMP in TBS and TBS-T) were compared. The M105 antibody was replaced and compared with HRP labelled non-*Salmonella* spp. specific IgG antibodies MIC 13/43 and MIC 13/133. Both antibodies were used at a 1/10,000 dilution in 1%BSA in TBS-T (determined by titration). The Prototype Dynabeads® were replaced by and compared with BSA coated M280 Dynabeads®.

Figure 3.11 shows the effect of antibody diluent on background absorbance readings. The lowest values were produced when the primary M105 antibody was diluted in 1% BSA

in TBS-T and the secondary antibody was diluted in 1% BSA in TBS. Blocking solution clearly affected background readings (Figure 3.12). Using 3% BSA in TBS-T as the blocking solution resulted in the lowest values. The comparison of IgG antibodies are shown in Figure 3.13. Both the MIC/43 and MIC/133 antibodies produced readings significantly lower than the M105 antibody ($P<0.05$). Figure 3.14 shows the effect of Dynabeads® on background absorbance readings. The readings produced by BSA coated 280 Dynabeads® were significantly lower than the Prototype Dynabeads® ($P<0.05$). Unusually high absorbance readings were produced by the Prototype Dynabeads® which were more than double the values reported in Figure 3.13. Despite this it is clear that the M105 antibody exhibited a limited cross-reaction with the antibodies coated on the Prototype Dynabeads®. This limited cross-reaction was not considered to be too problematical and resulted in the continued use of the Prototype Dynabeads®.

Unfortunately the changes in antibody diluent and blocking solutions for the HRP visualisation system did not significantly alter the background readings produced by the AP visualisation system. Mean absorbance readings remained high at OD_{405nm} 0.44.

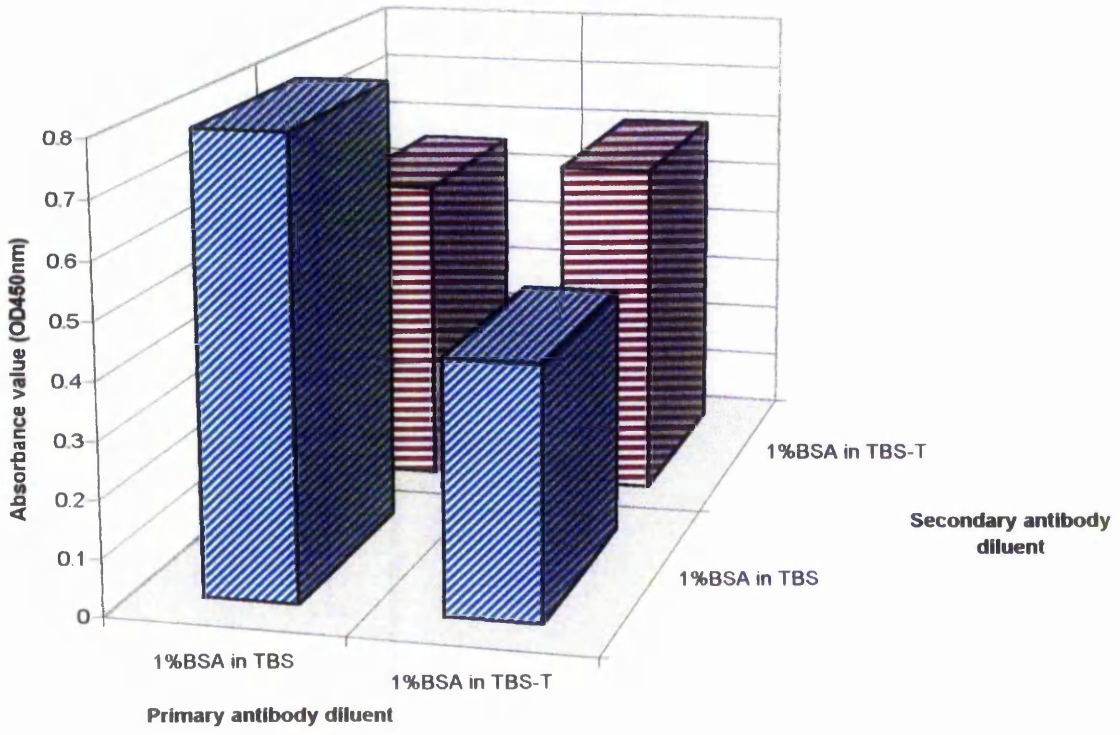


Figure 3.11: The effect of antibody diluent on background absorbance readings (where n=4)

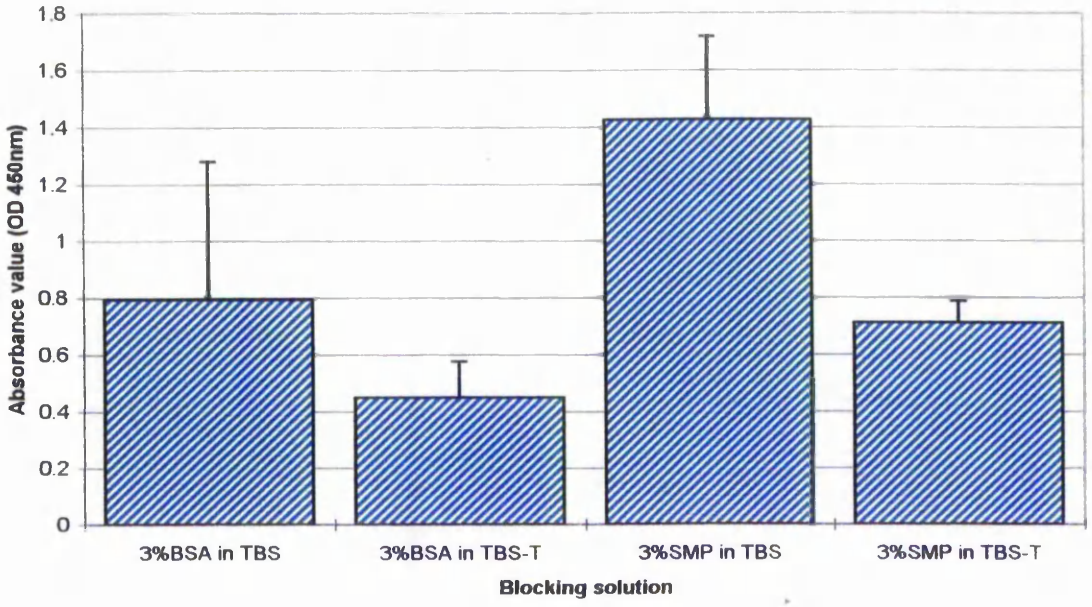


Figure 3.12: The effect of blocking solution on background absorbance readings (vertical bars represent 95% confidence limits, where n=5)

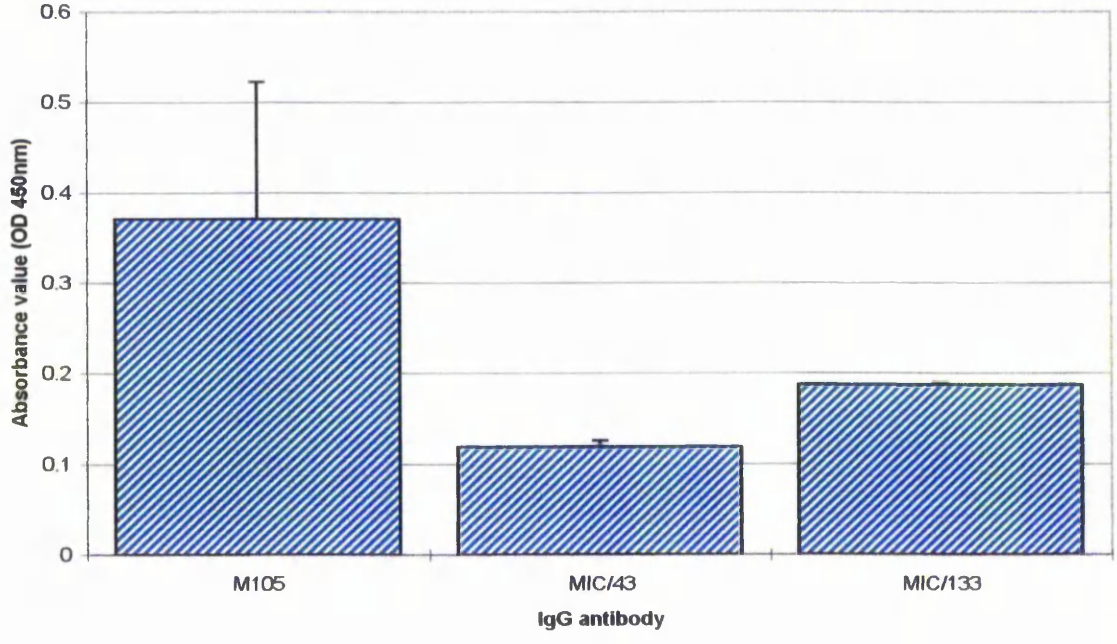


Figure 3.13: The effect of IgG antibodies on background absorbance readings (vertical bars represent 95% confidence limits, where n=3)

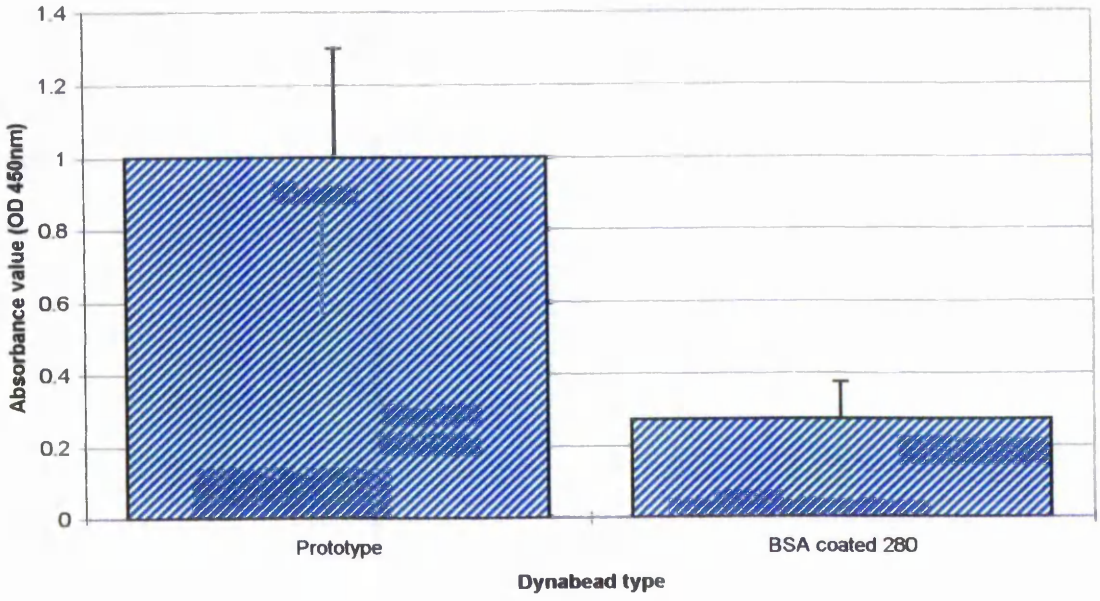


Figure 3.14: The effect of Dynabeads® on background absorbance readings (vertical bars represent 95% confidence limits, where n=3)

It can be seen from Figures 3.11-3.14 that the background values produced by the negative control were variable (OD_{405nm} 0.37-1.00). These results were significant with respect to calculating threshold values for the working IMS-ELISA protocol. This aspect is discussed later in Section 3.6.4.3 with reference to the AP visualisation system.

3.6.3.3 *Determination of substrate colour development time*

S.typhimurium cells were used to determine the substrate development time. Cultures were grown as standard and cell densities were adjusted in BPW. Cells were heat treated by boiling at 100°C for 30min. The IMS-ELISA protocol with AP visualisation (Section 2.11) was followed with the exception that substrate colour development of *S.typhimurium* and a TBS negative control was monitored over a period of 60min.

The substrate colour development cut off time was determined by calculating the difference in absorbance values produced by *S.typhimurium* and the negative control (Figure 3.15). The biggest difference was noted at 21 and 30min incubation. Although an optimum absorbance value of approximately OD_{405nm} 2.70 was reached after 30min, this value was not significantly different ($P<0.05$) from that obtained after 21min incubation. Hence a

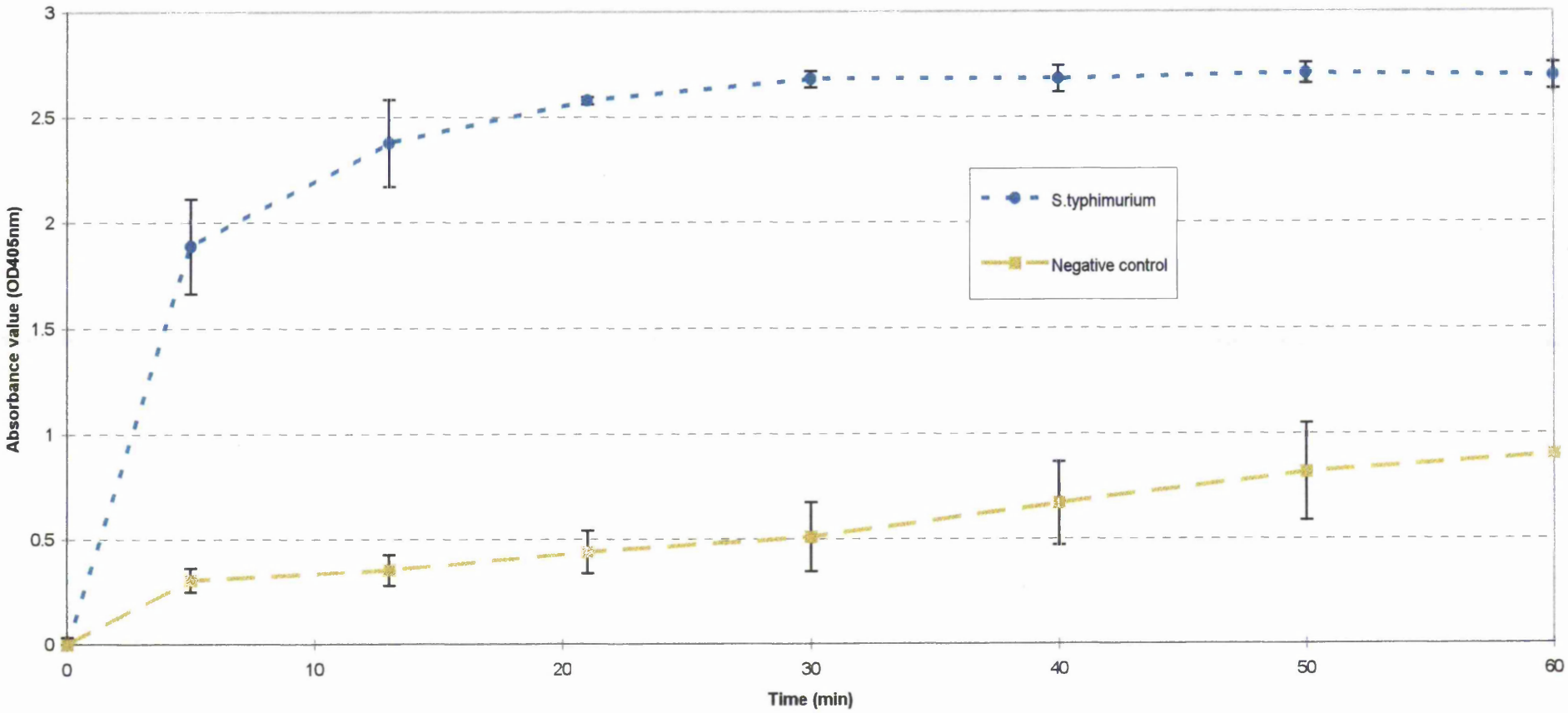


Figure 3.15: Monitoring the absorbance readings produced by *S.typhimurium* and a no antigen negative control as detected by IMS-ELISA (Vertical bars represent 95% confidence limits, where n= 10)

cut off time of 21min was chosen because near maximum absorbance had been reached with background levels under OD_{405nm} 0.5. Moreover the total test time of the IMS-ELISA would be reduced. The resultant working procedure is described in Section 2.11 and summarised in Figure 3.16.

3.6.4 *EVALUATION OF THE IMS-ELISA METHOD OF SALMONELLA DETECTION*

In the following section any reference to the 'IMS-ELISA' technique refers to the detection of salmonella antigens by the method summarised in Figure 3.16 and detailed in Section 2.11.

3.6.4.1 *Screening of Salmonella strains and other Enterobacteriaceae using the IMS-ELISA*

The AP visualisation system was used to further evaluate the IMS-ELISA technique by screening a range of *Salmonella* and non-*Salmonella* strains. Cultures were grown in BPW as standard. Cell densities were adjusted to 10^9 cfu/ml in BPW and heat treated (100°C, 30min) prior to analysis by IMS-ELISA.

Eighteen of the 22 *Salmonella* strains assayed produced mean absorbance values $\geq OD_{405nm}$ 1.40 (Table 3.13). However the remaining four strains (*S.adelaide*, both strains of *S.ealing* and *S.arizonae*, NTU) produced readings of $\leq OD_{405nm}0.60$. Cudjoe *et al.* (1995) similarly used the immunomagnetic separation technique in an ELISA based method. The system was referred to as the immunomagnetic particle (IMP)-ELISA. It used an alternative detection antibody (common structural antigen [CSA]-1 HRP labelled antibody) and pre-commercial Dynabeads® anti-Salmonella. Some of the strains analysed by Cudjoe in the IMP-ELISA were also tested in the present study by the IMS-ELISA method. These have been included in Table 3.13 for comparative purposes. With the exception of *S.agona* and *S.bredeney* the IMP-ELISA method produced higher absorbance readings than the IMS-ELISA.

All other non-salmonella Enterobacteriaceae gave mean absorbance values lower than OD_{405nm} 0.50 (Table 3.14). The top three strongest reactions given by the non-*Salmonella* spp. tested were roughly 94% (*E.coli*) and 82% (*C.freundii* NTU, 4/840) of the weakest *Salmonella* serotype when tested at the same cell density.

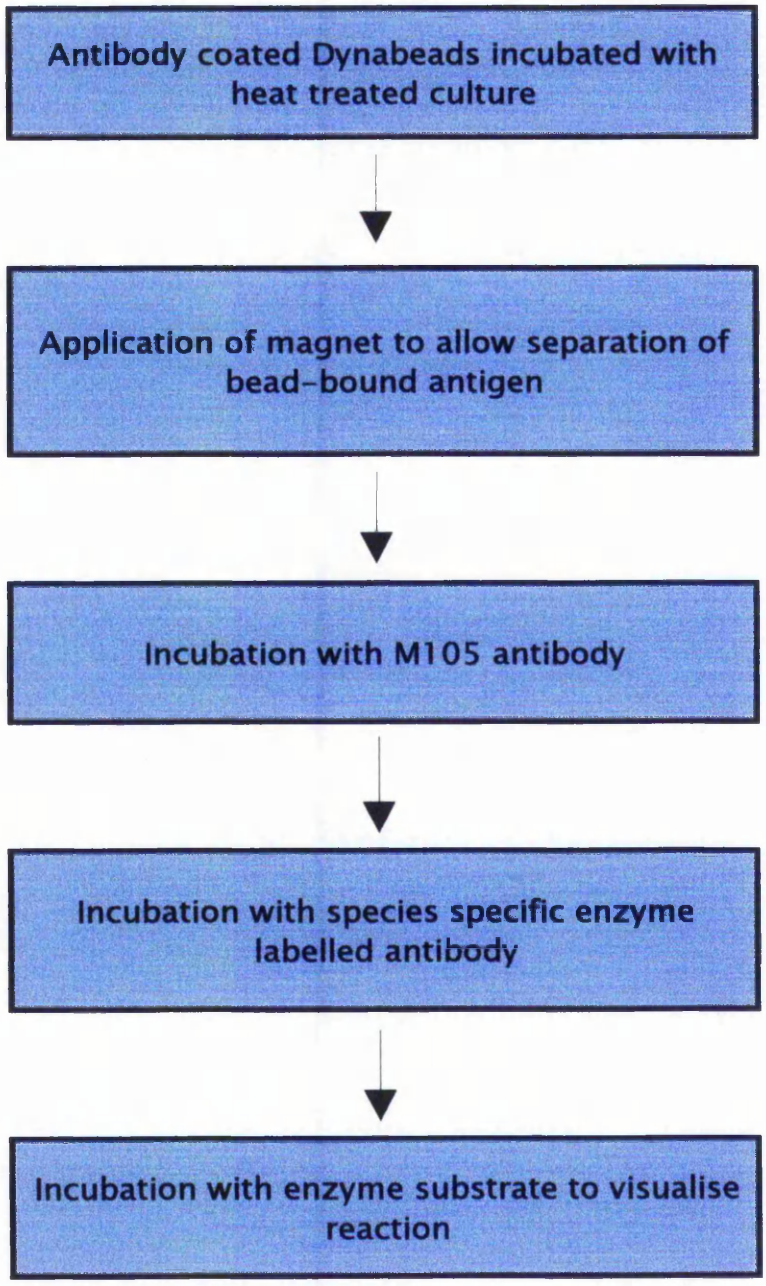


Figure 3.16: Summary of the IMS-ELISA salmonella detection procedure

Table 3.13: Reactivity of heat treated *Salmonella* strains as detected by the M105 antibody using the IMS-ELISA technique

Serogroup	Serotype	Absorbance reading at OD_{405nm}	
		IMS-ELISA[a] mean[b] value±SD (Outcome[c])	IMP-ELISA[d]
B	*S.agona*	2.53±0.07 (+)	2.36
	S.bredeney	2.49±0.07 (+)	2.06
	S.gallinarum	2.46±0.10 (+)	
	S.saint paul	2.41±0.11 (+)	
	S.typhimurium	2.57±0.09 (+)	3.02
C_1	*S.infantis*	1.67±0.19 (+)	3.31
	S.livingstone	1.79±0.19 (+)	
	S.virchow	1.45±0.30 (+)	2.62
C_2	*S.hadar*	2.11±0.14 (+)	
	S.manchester	2.23±0.13 (+)	
	S.newport	2.17±0.21 (+)	
C_3	*S.kentucky*	1.46±0.48 (+)	
D_1	*S.dublin*	2.63±0.05 (+)	
	S.enteritidis	2.64±0.00 (+)	3.31
	S.panama	2.60±0.06 (+)	
E_1	*S.give*	2.64±0.09 (+)	
E_4	*S.senftenberg*	2.66±0.07 (+)	
L	*S.minnesota*	1.40±0.35 (+)	
O	*S.adelaide*	0.56±0.21 (+)	
	S.ealing (NTU)	0.59±0.19 (+)	
	S.ealing (Canada)	0.50±0.16 (-)	
51	*S.arizonae* (NTU)	0.60±0.16 (+)	

[a], absorbance values based on bead bound antigen using M105 and an AP labelled secondary antibody system; [b], of 10 replicates;
[c], salmonella positive (+) and salmonella negative (-) results recorded based on a threshold value of OD_{405nm} 0.53; no antigen negative control mean absorbance value OD_{405nm} 0.46 where n=35;
[d]; Cudjoe *et al.*, 1995, absorbance values recorded by the immunomagnetic particle (IMP)-ELISA with detection by the common structural antigen (CSA)-1 HRP labelled antibody; SD, standard deviation

Table 3.14: Reactivity of heat treated non-salmonella *Enterobacteriaceae* as detected by the M105 antibody using the IMS-ELISA technique

Bacteria	Reference code	Mean[a] absorbance value at $OD_{405nm} \pm SD$	Outcome[b]
C.freundii	NTU	0.41±0.07	-
	4/840	0.41±0.09	-
	4/841	0.31±0.03	-
	4/1155	0.30±0.03	-
	4001	0.35±0.03	-
	4002	0.28±0.03	-
	5/308	0.37±0.03	-
	5/785	0.37±0.02	-
Enterobacter agglomerans		0.25±0.04	-
E.coli		0.47±0.08	-
Shigella flexneri		0.38±0.08	-

[a], of 5 replicates; [b], salmonella positive (+) and salmonella negative (-) results recorded based on a threshold value of OD_{405nm} 0.52; no antigen negative control mean absorbance value OD_{405nm} 0.45 where n=20; SD, standard deviation

3.6.4.2 *Determination of the IMS-ELISA sensitivity limit*

The sensitivity limit of the IMS-ELISA AP visualisation system was investigated. Representatives of the serogroups B (*S.typhimurium*), C (*S.virchow*), D (*S.enteritidis*) and E (*S.give*) were assayed. Cells were adjusted to 10^1-10^9 cfu/ml in BPW and heat treated at 100°C for 30min prior to analysis by IMS-ELISA.

Figure 3.17 clearly shows that all four *Salmonella* strains were detected at $\geq 10^6$ cfu/ml. Statistical analysis confirmed that the increase in mean absorbance values were significant ($P < 0.05$). Hence the sensitivity limit of the IMS-ELISA was between 10^5 and 10^6 cfu/ml.

3.6.4.3 *Determination of a threshold value for the IMS-ELISA*

There are a number of approaches to determining the threshold value. Choi *et al.* (1992) used the mean readings obtained from no antigen control experiments plus three times their standard deviation. Another approach is to double the mean background absorbance (Dr

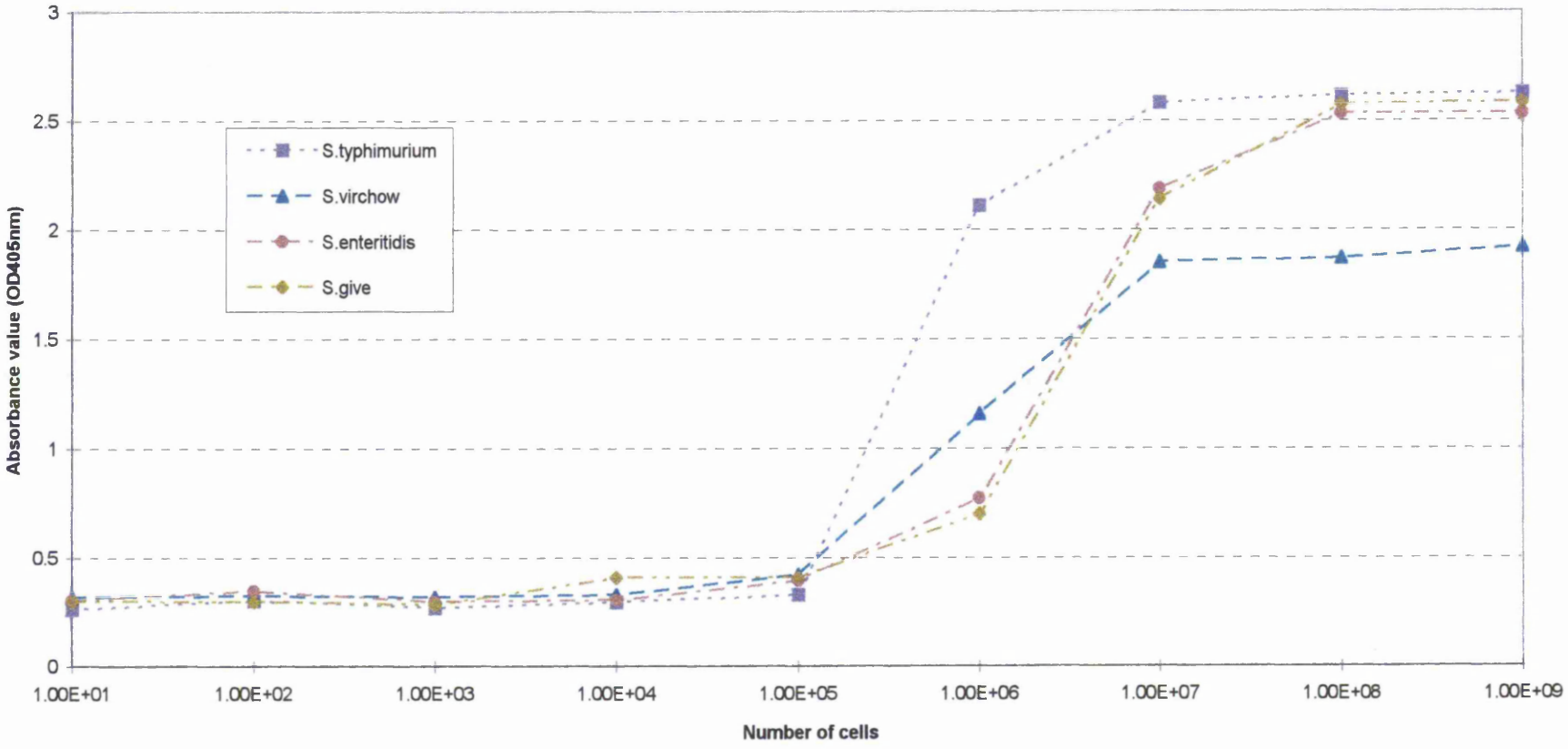


Figure 3.17: Sensitivity of the IMS-ELISA for the detection of heat treated *Salmonella* strains
(where n=4)

Ellen Billett, Immutec , The Nottingham Trent University; personal communication). The negative control of Cudjoe *et al.* (1995) consisted of a cocktail of three non-salmonella Enterobacteriaceae (*C.freundii*, *Ent.cloacae*, *E.coli*) in a ratio of 1:1:1 (total viable count 10^7 cfu/ml). Absorbance readings above the value produced by the boiled cells were considered as positive.

A fixed threshold value for reporting a test as positive was not determined for the present study. This was because statistical analysis of 155 negative control replicates showed the variation (OD_{405nm} 0.16-0.82) in absorbance readings to be significant ($P<0.05$). Moreover the mean absorbance values were relatively high at OD_{405nm} 0.44. Instead the threshold value was determined for each assay from the no antigen negative controls plus their least significant difference. Thus the threshold value for the screening of *Salmonella* and non-*Salmonella* strains was calculated at OD_{405nm} 0.53 and O.52 respectively. When such cut-off values were used, all *Salmonella* strains with the exception of *S.ealing* (Canada) were positive (Table 3.13) and all other Enterobacteriaceae were negative (Table 3.14). Using the method described above the threshold value for the sensitivity studies was calculated to be OD_{405nm} 0.47. When this threshold value was applied to the results of the sensitivity study the sensitivity limit remained the same.

3.6.4.4 *Investigation of maximum absorbance values*

The mean absorbance values produced by heat treated *Salmonella* strains appeared to reach a maximum of around OD_{405nm}2.50-2.60 (Table 3.13). Therefore experiments were undertaken to ascertain whether absorbance readings of greater than OD_{405nm}2.50 were an indication of system end point (i.e. maximum values) or a reflection of the limitations of the ELISA plate reader (i.e. optimum values). *S.typhimurium*, *S.virchow*, *S.newport*, *S.dublin*, *S.panama*, *S.give* and *S.senftenberg* cultures were grown as standard in BPW. Cell densities were adjusted to 10^9 cfu/ml in BPW and heat treated (100°C, 30min) prior to analysis by IMS-ELISA. After initial absorbance readings were recorded, solutions were diluted with an equal volume of buffer and further readings were taken. Samples were diluted in this manner until readings below the maximum were recorded. The absorbance values from diluted samples were multiplied by their dilution. These values were termed 'calculated' and were considered as true readings.

Recorded and calculated absorbance values are presented in Figure 3.18. The calculated absorbance values were significantly higher than the recorded optimum values ($P<0.05$).

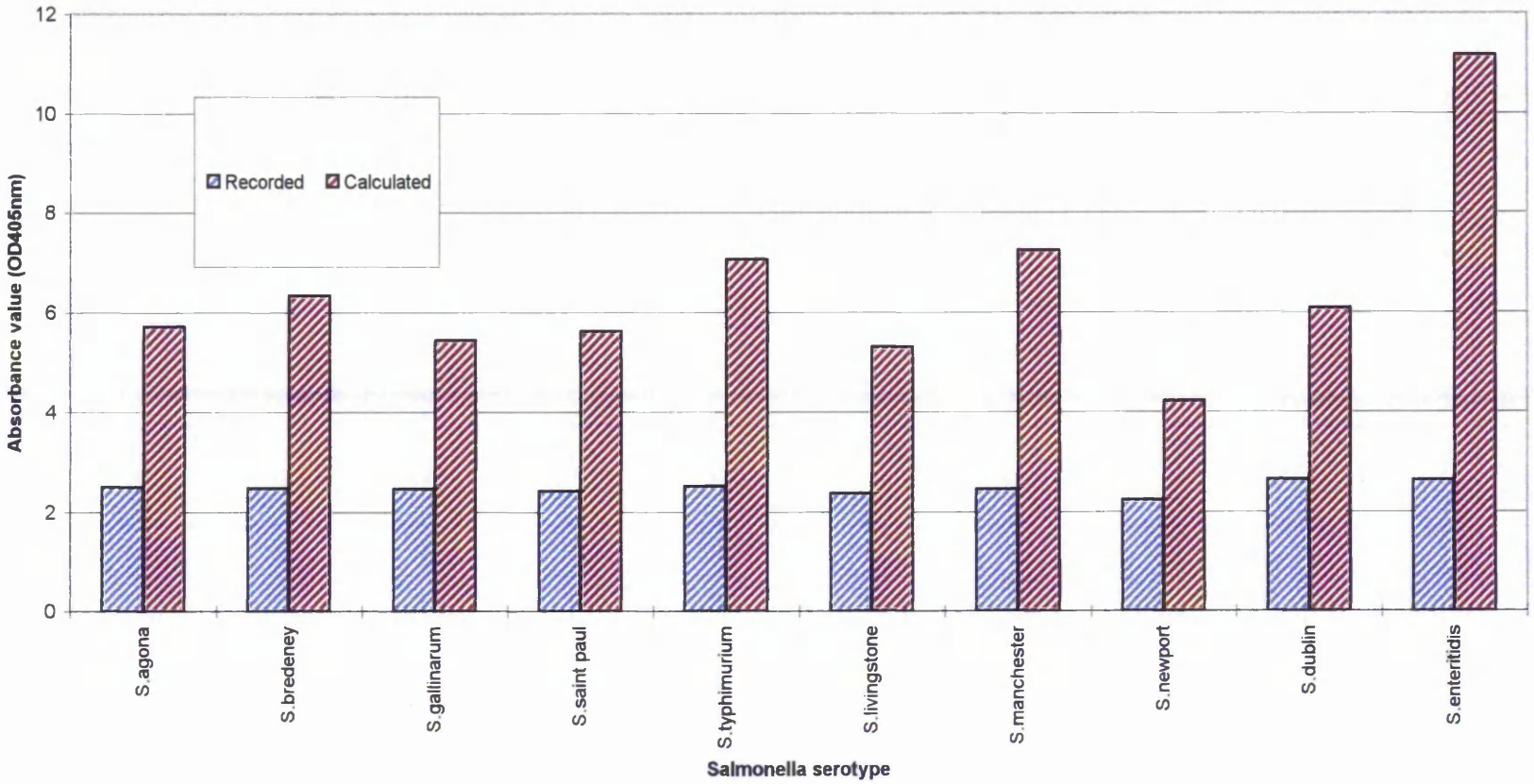


Figure 3.18: Recorded and calculated mean optimal absorbance values at OD_{405nm} for ten *Salmonella* strains using the IMS-ELISA technique (where n=10)

For example the highest recorded optimum reading for *S.enteritidis* was OD_{405nm}2.64. Whereas the calculated true reading was OD_{405nm}11.17. This confirmed instrument limitation.

3.6.4.5 *Investigation of low absorbance values*

Low absorbance readings of $OD_{405nm} < 0.60$ were recorded for 10^9 cfu/ml *S.adelaide*, both strains of *S.ealing* (NTU and Canada) and the NTU strain of *S.arizonae* (Table 3.13). Studies were therefore carried out to investigate whether low absorbance values were an indication of the low binding ability of heat treated cells to Prototype Dynabeads®. For this a *Salmonella* serogroup O specific antisera (unlabelled, raised in rabbit; Table 2.3) was used to detect the amount of bead-bound serogroup O *Salmonella*.

The reactivities of the core specific M105 antibody and the *Salmonella* serogroup O specific antisera were compared by analysing untreated and heat treated *S.ealing* (Canada strain; serogroup O). Cultures were grown and cell densities were adjusted to 10^9 cfu/ml in BPW. Cells were either untreated or heat treated (100°C, 30min) prior to analysis by the IMS-ELISA. Working antibody dilutions were determined by titration to be 1/500 for the serogroup O specific antisera and the AP labelled goat anti-rabbit secondary antibody. Heat treated *S.typhimurium* (10^9 cfu/ml) and TBS were run as positive and negative controls respectively. The results are presented in Table 3.15.

The absorbance readings produced by heat treated *S.ealing* as detected by the M105 antibody were 39% lower than previously described in Table 3.13. Moreover untreated cells produced readings lower than the negative control (OD_{405nm}0.34). The reasons for this are unknown.

Untreated and heat treated *S.ealing* reacted with the serogroup O specific antisera. This confirmed that both untreated and boiled cells were captured by Prototype Dynabeads®. Untreated *S.ealing* cells produced absorbance readings approximately double that of heat treated cells. This suggested that the antisera epitope was made inaccessible by boiling possibly because less antigen was bound to the beads. Hence the low absorbance values produced by *S.ealing* in the IMS-ELISA was probably due to a lack of the M105 epitope. This aspect is investigated further in Section 3.9.2.

Table 3.15: Reactivity of untreated and heat treated[a] *S.ealing* (Canada) using the IMS-ELISA method and anti-serogroup O specific antisera compared with the M105 antibody

Serotype	Treatment	Salmonella detection antibody	
		Anti-serogroup O specific antisera	M105
S.ealing	Untreated	1.72±0.16	0.34±0.01
S.ealing	Heat treated	0.88±0.11	0.36±0.04
Positive control[b]	Heat treated	0.46±0.03	2.47±0.05
Negative control[c]	na	0.56±0.06	0.45±0.10

[a], 100°C, 30min; [b], heat treated *S.typhimurium* (10^9 cfu/ml); [c], no antigen; mean absorbance readings of 3 replicates ± standard deviation; na, not applicable

3.6.4.6 *Investigations into the requirement of heat treatment*

It had been decided, during the course of the dot blot assay experiments, that a standard heat treatment of boiling cells at 100°C for 30min would produce non-viable cells (Section 3.4.1). This would also help to reduce the risk of cross-contamination in the food testing laboratory. However the IMS-ELISA results from the investigation of low absorbance values of *S.ealing* (Canada; Section 3.6.4.5) suggested that the O-chain antisera epitope was in fact made inaccessible by boiling. Studies were therefore undertaken to ascertain whether heat treatment affected the M105 core epitope in the same manner. Strains of *S.ealing* could not be analysed since the absorbance values produced by 10^9 cfu/ml were very low. Hence representative serotypes from serogroups B (*S.typhimurium*), C (*S.virchow*), D (*S.enteritidis*) and E (*S.give*) were assayed by the IMS-ELISA system. The level of salmonellas following pre-enrichment may vary considerably and is generally low. Therefore a range of salmonella cell densities were assayed (10^1-10^9 cfu/ml). Cultures were prepared as standard in BPW with the exception that cells were not heat treated prior to analysis by IMS-ELISA.

The IMS-ELISA detection limit for untreated cells was 10^6-10^7 cfu/ml (Figure 3.19). This was tenfold less sensitive than the detection limit for heat treated cells (10^5-10^6 cfu/ml; Figure 3.17). Hence the IMS-ELISA could detect lower levels of heat treated *Salmonella* strains. In addition the absorbance values produced by untreated cells were (in most cases) less than half that produced by heat treated cells. For example untreated *S.typhimurium* cells produced values of OD_{405nm}0.44, whereas heat treated cells produced values of

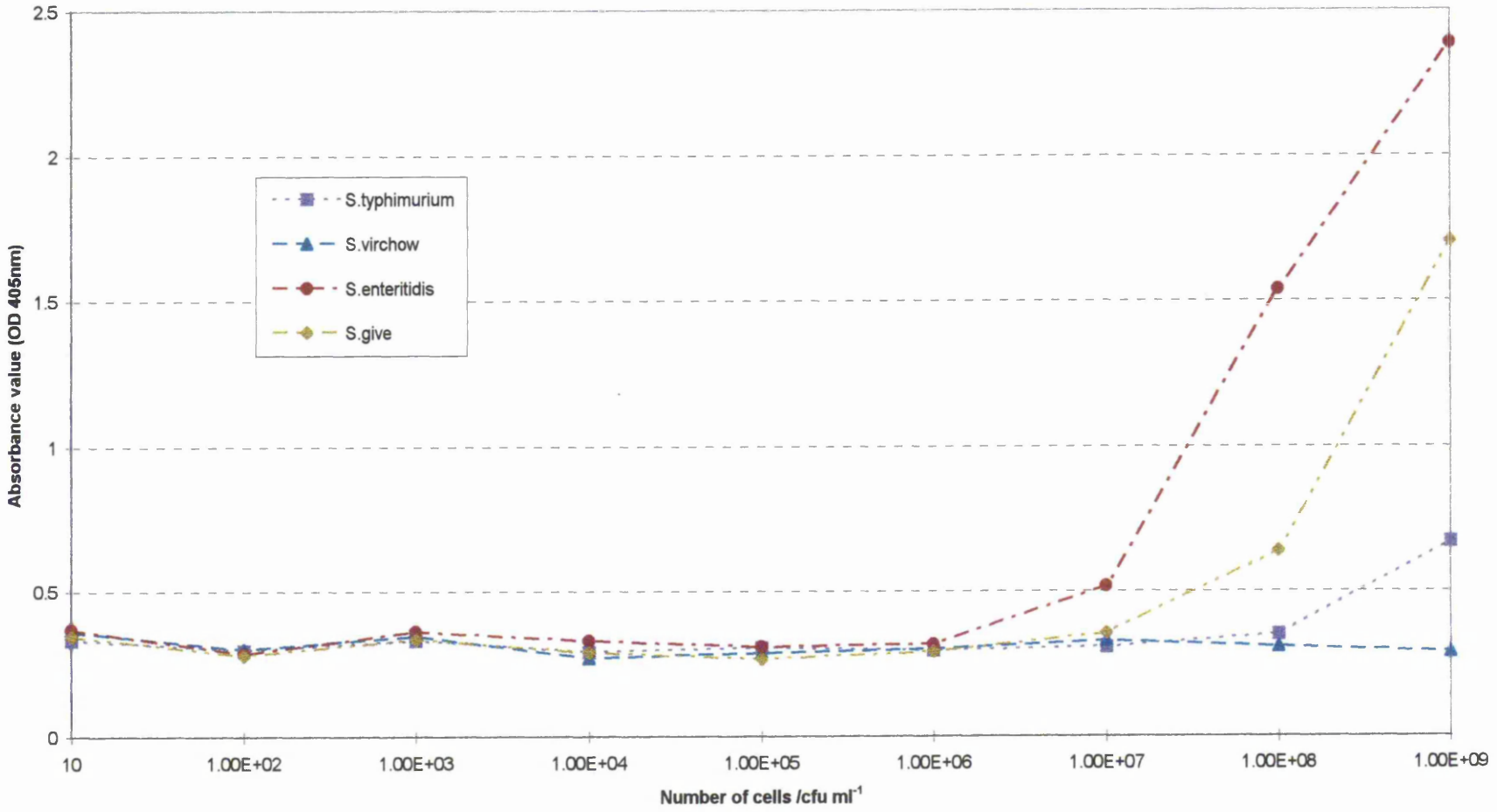


Figure 3.19: Sensitivity of the IMS-ELISA for the detection of untreated *Salmonella* strains (where n=3)

OD_{405nm}1.59. Therefore it could be concluded that heat treatment of cell was required to increase the sensitivity of the IMS-ELISA.

3.7 EVALUATION OF THE SINGLE BEAD PROTOCOL OF SALMONELLA ISOLATION WITH IMS-ELISA DETECTION BY PRELIMINARY FOOD TRIAL

The IMS-ELISA was developed to test for the presence of *Salmonella* spp. in food. Hence the IMS-ELISA was evaluated in a food trial situation. It was anticipated that if from the results of this preliminary study the IMS-ELISA system showed potential, a larger more indepth food trial would be undertaken.

For the following section the reader is reminded of the BSI approved method of salmonella isolation and detection. According to this traditional culture method a food sample is pre-enriched in BPW, selectively enriched in broth (e.g. Rappaport-Vassiliadis; RV) and on agar (e.g. XLD) to produce presumptive positive salmonella colonies (Figure 1.8). Further biochemical and serology tests are used to confirm results.

The reader is also reminded of the terms RV-XLD and IMS-XLD. RV-XLD refers to the traditional method of salmonella isolation in RV broth and detection by growth on XLD plates. The term IMS-XLD relates to the standard IMS capture and visualisation of salmonella by growth on XLD agar plates. The 'single bead protocol' is summarised in Figure 3.20. Simply, an aliquot of traditionally enriched food sample is removed for heat treatment. Heat released antigen is detected by the IMS-ELISA technique. If successful this procedure would enable presumptive salmonella positive samples to be detected one day earlier than the IMS method and two days earlier than the traditional culture method. The preliminary food trial was set up according to the procedure described in Section 2.21.2. *S.typhimurium*, *S.virchow*, *S.enteritidis* and *S.give* representing serogroup B, C, D and E respectively were artificially inoculated into SMP and raw chicken samples. SMP represented a food likely to contain low levels of salmonellas. Whereas raw chicken represented a food type likely to contain high numbers of non-salmonella organisms. In addition two serotypes which produced very low absorbance readings were tested: *S.ealing* (NTU) and *S.arizonae* (NTU). To imitate a low level contamination and simulate naturally contaminated samples, foods were inoculated with a tenfold dilution series (1, 10 and 100 cfu/ml) of aged salmonellas. *Salmonella* strains were recovered from pre-enrichment broths by the IMS-ELISA technique, IMS-XLD and traditional RV-XLD. The results are presented in Tables 3.16 and 3.17 for SMP and raw chicken samples respectively. Each

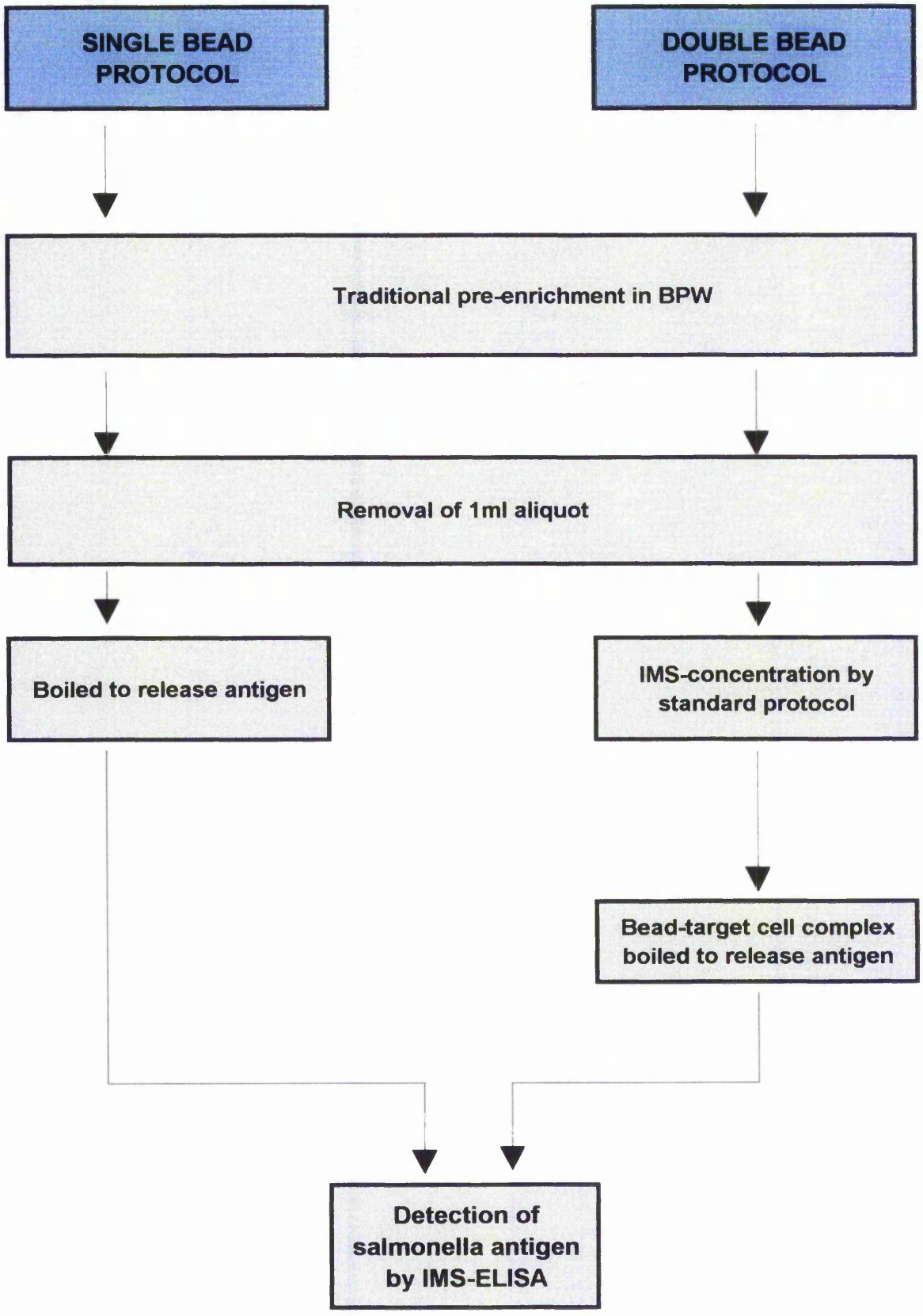


Figure 3.20: Summary of single bead and double bead (without enrichment) salmonella isolation and detection protocols

presents data for the six *Salmonella* serotypes according to predicted inoculum size and detection procedure. By combining the results of all the detection methods, all SMP samples with the exception of the negative control were positive for *Salmonella* spp. (Table 3.16). Traditional RV-XLD and standard IMS-XLD detected the presence of *Salmonella* spp. in all artificially inoculated SMP samples and the absence of *Salmonella* spp. from non-inoculated samples. Based on a threshold value of OD_{405nm} 0.35, the IMS-ELISA detected *Salmonella* spp. in all 18 positive samples. However two false positives were recorded for the uninoculated SMP controls. These M105 antibody cross-reactive bacteria could not be identified because samples were destroyed by boiling in order to release the antigen for the IMS-ELISA detection.

By combining the results of all the detection methods all raw chicken samples with the exception of the negative controls were positive for *Salmonella* spp. (Table 3.17). The IMS-XLD method detected *Salmonella* spp. in all artificially inoculated raw chicken samples (18/18). However two false positive results were recorded when *Proteus mirabilis* was recovered by the IMS-XLD technique from unspiked samples. Traditional enrichment in RV broth detected *Salmonella* spp. in 17/18 artificially inoculated samples but failed to detect low levels of *S.arizonae* (NTU). Moreover the RV-XLD method did not detect salmonella in the uninoculated controls. A 48h enrichment in RV broth was required to detect *S.enteritidis* at 1 cell inoculum size and *S.enteritidis* and *S.arizonae* (NTU) 10 cell inoculum size. The IMS-ELISA detected *S.give* at all three inoculum levels. In addition *S.typhimurium* and *S.virchow* were detected at 100 and 10 cell inoculum sizes; and *S.enteritidis* at 100 cell inoculum size. However 10/18 false negative results were recorded. It should be noted that aliquots of pre-enriched samples congealed on boiling. This meant that the beads could not move freely through the semi-solid material and could not be concentrated by application of the magnet. Thus the majority of the beads were discarded in the supernatant. This confirms that the single bead IMS-ELISA protocol could not be used to recover *Salmonella* spp. from raw chicken (pre-enriched in BPW) samples or presumably any food with a high fat content which was likely to congeal on boiling.

Table 3.16: The isolation of *Salmonella* strains from skimmed milk powder-buffered peptone water pre-enrichment broths by IMS-ELISA, IMS-XLD and RV-XLD

Serotype	Predicted inoculum size (cfu/250ml)	Salmonella detection procedure		
		IMS-ELISA (OD_{405nm}[a])	IMS-XLD	RV-XLD 24h (48h)
S.typhimurium	100	+ (1.83)	+	+
	10	+ (1.87)	+	+
	1	+ (1.82)	+	+
S.virchow	100	+ (1.81)	+	+
	10	+ (1.83)	+	+
	1	+ (0.93)	+	+
S.enteritidis	100	+ (1.82)	+	+
	10	+ (1.79)	+	+
	1	+ (1.42)	+	+
S.give	100	+ (1.86)	+	+
	10	+ (1.84)	+	+
	1	+ (1.61)	+	+
S.ealing (NTU)	100	+ (0.61)	+	+
	10	+ (0.44)	+	+
	1	+ (0.44)	+	+
S.arizonae (NTU)	100	+ (0.92)	+	+
	10	+ (0.75)	+	+
	1	+ (0.63)	+	+
Uninoculated control	0	+ (0.74)[b]	-	- (-)
	0	+ (0.53)[b]	-	- (-)

[a], mean of 2 replicates; [b], unidentified cross-reactive bacteria; +, salmonella positive; -, salmonella negative; + and - IMS-ELISA results recorded based on a threshold value of OD_{405nm} 0.35; no antigen negative control mean absorbance value OD_{405nm} 0.23 where n=2; positive control (10^9 cfu/ml heat treated *S.typhimurium*) OD_{405nm} 1.80 where n=2

Table 3.17: The isolation of *Salmonella* strains from raw chicken-buffered peptone water pre-enrichment broths by IMS-ELISA, IMS-XLD and RV-XLD

Serotype	Predicted inoculum size (cfu/250ml)	Salmonella detection procedure		
		IMS-ELISA (OD_{405nm}[a])	IMS-XLD	RV-XLD 24h (48h)
S.typhimurium	100	+ (0.83)	+	+
	10	+ (0.67)	+	+
	1	- (0.29)	+	+
S.virchow	100	+ (0.44)	+	+
	10	+ (0.37)	+	+
	1	- (0.28)	+	+
S.enteritidis	100	+ (0.72)	+	+
	10	- (0.32)	+	- (+)
	1	- (0.34)	+	- (+)
S.give	100	+ (1.14)	+	+
	10	+ (1.12)	+	+
	1	+ (0.36)	+	+
S.ealing (NTU)	100	- (0.24)	+	+
	10	- (0.26)	+	+
	1	- (0.24)	+	+
S.arizonae (NTU)	100	- (0.27)	+	+
	10	- (0.24)	+	- (+)
	1	- (0.34)	+	- (-)
Uninoculated control	0	- (0.23)	+[b]	- (-)
	0	- (0.20)	+[b]	- (-)

[a], mean of 2 replicates; [b], false positive confirmed as *Proteus mirabilis*; +, salmonella positive; -, salmonella negative; + and - IMS-ELISA results recorded based on a threshold value of OD_{405nm} 0.35; no antigen negative control mean absorbance value OD_{405nm} 0.23 where n=2; positive control (10^9 cfu/ml heat treated *S.typhimurium*) OD_{405nm} 1.80 where n=2

3.8 THE DEVELOPMENT OF A DOUBLE BEAD PROTOCOL

3.8.1 *THE DOUBLE BEAD (WITHOUT ENRICHMENT) PROTOCOL*

Preliminary food trial results indicated that pre-enriched samples such as raw chicken which have a high fat content could not be boiled (heat treated) directly. Therefore a double bead system was developed whereby the IMS technique was first used to capture live *Salmonella* spp.. Subsequently the bead bound antigen was released by heat treatment and detected by the IMS-ELISA method.

Representatives of serogroup B (*S.typhimurium*), C (*S.virchow*), D (*S.enteritidis*), E (*S.give*), O (*S.ealing*; NTU) and 51 (*S.arizonae*; NTU) were grown in BPW as standard. Cells, decimally diluted in BPW to 10^2-10^9cfu/ml, were assayed as per the double bead (without enrichment) protocol summarised in Figure 3.20. In brief, a 1ml aliquot was removed for IMS. Captured cells were concentrated into a 50μl volume. From this, a 25μl volume of bead bound salmonellas were plated onto XLD as per the standard IMS-XLD method. The remaining 25μl was diluted with 75μl TBS and heat treated by boiling at 100°C for 30min to release antigen. Heat treated beads were concentrated by application of the magnet and the supernatant was analysed by the IMS-ELISA with AP visualisation (Section 2.11.2). The positive and negative IMS-ELISA controls were boiled *S.typhimurium* cells (10^9 cfu/ml) and TBS respectively.

Table 3.18 presents data for the six *Salmonella* serotypes according to predicted inoculum size and detection method. The threshold value was calculated at OD_{405nm} 0.44. Hence the sensitivity limit for the double bead IMS-ELISA was determined to be $\geq 10^8$ cfu/ml depending upon serotype. *S.ealing* and *S.arizonae* were not detected at any cell level. The IMS-XLD method confirmed that *Salmonella* strains were captured at all cell levels by IMS with the exception of *S.enteritidis* 10^2 cfu/ml. These results indicate that heat treatment did not release all bead bound antigen from the first bead. Hence the antigen was not present in sufficient amounts in the supernatant to be detected by the IMS-ELISA.

Table 3.18: The detection of IMS captured *Salmonella* strains by ELISA and XLD to evaluate the double bead (without enrichment) protocol

	S.typhimurium		*S.virchow*		*S.enteritidis*		*S.give*		*S.ealing* (NTU)		*S.arizonae* (NTU)	
	Detection method		Detection method		Detection method		Detection method		Detection method		Detection method	
Predicted cell number (cfu/ml)	ELISA (OD_{405nm})	XLD	ELISA (OD_{405nm})	XLD	ELISA (OD_{405nm})	XLD	ELISA (OD_{405nm})	XLD	ELISA (OD_{405nm})	XLD	ELISA (OD_{405nm})	XLD
10^9	+ (0.98)	+	+ (0.48)	+	+ (0.58)	+	+ (0.73)	+	- (0.40)	+	- (0.33)	+
10^8	- (0.37)	+	- (0.18)	+	+ (0.46)	+	+ (0.45)	+	- (0.26)	+	- (0.29)	+
10^7	- (0.24)	+	- (0.33)	+	- (0.27)	+	- (0.30)	+	- (0.27)	+	- (0.24)	+
10^6	- (0.15)	+	- (0.15)	+	- (0.22)	+	- (0.17)	+	- (0.17)	+	- (0.18)	+
10^5	- (0.18)	+	- (0.18)	+	- (0.21)	+	- (0.26)	+	- (0.21)	+	- (0.20)	+
10^4	- (0.19)	+	- (0.15)	+	- (0.20)	+	- (0.22)	+	- (0.17)	+	- (0.20)	+
10^3	- (0.18)	+	- (0.18)	+	- (0.18)	+	- (0.23)	+	- (0.20)	+	- (0.21)	+
10^2	- (0.28)	+	- (0.30)	+	- (0.18)	-	- (0.26)	+	- (0.22)	+	- (0.21)	+

+, salmonella positive; -, salmonella negative; + and - IMS-ELISA results recorded based on a threshold value of OD_{405nm} 0.44; no antigen negative control mean absorbance value OD_{405nm} 0.27 where n=8; positive control (10^9 cfu/ml heat treated *S.typhimurium*) OD_{405nm} 1.46 where n=8

3.8.1.1 *Investigations into the effect of heat treatment on the release of bead bound antigen*

The IMS-ELISA method detected $\geq 10^8$cfu/ml salmonella cells using the double bead (without enrichment) protocol (Table 3.18). However preliminary investigations had shown that the IMS-ELISA detection method was capable of detecting $\geq 10^6$ cfu/ml (Figure 3.17). Thus the inclusion of an additional batch of beads reduced the sensitivity 100-fold. These results indicated that heat treatment of bead captured salmonella did not release all antigen into the supernatant to be detected by the IMS-ELISA. Therefore studies were carried out to investigate how much antigen remained attached to the primary batch of beads after treatment. The investigation was restricted to two *Salmonella* serotypes. *S.typhimurium* and *S.give* were analysed because they produced the two highest absorbance values in the double bead (without enrichment) protocol (Table 3.18).

Traditionally enriched cells were adjusted to 10^9 cfu/ml in TBS and assayed according to the double bead (without enrichment) protocol (Figure 3.20). Bead captured salmonella were resuspended in 200μl TBS and divided into two prior to heat treatment (100°C for 30min). Beads were concentrated by application of the magnet and the supernatant removed. Antigen that remained attached to the heat treated beads was detected with M105 antibody by ELISA. Heat released antigen present in the supernatant was captured by a second batch of beads and analysed by the IMS-ELISA with AP visualisation.

Similar absorbance readings were produced by the bead bound and heat released antigen (Table 3.19). These results suggested that approximately 50% of the antigenicity remained attached to the primary batch of beads despite heat treatment. The sum of the absorbance values from the bead bound and heat released antigen exceeded that of the positive control for both serotypes. This was probably due to the limitations of the ELISA plate reader. Previous experimentation has shown that absorbance readings of OD_{405nm} 2.50 were maximal due to the limitations of the plate reader (Figure 3.18). It should also be noted that the readings produced by the heat released antigen (OD_{405nm} 1.67 and 1.96 for *S.typhimurium* and *S.give* respectively) was approximately double that of the previous experiment for (OD_{405nm} 0.98 and 0.73 for *S.typhimurium* and *S.give* respectively). Since the level of antigen was the same (10^9 cfu/ml) and experiments were not carried out over a period of years, the reason for the difference is unclear.

Table 3.19: The effect of boiling on the release of bead bound M105 antigen as detected by the IMS-ELISA technique

Serotype	Positive control[a]	Mean[b] absorbance value$_{405nm}$ for	
		Bead bound antigen[c]	Heat released antigen[d]
S.typhimurium	2.54±0.06	1.72±0.59	1.67±0.38
S.give	2.48±0.04	1.88±0.30	1.96±0.40

[a], 10^9 cfu/ml heat treated cells prior to incubation with Prototype beads; [b], of 12 replicates; [c], the antigenicity that remains attached to the beads after boiling; [d], antigen released by boiling (supernatant) and captured by a second aliquot of Prototype beads; ± standard deviation

3.8.2 *DEVELOPMENT OF A DOUBLE BEAD (WITH ENRICHMENT) PROTOCOL*

3.8.2.1 *Investigations to establish a post enrichment incubation time*

The detection limit for the IMS-ELISA employed in the double bead (without enrichment) protocol was $\geq 10^8$ cfu/ml depending on the serotype tested (Table 3.18). When food testing, the number of salmonellas in the pre-enrichment broth may vary considerably and would never realistically exceed 10^9 cfu/ml. Hence a commercial double bead (without enrichment) protocol would produce unacceptably high numbers of false negative results. Therefore investigations were carried out to ascertain whether the inclusion of an enrichment step would improve the efficacy of the double bead system. Glucose nutrient (GN) broth with novobiocin (nGN broth) was used as the enrichment medium to promote the growth of salmonella and inhibit the growth of non-salmonella organisms such as *Citrobacter* spp..

A single *Salmonella* serotype (*S.typhimurium*) was assayed. Cultures were grown in BPW as standard. Cell numbers were adjusted 10^2, 10^3 and 10^4 cfu/ml in nGN broth and confirmed by plate count on TSA. All nGN broths were incubated at 42°C according to the manufacturer's instructions. After 2, 4 and 6h aliquots were removed for plate count on TSA. The results are presented in Figure 3.21. A 6h post enrichment in nGN broth as required for an initial inoculum of 10^2 salmonella cells to exceed the 10^5-10^6 cfu/ml detection limit of the IMS-ELISA (Figure 3.17) and was therefore chosen as the incubation time for the double bead with enrichment protocol. A summary of the final proposed

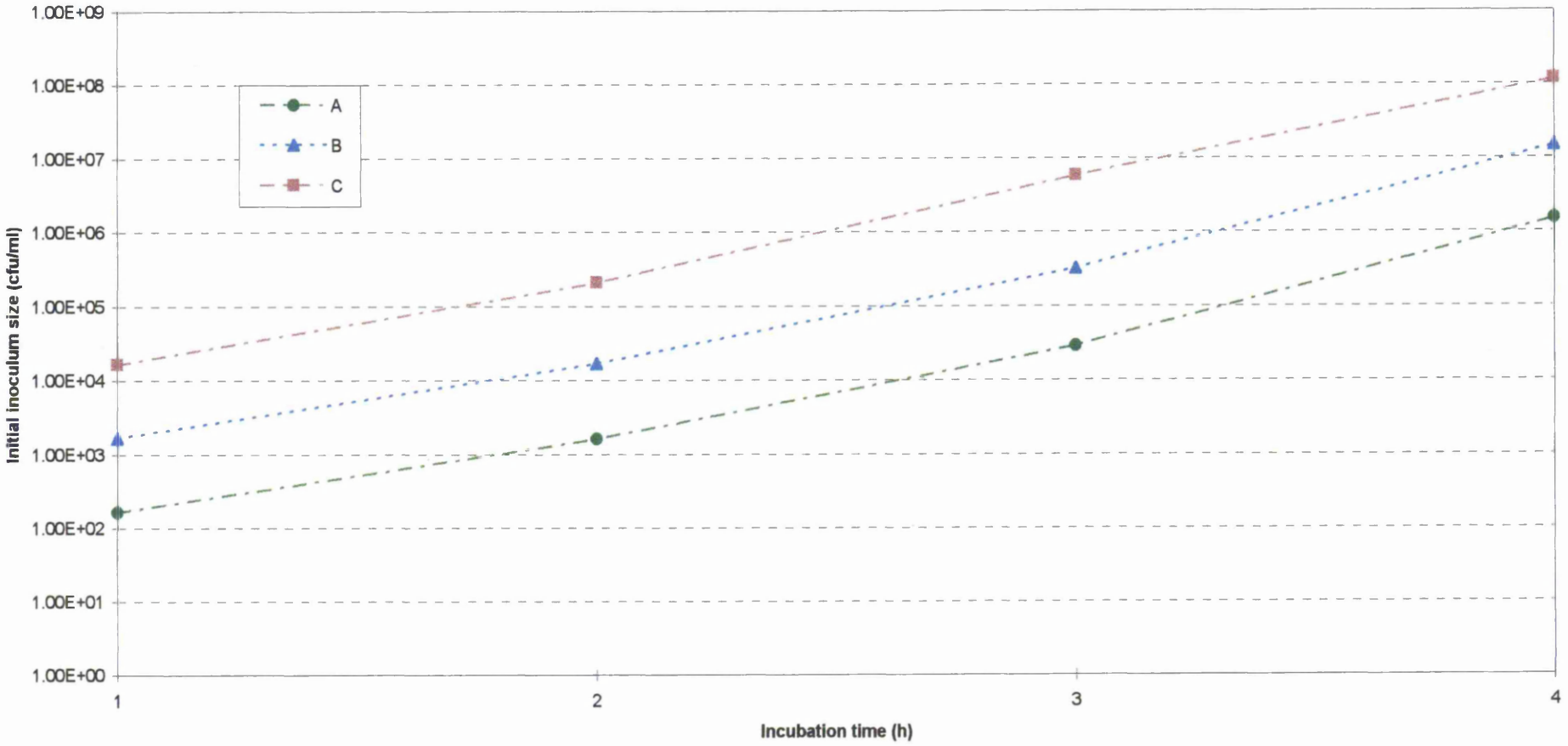


Figure 3.21: The growth of *S.typhimurium* in nGN broth inoculum size 10^2 (A), 10^3 (B) and 10^4(C) cfu/ml (Mean of 3 replicates)

double bead protocol including a 6h enrichment in nGN broth, referred to as the double bead (with enrichment) protocol, is shown in Figure 3.22.

3.8.2.2 *Evaluation of the double bead (with enrichment) protocol of salmonella isolation with IMS-ELISA detection by preliminary food trial*

The design of the preliminary food trial was the same as for the evaluation of the single bead protocol with respect to the *Salmonella* serotypes and inoculum levels assayed. To save time only raw chicken samples, which represented the more difficult food type, was tested. If the double bead (with enrichment) protocol was successful at isolating and detecting very low levels of salmonella cells from the raw chicken-BPW samples, a large scale food trial would be undertaken which would test a wide variety of food types including SMP.

Cultures were grown as standard and cell numbers were adjusted in BPW. *S.typhimurium*, *S.virchow*, *S.enteritidis*, *S.give*, *S.ealing* (NTU) and *S.arizonae* (NTU) were artificially inoculated into raw chicken samples at three inoculum levels (100, 10 and 1 cell). *Salmonella* strains were recovered from raw chicken-BPW by traditional RV-XLD, standard IMS-XLD and the double bead (with enrichment) protocol with IMS-ELISA and IMS-XLD detection (Section 2.21.3): in brief, a 1ml aliquot of pre-enriched raw chicken-BPW was removed for IMS. Captured cells were concentrated into a 50μl volume. From this, a 25μl volume of bead bound salmonellas were plated onto XLD. The remaining 25μl was post-enriched in nGN broth. After a 6h incubation, a 100μl volume was removed and heat treated by boiling. Heat released antigen was detected by the IMS-ELISA.

Table 3.20 presents data for the recovery of six *Salmonella* serotypes according to predicted inoculum size and detection procedure. By combining the results of all methods it was shown that all samples with the exception of *S.enteritidis* 1 cell inoculum size and the two negative controls were positive for *Salmonella* spp. (i.e. 17/20). The traditional RV-XLD method detected *Salmonella* spp. in 14/17 samples. Three false negative results were recorded: *S.enteritidis* 100 cells and *S.arizonae* 10 and 100 cell inoculum sizes. The IMS-XLD method detected *Salmonella* spp. in 16/17 samples, failing to detect *S.arizonae* at the 1 cell inoculum size. Four false positives (confirmed to be *Proteus mirabilis*) were

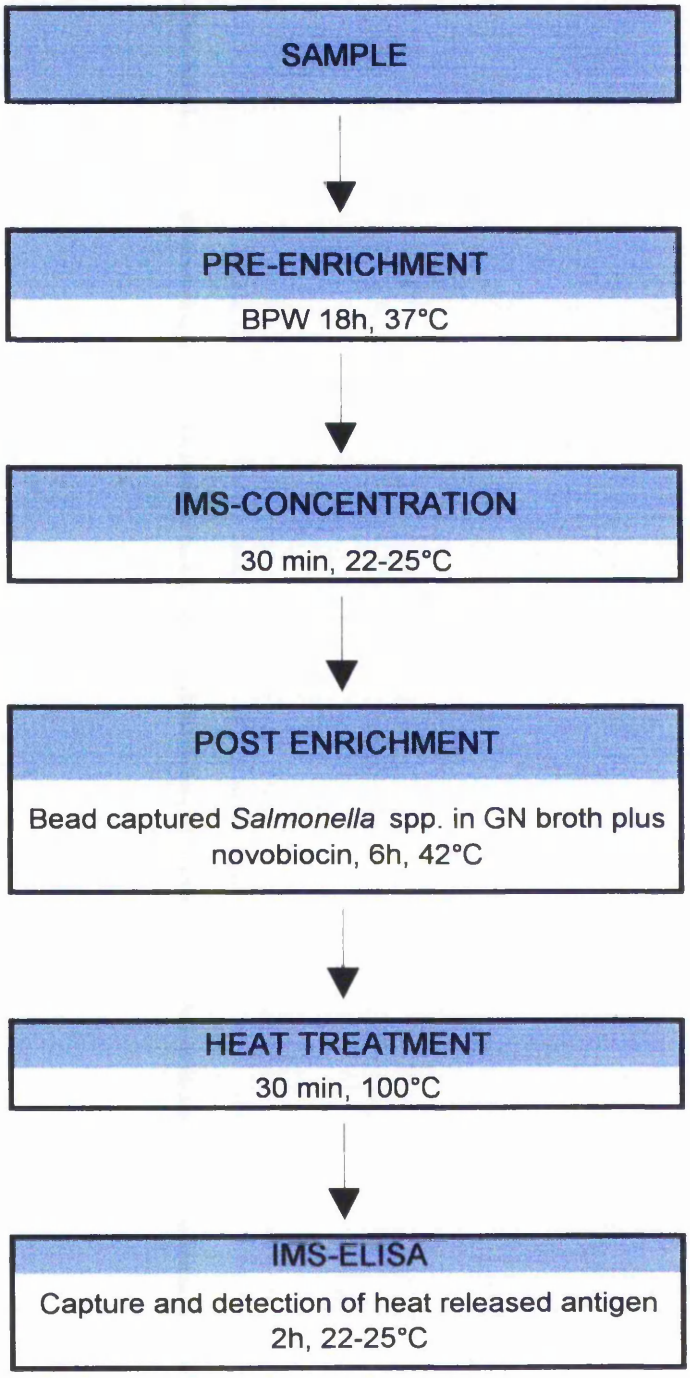


Figure 3.22: Final proposed double bead (with enrichment) protocol for the isolation and detection of salmonella from ready-to-eat foods

Table 3.20: The isolation and detection of *Salmonella* strains from raw chicken-buffered peptone water pre-enrichments broths by IMS-ELISA (following IMS and nGN enrichment), IMS-XLD and RV-XLD

		Isolation, capture and detection procedure					
		Following IMS nGN enrichment		IMS-XLD	RV[a]-XLD		All methods
Serotype	Predicted inoculum size	IMS-ELISA (OD_{405nm})	IMS-XLD		24h	(48h)	
S.typhimurium	100	+ (1.54)	+	+	+		+
	10	+ (1.25)	+	+	+		+
	1	+ (1.40)	+	+	+		+
S.virchow	100	+ (0.40)	+	+	+		+
	10	- (0.27)	+	+	+		+
	1	- (0.18)	+	+	+		+
S.enteritidis	100	+ (1.46)	+	+[b]	-	(-)	+
	10	+ (1.04)	+	+	+		+
	1	- (0.23)	-	+[c]	-	(-)	-
S.give	100	+ (1.73)	+	+	+		+
	10	+ (1.76)	+	+	+		+
	1	+ (0.75)	+	+	+		+

Serotype (Source)	Predicted inoculum size	Isolation, capture and detection procedure					
		Following IMS nGN enrichment		IMS-XLD	RV-XLD		All methods
		IMS-ELISA (OD_{405nm})	IMS-XLD		24h	(48h)	
S.ealing (NTU)	100	- (0.25)	+	+	+		+
	10	- (0.25)	+	+	+		+
	1	- (0.27)	+	+	+		+
S.arizonae (NTU)	100	- (0.19)	+	+[b]	-	(-)	+
	10	- (0.26)	+	+[b]	-	(-)	+
	1	- (0.22)	+	+[c]	+[b]		+
Uninoculated control	0	- (0.25)	-	-	-	(-)	-
	0	- (0.26)	-	-	-	(-)	-

[a], RV restreaked after 48h incubation only if 24h streak was negative for salmonella; [b], confirmed salmonella positive; [c], confirmed false positive *Proteus mirabilis*; +, salmonella positive; -, salmonella negative; + and - IMS-ELISA results recorded based on a threshold value of OD_{405nm} 0.30; no antigen negative control mean absorbance value OD_{405nm} 0.23 where n=2; positive control (10^9 cfu/ml heat treated *S.typhimurium*) OD_{405nm} 1.70 where n=2

also recorded. The threshold value for the IMS-ELISA was determined at OD_{405nm} 0.30. Hence the IMS-ELISA detected *Salmonella* strains in 9/17 samples. False negatives were produced by *S.virchow*, *S.ealing* and *S.arizonae* at all three inoculum levels. Growth of typical salmonella colonies on XLD plates from loop inoculated nGN broths confirmed the presence of *Salmonella* spp. in all nGN broths with the exception of *S.enteritidis* 1 cell inoculum size and the two negative controls. This suggested that the number of salmonellas in the nGN broths were below the 10^5 cfu/ml detection limit of the IMS-ELISA.

3.9 LOCALISATION OF THE M105 EPITOPE WITHIN THE LPS MOLECULE

Although similar numbers of cells were analysed the M105 antibody exhibited a wide range of reactivities with the *Salmonella* strains tested by dot blot assay (Table 3.9). This could not be attributed to large differences in the amount of LPS isolated from cells, since this is reportedly small (3-8% of the dry weight; Rietschel *et al.*, 1984). Based on the assumption that all *Salmonella* spp. share a common core (Jansson *et al.*, 1981; Westphal *et al.*, 1983; Jann and Jann 1984), the range of M105 antibody reactivity may have been due to differences in the O-chain length and sugar type causing a steric hindrance to M105 antibody binding. Therefore LPS isolated from *Salmonella* strains were analysed by SDS-PAGE and immunoblot assay. The SDS-PAGE technique was used to observe the heterogeneity of LPS O-chain length. This method coupled with the immunoblot assay of the electrophoretically transferred LPS profiles enabled the localisation of the M105 antibody epitope with respect to LPS of varying O-chain length.

LPS was extracted and solubilised from whole cell lysates of *Salmonella* and non-*Salmonella* strains by the proteinase K method (Section 2.12.1). Isolated purified LPS were obtained from Sigma and Dr Otto Holst of the Forschungs Zentrum, Borstel, Germany. The isolated LPS samples were solubilised according to the Public Health Laboratory procedure (Section 2.12.1). All solubilised LPS samples were analysed by SDS-PAGE (Section 2.12.2). The volume of solubilised LPS analysed was ascertained by titration. Purified LPS from *S.typhimurium* wild type and Ra mutant strains were run as standards in each gel to ensure adequate colour development and to assess the reproducibility of the techniques. The LPS profiles were carbohydrate silver stained by a modified Tsai and Frasch (1982) method described in Section 2.13.1. The electrophoresed LPS was transferred to nitrocellulose membrane by a semi-dry transfer method (Section 2.14) and analysed by immunoblot assay (Section 2.9).

3.9.1 *ANALYSIS OF LPS FROM SALMONELLA STRAINS AND OTHER ENTEROBACTERIACEAE BY SDS-PAGE*

The SDS-PAGE analysis of bacterial strains produced unique LPS banding profiles. Examples of which are shown in Figure 3.23. The results of the SDS-PAGE analysis of *Salmonella* strains have been summarised with respect to the number and pattern of bands in the profile. QuantiScan software was used to analyse the LPS banding profiles. From this analysis the number of bands in a profile could be tentatively determined and varied from 10 to >30. The *Salmonella* strains could be divided into groups based on the number of LPS bands present in a profile. The results are shown in Table 3.21. The majority of strains (26) produced profiles with typically 21-30 bands. Eleven strains produced profiles with 10-20 bands. Of the remaining five strains, four exhibited profiles with greater than 30 bands. Whereas the profile of a strain of *S.cerro* was unclear due to overloading. Time restrictions prevented further analysis.

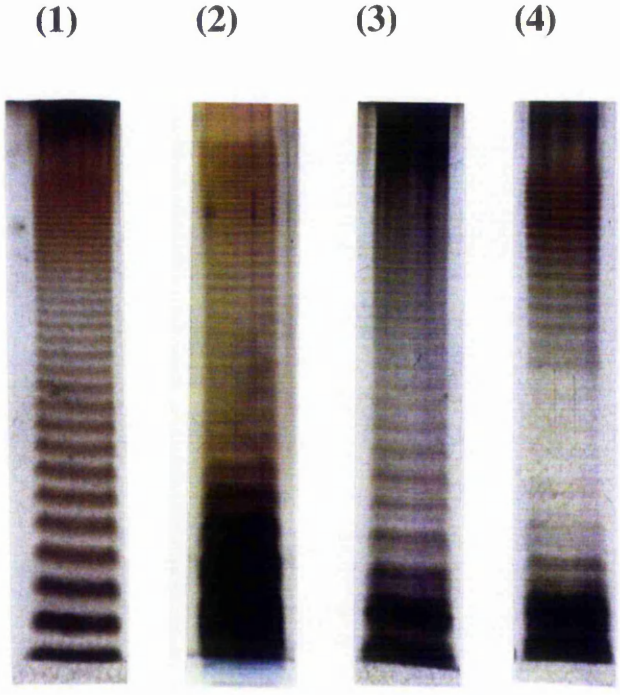


Figure 3.23: SDS-PAGE of LPS from *S.typhimurium* (Profile A; Lane 1); *S.agona* (Profile B; Lane 2); *S.adelaide* (Profile C; Lane 3) and *S.arizonae* (NTU; Profile D; Lane 4)

The LPS profiles could be divided into groups designated profile A, B, C and D (Table 3.22). Examples of each profile are shown in Figure 3.23. In summary profile A

Table 3.21: The number of bands in the LPS profiles of *Salmonella* strains

Serogroup	Serotype (reference)	Number of bands[a]
B	*S.abortis equi* (L-5886)	21-30
	S.agona	21-30
	S.bredeney	21-30
	S.gallinarum	21-30
	S.saint paul	21-30
	S.typhimurium	>30
	S.typhimurium (L-6511)	21-30
	S.typhimurium (1135)	21-30
	S.typhimurium (1835)	21-30
C_1	*S.infantis*	21-30
	S.livingstone	21-30
	S.virchow	21-30
C_2	*S.hadar*	21-30
	S.manchester	>30
	S.newport	10-20
C_3	*S.kentucky*	>30
D_1	*S.dublin*	10-20
	S.enteritidis	10-20
	S.enteritidis (L-6011)	21-30
	S.panama	>30
E_1	*S.give*	10-20

Serogroup	Serotype (reference)	Number of bands[a]
E_4	*S.senftenberg*	21-30
F	*S.arizonae* (1823)	21-30
	S.arizonae (10455)	21-30
G	*S.arizonae* (10466)	21-30
K	*S.cerro* (10390)	unclear
L	*S.minnesota*	21-30
	S.minnesota (L-6261)	21-30
N	*S.godesberg* (1140)	10-20
	S.godesberg (1431)	10-20
O	*S.adelaide*	21-30
	S.adelaide (1464)	21-30
	S.ealing (NTU)	21-30
	S.ealing (Canada)	21-30
U	*S.milwaukee* (1139)	10-20
V	*S.nirembe* (1660)	10-20
X	*S.bere* (10392)	10-20
	S.bere (10427)	10-20
Y	*S.djakarta* (10403)	21-30
51	*S.arizonae* (NTU)	21-30
	S.arizonae (1803)	10-20
63	*S.arizonae* (10463)	21-30

[a], number of bands determined by QuantiScan analysis of silver stained LPS profiles; unclear, due to overloading

Table 3.22 : The LPS profile type of salmonella LPS

Serogroup	Serotype (reference)	LPS profile type[a]
B	*S.abortis equi*	D
	S.agona	B
	S.bredeney	B
	S.gallinarum	C
	S.saint paul	B
	S.typhimurium	A
	S.typhimurium (L-6511)	A
	S.typhimurium (1135)	A
	S.typhimurium (1835)	A
C_1	*S.infantis*	D
	S.livingstone	D
	S.virchow	D
C_2	*S.hadar*	A
	S.manchester	C
	S.newport	C
C_3	*S.kentucky*	C
D_1	*S.dublin*	C
	S.enteritidis	C
	S.enteritidis (L-6011)	B
	S.panama	D
E_1	*S.give*	C

Serogroup	Serotype (reference)	LPS profile type[a]
E_4	*S.senftenberg*	B
F	*S.arizonae* (1823)	D
	S.arizonae (10455)	D
G	*S.arizonae* (10466)	D
K	*S.cerro* (10390)	unclear
L	*S.minnesota*	D
	S.minnesota (L-6261)	D
N	*S.godesberg* (1140)	C
	S.godesberg (1431)	C
O	*S.adelaide*	B
	S.adelaide (1464)	B
	S.ealing (NTU)	B
	S.ealing (Canada)	B
U	*S.milwaukee* (1139)	B
V	*S.nirembe* (1660)	C
X	*S.bere* (10392)	D
	S.bere (10427)	D
Y	*S.djakarta* (10403)	C
51	*S.arizonae* (NTU)	D
	S.arizonae (1803)	C
63	*S.arizonae* (10463)	D

[a], LPS profile type: A, bands throughout gel of similar intensity; B, more intense bands in the lower half of the gel; C, concentration of bands at the bottom of the gel; D, concentration of bands at the bottom and top of the gel;
unclear, due to over loading

included five strains which expressed LPS of short, medium and long chains (similarly intense bands throughout the profile; Lane 1). Those strains whose LPS profiles were made up of predominantly short to medium chains were termed B (more intense bands in the lower half of the gel; Lane 2; ten strains). Twelve strains exhibited profile C with a concentration of short chains (bands at the bottom of the gel; Lane 3). Finally profile D strains produced LPS which was predominantly short chain with a concentration of long chains (bands that were concentrated at the bottom and towards the top of the gel; Lane 4; 14 strains). The profile of a strain of *S.cerro* was not designated a group letter because the gel was overloaded.

LPS molecular weight markers are not commercially available. Although there are a number of reports which have used protein molecular weight markers, their use is not routine. Tsai and Frasch (1982) suggested that protein molecular weight markers were not suitable for estimating the size of LPS in SDS-PAGE. In the SDS-PAGE of proteins separation is dependent on the molecular size of the protein. For LPS, migration and separation is probably due to differences in their charge density as well as their molecular size (Duchesne *et al.*, 1988). Hence in the present study protein molecular weight markers were not used, subsequently the molecular weight of individual LPS bands could not be calculated. For the purpose of the present study, estimating the molecular weight of the LPS bands was not a priority. However it was important to realise that minor differences in the structure of the core oligosaccharide of rough mutant strains, resulted in a differential migration of the R-type LPS. Rough mutant strains are defective in core biosynthesis and as such contain various portions of core linked to lipid A (Figure 1.3).

Figure 3.24 shows the differential movement of rough mutant strains of *S.typhimurium* (Ra, Rb_1, Rc, Re). The LPS banding profile of wild type *S.typhimurium* has been included for comparison. By observing the fastest moving band only, the more incomplete the LPS core, the further down the gel it migrated. The LPS from the Re mutant migrating the furthest. The R-type LPS from the wild and the Ra rough mutant strains migrated the same amount suggesting that the R-type LPS of wild type *S.typhimurium* was composed of Ra type core. It should also be noted that the rough mutant strains produced multiple band profiles instead of the expected single band. However this phenomenon was not investigated further.

Doublet bands were not present in the LPS profiles of all *Salmonella* strains tested. LPS

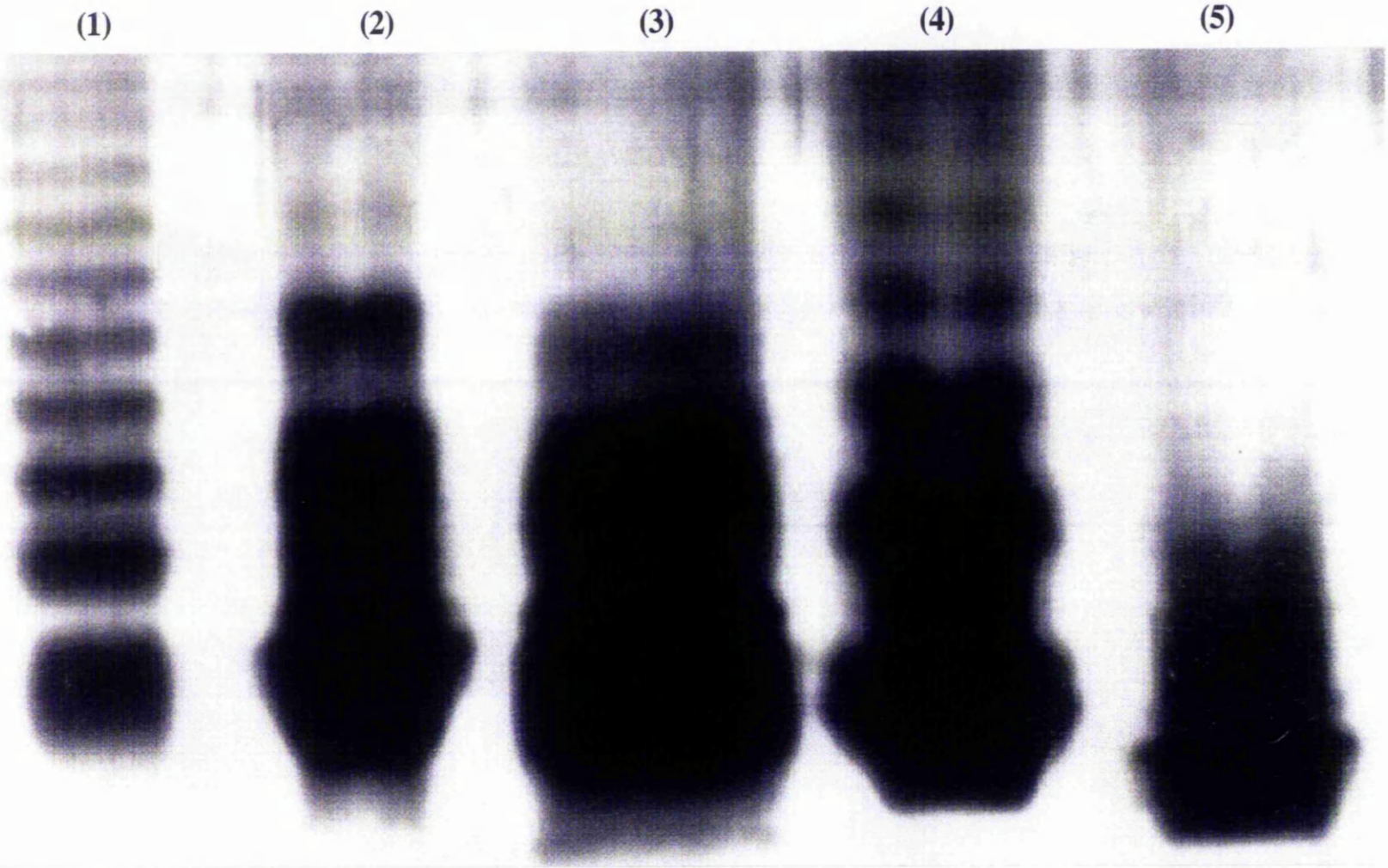


Figure 3.24: Differential migration of the R-type LPS from wild type (Lane 1) and rough mutant type Ra (Lane 2), Rb_1 (Lane 3), Rc (Lane 4) and Re (Lane 5) *S.typhimurium*

isolated from *S.arizonae* (NTU), *S.ealing* (NTU and Canada), *S.hadar*, *S.infantis*, *S.virchow*, *S.gallinarum*, *S.kentucky*, *S.livingstone* and *S.manchester* showed doublet bands. However the doublets were not visible on every gel. In overloaded gels the doublets merged into a single band (Figure 3.25, Lane 1). When a smaller amount of LPS was applied to the gel both the R- and SR-type LPS were clearly made up of doublet bands (Figure 3.25, Lane 2). The doublet bands were stained the same red/brown colour as the non-doublet bands. In most instances the lower band of the doublet was darker and thicker than the upper band.

The M105 antibody exhibited a limited cross-reaction with 11 non-*Salmonella* strains tested by dot blot assay (Table 3.10). Therefore it was plausible that these strains shared a common core structure. Hence, in addition to *Salmonella* strains, the LPS from other *Enterobacteriaceae* were analysed by SDS-PAGE and immunoblot assay. These included eight strains of *C.freundii*, and a strain each of *Enterobacter agglomerans*, *E.coli* and *Shigella flexneri*. Examples of the SDS-PAGE profiles of non-salmonella *Enterobacteriaceae* are shown in Figure 3.26. Unique SDS-PAGE banding patterns were produced with the exception that identical profiles were produced by *C.freundii* reference number 4/840 (API 20E 1744553) and 4/841 (API 20E 1704553) (Figure 3.27). Although these *C.freundii* were isolated from two different food sources, it seemed likely that they were in fact the same strain. The LPS profiles from the eight *C.freundii* strains tested could be divided into three distinct patterns (Figure 3.27). The NTU laboratory strain expressed LPS that was made up of short chains (Lane 1). Five strains (4/840, 4/841, 4/1155, 4001, 4002) expressed LPS which was both long and short chain (Lanes 2 and 3). Whereas strains 5/308 and 5/785 expressed LPS which was predominantly short chain with some medium to long chain LPS (Lane 4).

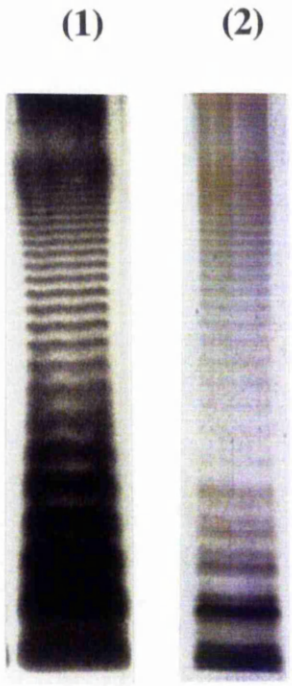


Figure 3.25: SDS-PAGE of LPS from *S.hadar* showing the absence (Lane 1) and presence (Lane 2) of doublet bands

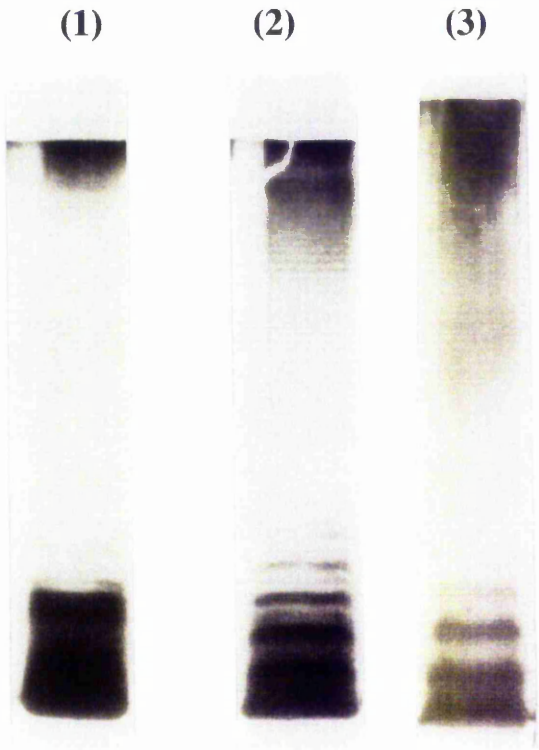


Figure 3.26: SDS-PAGE of LPS from *E.coli* (Lane 1), *Enterobacter agglomerans* (Lane 2) and *Shigella flexneri* (Lane 3)

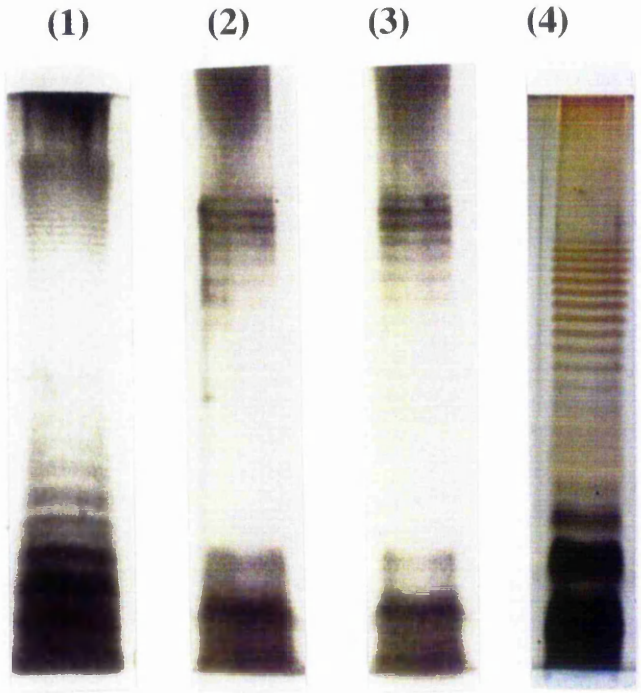


Figure 3.27: SDS-PAGE of LPS from *C.freundii* reference code NTU (Lane 1), 4/840 (Lane 2), 4/841 (Lane 3) and 5/308 (Lane 4)

3.9.2 *THE IMMUNOBLOT ASSAY OF SDS-PAGE DIFFERENTIATED ELECTROBLOTTED LPS DETECTED BY THE M105 ANTIBODY*

To help understand the next section the reader is reminded that the LPS from wild strains of bacteria can be divided into R-type LPS, SR-type LPS and S-type LPS. R-type LPS is made up of lipid A and core. SR-type LPS is made up of lipid A, core and a single O-chain repeat unit. Whereas S-type LPS is made up lipid A, core and two or more O-chain repeat units.

The M105 antibody reacted with all *Salmonella* strains but did not react with all LPS molecules (SR- and S-type LPS). The types of reaction that were exhibited could be classified into three groups as follows:

Reaction Type 1:

The M105 antibody reacted with all LPS molecules regardless of O-chain length or exhibited limited reaction with the LPS of very long O-chain length.

Reaction Type 2:

The M105 antibody reacted strongly with R-type LPS and weakly with SR-type LPS or short chain LPS. This weak reaction was often expressed as a smear rather than a band of reaction.

Reaction Type 3:

The M105 antibody reacted weakly with R-type LPS.

Table 3.23 shows the immunoblot reaction types of all *Salmonella* strains tested. Examples of each reaction type are shown in Figure 3.28 with the corresponding silver stained profile. All serotypes belonging to serogroup C_2, C_3, D and E expressed reaction type 1. *Salmonella* serogroup C_1, F and L reacted with short chain LPS only in reaction type 2. While strains belonging to serogroup G, K, O, U, V, X and 63 expressed the third reaction type. Strains of the same serotype in some cases produced different reactions. For example the 1140 strain of *S.godesberg* reacted with R-type LPS only, while the 1431 strain reacted with SR- and R-type LPS. The reduced reactivity of the 1140 strain was not a consequence of insufficient loading of the LPS sample to the SDS-PAGE gel. The NTU strain of serogroup 51 *S.arizonae* reacted with R-type LPS whereas the other strain (1803) failed to react. Moreover M105 antibody did not react with LPS from *S.djakarta*.

In addition to the M105 antibody immunoblot results the reaction with the monoclonal antibody T6 (Tsang *et al.*, 1991b) has been included in Table 3.23 for comparative purposes. While observing these results the reader should bare in mind that, in addition to Ra type core LPS, the M105 antibody reacts with Rb_1 but with a reduced reactivity. In contrast the T6 antibody has been reported to react with Ra type core only (Tsang *et al.*, 1987). Hence by comparing the immunoblot reactivities of the M105 and T6 antibodies the possible core structure (with respect to Ra and Rb_1) of individual strains can be determined. From the results it can be inferred that the LPS core structure of representatives of the most commonly isolated *Salmonella* serogroups B-E were of the Ra or Rb_1 type. Since the immunoblot reaction of individual bands was strong, it is likely that these strains possess typical Ra type core structures. The identity of the core of some strains could be tentatively identified. For example *S.arizonae* (1823) and *S.cerro* (10390) reacted with M105 and T6 antibodies and therefore have Ra type core structures. Both

Table 3.23: The reactivities of a range of *Salmonella* strains with M105 and T6 antibodies by dot blot and immunoblot assay

Serogroup	Serotype (reference)	Immunoblot reaction type		Possible core structure
		M105 antibody	T6 antibody[a]	
B	*S.abortis equi* (L-5886)	1	nd	Ra or Rb_1
	S.agona	1	nd	Ra or Rb_1
	S.bredeney	1	nd	Ra or Rb_1
	S.gallinarum	1	nd	Ra or Rb_1
	S.saint paul	1	nd	Ra or Rb_1
	S.typhimurium	1	nd	Ra or Rb_1
	S.typhimurium (L-6511)	1	nd	Ra or Rb_1
	S.typhimurium (1135)	1	nd	Ra or Rb_1
	S.typhimurium (C5)	1	nd	Ra or Rb_1
C_1	*S.infantis*	2	nd	Ra or Rb_1
	S.livingstone	2	nd	Ra or Rb_1
	S.virchow	2	nd	Ra or Rb_1
C_2	*S.hadar*	1	nd	Ra or Rb_1
	S.manchester	1	nd	Ra or Rb_1
	S.newport	1	nd	Ra or Rb_1
C_3	*S.kentucky*	1	nd	Ra or Rb_1

Serogroup	Serotype (reference)	Immunoblot reaction type		Possible core structure[b]
		M105 antibody	T6 antibody[a]	
D_1	*S.dublin*	1	nd	Ra or Rb_1
	S.enteritidis	1	nd	Ra or Rb_1
	S.enteritidis (L-6011)	1	nd	Ra or Rb_1
	S.panama	1	nd	Ra or Rb_1
E_1	*S.give*	1	nd	Ra or Rb_1
E_4	*S.senftenberg*	1	nd	Ra or Rb_1
F	*S.arizonae* (1823)	2	+	Ra
	S.arizonae (10455)	2	nd	Ra or Rb_1
G	*S.arizonae* (10466)	3	nd	Ra or Rb_1
K	*S.cerro* (10390)	3	+	Ra
L	*S.minnesota*	2	nd	Ra or Rb_1
	S.minnesota (L-6261)	2	nd	Ra or Rb_1
N	*S.godesberg* (1140)	3	-	Rb_1
	S.godesberg (1431)	2	-	Rb_1

[a], results from Tsang *et al.*, 1991b; [b], the monoclonal antibodies T6 and M105 react with Salmonella Ra type core. In addition the M105 anibody reacts with Salmonella Rb_1 core with reduced reactivity. By comparing the immunoblot reactivity of the T6 and M105 antibodies the possible core structure (with respect to Ra and Rb_1) of individual strains can be determined; nd, not done; immunoblot reaction: 1, type one; 2, type two; 3, type three; +, T6 antibody reacted with the LPS isolated from these strains; -, T6 antibody did not react

Table 3.23 continued

Serogroup	Serotype (reference)	Immunoblot reaction type		Possible core structure[b]
		M105 antibody	T6 antibody[a]	
O	*S.adelaide*	3	nd	Ra or Rb_1
	S.adelaide (1464)	3	-	Rb_1
	S.ealing (NTU)	3	nd	Ra or Rb_1
	S.ealing (Canada)	3	nd	Ra or Rb_1
U	*S.milmaukee* (1139)	3	-	Rb_1
V	*S.nirembe* (1660)	3	-	Rb_1
X	*S.bere* (10392)	3	-	Rb_1
	S.bere (10427)	3	-	Rb_1
Y	*S.djakarta* (10403)	no reaction	-	not Ra or Rb_1
51	*S.arizonae* (NTU)	3	nd	Ra or Rb_1
	S.arizonae (1803)	no reaction	-	not Ra or Rb_1
63	*S.arizonae* (10463)	3	-	Rb_1

[a], results from Tsang *et al.*, 1991b; [b], the monoclonal antibodies T6 and M105 react with Salmonella Ra type core. In addition the M105 anibody reacts with Salmonella Rb_1 core with reduced reactivity. By comparing the immunoblot reactivity of the T6 and M105 antibodies the possible core structure (with respect to Ra and Rb_1) of individual strains can be determined; nd, not done; immunoblot reaction: 1, type one; 2, type two; 3, type three; +, T6 antibody reacted with the LPS isolated from these strains; -, T6 antibody did not react

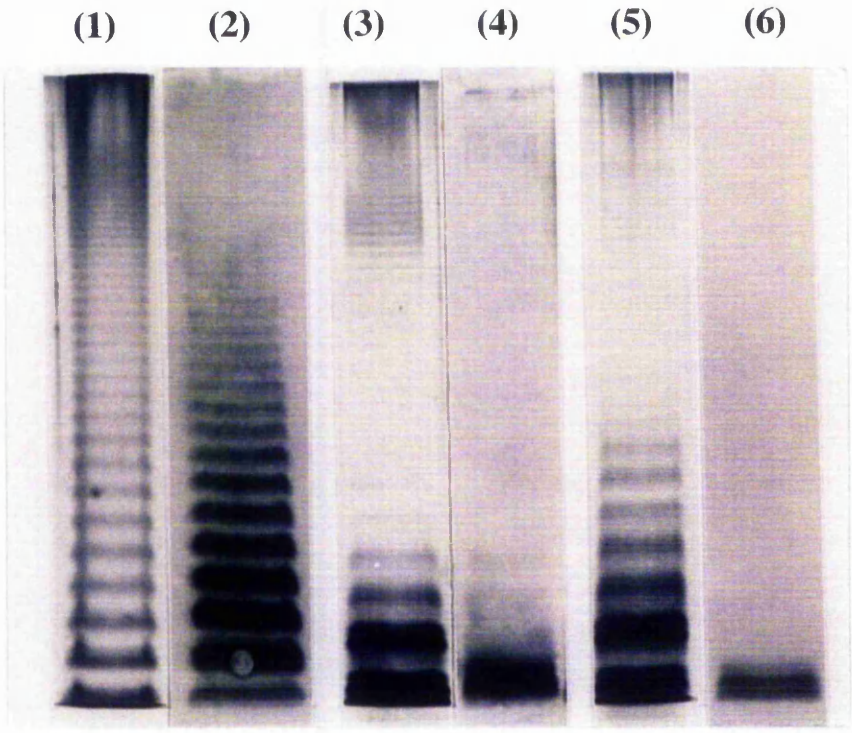


Figure 3.28: The silver stained LPS profiles and corresponding immunoblot assay of *S.typhimurium* (Lane 1 and 2; reaction type 1); *S.virchow* (Lane 3 and 4; reaction type 2) and *S.ealing* (Lane 5 and 6; reaction type 3) (from Mansfield *et al.*, 1996)

strains of *S.godesberg* (1140 and 1431) and *S.arizonae* (10463) reacted with M105 only and hence possess atypical Rb_1 type cores. *S.djakarta* (10403) and *S.arizonae* (1803) failed to react with either antibody. These results confirm the presence of non Ra and Rb_1 type core structures.

M105 antibody failed to react with the LPS profiles of any of the non-salmonella *Enterobacteriaceae* analysed.

3.9.2.1 *Investigations into the effective transference of differentiated LPS from S.ealing*

The lack of M105 antibody reactivity with the S-type LPS of some strains could have been due to non-effective transference. Therefore the transference of differentiated LPS needed to be confirmed. This was achieved by using *Salmonella* serogroup O specific antisera (raised in rabbit) with an AP labelled secondary antibody (goat anti-rabbit).

Proteinase K digested whole cell lysates of wild type strains of *S.ealing* (NTU) and *S.arizonae* (NTU) and pure *S.typhimurium* Ra mutant LPS were differentiated by SDS-

PAGE (Section 2.12.2) and electrophoretically transferred onto nitrocellulose membrane (Section 2.14). Analysis by immunoblot assay was carried out as standard with the core specific M105 antibody (Section 2.9). In addition the *Salmonella* serogroup O specific antisera was tested. Antisera and secondary antibody concentrations were predetermined by titration to be 1/1000 (diluted in 1% BSA in TBS-T).

The LPS gel profile and immunoblot are shown in Figure 3.29. Antisera reacted with all S-type LPS of *S.ealing* confirming that S-type LPS was transferred onto nitrocellulose. No reaction was noted with *S.arizonae* and *S.typhimurium* LPS confirming that the antisera was serogroup O specific.

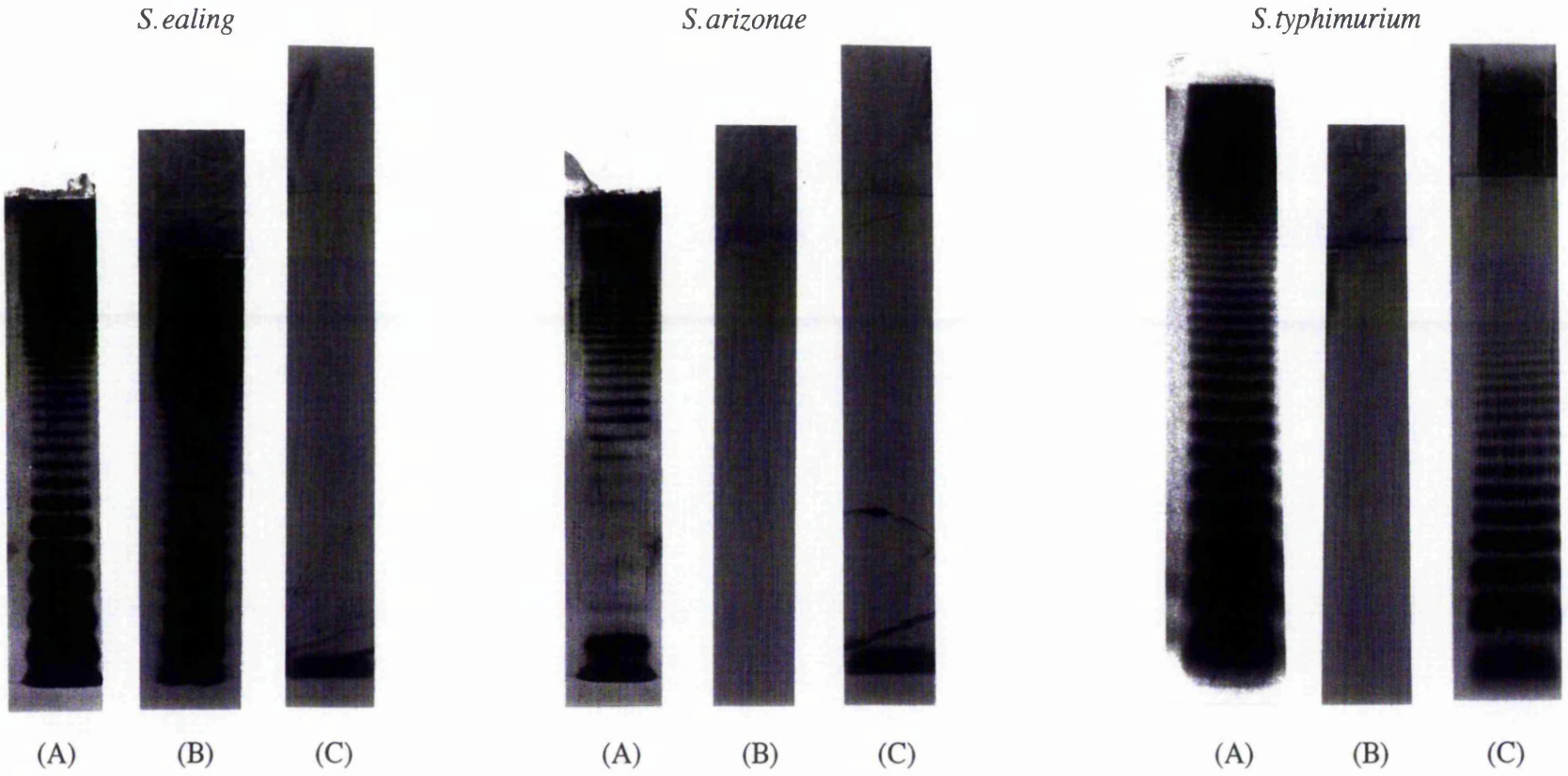


Figure 3.29: SDS-PAGE (A) and immunoblots of LPS from *S.ealing* (NTU), *S.arizonae* (NTU) and *S.typhimurium* with anti-serogroup O specific antisera (B) and M105 antibody (C)

3.10 CHEMICAL ANALYSIS OF LPS AND LPS CORE STRUCTURE

Dot blot assay results showed that *Salmonella* serogroup O strains and *S.arizonae* (NTU) had a weak reaction with the M105 antibody (Table 3.9). Results from the immunoblot assay revealed that the M105 antibody did not react with the S-type LPS from these wild type strains. Moreover the reaction with the R-type LPS was very weak (immunoblot reaction type 3, Lane 6; Figure 3.28). The relative intensity and thickness of a silver stained LPS band reflects the number of LPS molecules of a particular O-chain length, the more intense the band, the greater the number of LPS molecules. Silver stained LPS profiles were compared visually. The amount of R-type LPS, in the M105 antibody weakly reactive strains, was not significantly different to the strongly reactive strains (comparing *S.virchow*, Lane 3 and *S.ealing*, Lane 5; Figure 3.28). Thus R-type LPS from the M105 antibody weakly reactive strains was not limited. Preliminary investigations to determine the M105 antibody epitope site had shown that, in addition to the Ra type core, the M105 antibody reacted with Rb_1 type core with reduced reactivity (Table 3.3). Therefore the weak M105 antibody reactivity could have indicated the presence of non-Ra type core structures. It was possible that the R-type LPS of the M105 antibody weakly reactive strains could be made up of a mixture of complete Ra type core and partial core corresponding to the Rb_1 type structure. Alternatively the R-type LPS could be entirely made up of Rb_1 type core. The LPS from *S.arizonae* (NTU; serogroup 51) and *S.ealing* (NTU) a representative of *Salmonella* serogroup O were chosen to be chemically analysed to identify their LPS core structures.

There is no published data on the O-chain repeat units of *S.ealing* and *Salmonella* serogroup 51. Therefore in addition to the core structure, the O-chain repeat units of *S.ealing* and *S.arizonae* were analysed. Purified LPS is required for the purpose of analysis. Hence the LPS from *S.ealing* and *S.arizonae* were extracted from whole cells (Section 3.10.1) prior to compositional analysis (Section 3.10.2).

3.10.1 *LPS EXTRACTION*

S.ealing and *S.arizonae* were grown up in LB-medium according to the 'in-house' procedure of the Microbiology Department, Forschungs Zentrum, Borstel, Germany (Section 2.5.3). The cells were pelleted by centrifugation, dried and the LPS extracted according to the hot phenol/water method of Galanos *et al.* (1969). Crude LPS extract was purified by ultracentrifugation and freeze dried for storage at room temperature (Section 2.7). The weight of cells and the percentage of LPS extracted is shown in Table

3.24. The LPS isolated from the NTU strain of *S.arizonae* comprised 1.83% of the freeze dried weight of the cells.

A proportion of lyophilised LPS was dissolved in deionised distilled water to a final concentration of 5mg/ml for further analysis. The purity of the LPS preparations was assessed from the UV absorbance at 260nm for DNA and RNA. Negligible impurities were recorded. Hence no further purification was required.

On occasions it has been observed that LPS from wild type bacteria separate into the phenol phase during hot phenol/water extraction due to the strong association with cellular proteins (Lüderitz *et al.*, 1982; Chart 1995). Therefore to ensure that all LPS had been extracted into the aqueous phase, the phenol phase was analysed. The phenol phase of LPS isolated from *S.ealing* and *S.arizonae* was dialysed against water and freeze dried as per the aqueous phase (Section 2.7.2). When analysed by SDS-PAGE the extracted material did not silver stain and was therefore not LPS. Hence no LPS was separated into the phenol phase of the hot phenol/water method.

Table 3.24: The weight of cells and the percentage of LPS extracted from *S.ealing* (NTU) and *S.arizonae* (NTU)

	Weight / g				
	Cells			LPS	
Serotype	**Wet**	**Dry**	**Freeze dried**	**Freeze dried**	**%**
S.ealing (NTU)	23.00	5.37	nd	nd	nd
S.arizonae (NTU)	21.00	5.01	4.90	0.09	1.83

nd, not done

3.10.2 *THE COMPOSITIONAL ANALYSIS OF PURIFIED LPS*

In the following section the reader should bear in mind that LPS isolated from wild type bacteria by the hot phenol/water method of Galanos *et al.* (1969) is made up of a heterogenous population of varying O-chain lengths. Moreover the O-chain comprises a greater proportion of the LPS than the core oligosaccharide. Compositional analysis of such LPS provides vital information of what residues may be present or indeed absent from the expected salmonella Ra core. Furthermore these studies produce essential

information on the composition of the O-chain and O-chain linkage to the core. Hence more informed decisions can be made regarding the approach to the isolation of core oligosaccharide.

The lipid A-core region of salmonella LPS is shown in Figure 1.3. It is made up of fatty acids, phosphate (P) and monosaccharides including glucose (Glc), galactose (Gal), glucosamine and N-acetyl glucosamine (GlcN), heptose (Hep) and 3-deoxy-D-manno-octo-2-ulopyranosonic acid (Kdo).

To aid the understanding of the compositional analysis results the expected composition of the core and lipid A are described below. The expected monosaccharide ratios are shown in Table 3.25 for Ra, Rb_1 and Rb_2 type core structures. In addition to the two GlcN residues mentioned in Table 3.25, the lipid A of salmonella LPS is made up of four residues of (R)-3-hydroxytetradecanoic acid [14:O (3-OH)]. These hydroxy fatty acids can carry additional non-hydroxylated fatty acids. These include one decanoic acid (12:O, chain length 12 carbon atoms), one tetra decanoic acid (14:O) and one hexadecanoic acid (16:O). The hexadecanoic acid is nonstoichiometric and therefore the amount of fatty acid may vary. Also the amount of phosphate in the lipid A-core region may vary since it too is present in nonstoichiometric amounts (Knirel and Kochetkov 1993b).

Table 3.25: The expected monosaccharide composition of Ra, Rb_1 and Rb_2 type salmonella LPS

Core type	Monosaccharide				
	Glc	Gal	Hep	Kdo[a]	GlcN
Ra	2	2	3	2/3	3[b]
Rb_1	2	2	3	2/3	2
Rb_2	1	2	3	3	2

[a], substitution of Kdo II by Kdo III was nonstoichiometric in all the LPS studied so far except *S.minnesota* Rb_2 strain R345 (Holst and Brade 1992);
[b], total glucosamine includes GlcNAc and GlcN from lipid A; Glc, glucose; Gal, galactose; Hep, heptose; Kdo, 3-deoxy-D-manno-octo-2-ulopyranosonic acid; GlcN; glucosamine

The compositional analysis of the LPS isolated from *S.ealing* and *S.arizonae* involved the identification and quantitative determination of LPS constituents by methods described in Section 2.15. The analysis of GlcN, Kdo and P were carried out spectrophotometrically. Neutral monosaccharides (Gal, Glc and Hep) were analysed as alditol derivatives using gas liquid chromatography (GLC) and further analysed by mass spectrometry (MS). Fatty acid derivatives (methyl esters) were analysed by GLC-MS. The LPS preparation of the rough mutant *S.minnesota* R345 (Rb_2 type) was analysed as a standard to monitor each procedure.

The spectrophotometric and GLC results for *S.ealing*, *S.arizonae* and the standard *S.minnesota* R345 are presented in Table 3.26. The monosaccharide and P compositions are shown within the brackets as nmol per mg of LPS ($nmolmg^{-1}$). GlcNAc, Glc and Gal resides are present in the outer core region of salmonella LPS. Variations within the core structure of salmonella LPS have been identified in the outer core region (Tsang *et al.*, 1992; Hoofdvak and Olsthoorn 1995). Hence these monosaccharide residues were not used to calculate the molar ratios. Hep is confined to the inner core region (Figure 1.3). Therefore the molar ratio of each residue was calculated based on an expected 3 mol Hep per mol of LPS (Table 3.25).

Table 3.26: Monosaccharide and phosphate compositions of wild type *S.ealing* (NTU), *S.arizonae* (NTU) and rough mutant *S.minnesota* R345 LPS expressed as a molar ratio[a] and nmol/mg (within brackets)[b]

	Component					
LPS source	**Glc**	**Gal**	**Hep**	**Kdo**	**GlcN[c]**	**P**
S.ealing (NTU)	15.4 *(1420)*	11.1 *(1017)*	3 *(276)*	5.5 *(503)*	10.9 *(1005)*	9.7 *(890)*
S.arizonae (NTU)	87.5 *(17591)*	0.2 *(44)*	3 *(603)*	0.3 *(60)*	2.9 *(578)*	1.9 *(390)*
S.minnesota R345	3 *(571)*	2.3 *(438)*	3 *(565)*	2.5 *(470)*	2.4 *(448)*	4.6 *(868)*

[a], molar ratio based on 3 mol Hep/mol LPS; [b], mean of 2 replicates; [c], GlcN represents both glucosamine and N-acetyl glucosamine; Glc, glucose; Gal, galactose; Hep, heptose; Kdo, 3-deoxy-D-manno-octo-2-ulopyransonic acid; GlcN, glucosamine; P, phosphate

The expected monosaccharide ratios for an 'Rb_2 type core' was 2:3:3:2 for Gal, Hep, Kdo and GlcN residues respectively (Table 3.25). Although the analysis of the Rb_2 type rough

mutant strain of *S.minnesota* R345 did not produce an 'ideal' set of results, the ratios were similar (2.3:3:2.5:2.4). However instead of one Glc residue the calculations suggested that three residues were present per mol of LPS. This is clearly incorrect. It is possible that this anomoly was due to experimental error. It is also possible that the Glc values produced by the two test strains *S.ealing* and *S.arizonae* were higher than they should have been. This was taken into consideration when interpreting the results.

A mean value of 565nmolmg^{-1} Hep was recorded for the standard *S.minnesota* R345. A similar value was reported for *S.arizonae* (603nmolmg^{-1}). In comparison the LPS isolated from *S.ealing* was composed of 276nmol Hep per mg of LPS. Since the monosaccharide and phosphate ratios were based on three mol of Hep per mol of LPS the relatively low value produced by *S.ealing* had a significant affect on the final ratios. Therefore the amount of monosaccharide residue, as well as the ratios of LPS components was taken into consideraton for the interpretation of results. Moreover the amount of each residue was compared with the standard *S.minnesota* R345.

For *S.ealing* LPS high levels of Glc (1420nmolmg^{-1}), Gal (1017nmolmg^{-1}) and GlcN (1005nmolmg^{-1}) were recorded compared with the standard *S.minnesota* R345 (571, 438 and 448nmolmg^{-1}). Bearing in mind that the results for Glc may have been high. These results still indicated that Glc, Gal and GlcN residues were present in the O-chain repeat unit of *S.ealing* LPS. Hep and Kdo form part of the structure of the inner core region and lipid A. Their ratios were expected to be similar (2:2 or 3), however the amount of Hep (276nmolmg^{-1}) present in *S.ealing* LPS was approximately half the amount of the Kdo (503nmolmg^{-1}). Moreover the amount of Hep was lower than for *S.minnesota* R345 (565nmolmg^{-1}). Similar levels of P were recorded for *S.minnesota* R345 (868nmolmg^{-1}) and *S.ealing* (890 nmolmg^{-1}). The GLC-MS results indicated that the O-chain of *S.ealing* in part comprised of 3,6-dideoxyhexose. Based on previous studies of another serogroup O strain *S.adelaide*, it is plausible that this 3,6-dideoxyhexose was colitose (Kenne *et al.*, 1983).

For *S.arizonae* very high levels of Glc were recorded (17591nmolmg^{-1}). This suggested that the O-chain repeat unit of *S.arizonae* was made up of a significant amount of Glc. The results suggested that 0.2mol Gal and 0.3mol Kdo were present per mol of LPS. This implied that the structure of both the inner and the outer core regions of *S.arizonae* LPS were not the same as the typical salmonella Ra core. Furthermore low levels of P

(390nmolmg^{-1}) were recorded compared with the standard *S.minnesota* R345 (868nmolmg^{-1}). According to the literature Kdo and P are present in nonstoichiometric amounts (Figure 1.3). However this alone cannot account for the unexpectedly low levels of Kdo and P for *S.arizonae*. The results in Table 3.26 show that the ratio of GlcN was as expected (approximately 3 mol) for an Ra type core structure. However the reader should bare in mind that S-form LPS was analysed. So that the GlcN may have been present in the O-chain. The GLC and MS results, according to the expert opinion of Dr Otto Holst (the Forschungs Zentrum, Borstel, Germany), indicated that the GlcNAc residue was in fact absent in the LPS from *S.arizonae*. Evidence for the absence of GlcNAc in the core region and the presence of GlcN in the O-chain repeat unit of *S.arizonae* could only come from the analysis of the separate R- and S-type LPS portions.

The total fatty acid composition of the LPS isolated from the two test strains *S.ealing* and *S.arizonae* was similar to that of the *S.minnesota* R345 standard. Both test strains contained a high amount of (R)-3-hydroxytetradecanoic acid [14:O (3-OH)]. From these results it can be inferred that the composition of the lipid A of *S.ealing* and *S.arizonae* were typical (Dr Simon Haseley, the Forschungs Zentrum, Borstel, Germany; personal communication).

3.10.3 *THE FRACTIONATION OF LPS*

As previously stated S-type LPS is made up of lipid A, core and O-chain (Section 1.4.1). The O-chain comprises typically of up to 20-40 repeating units (Rieschel and Brade 1992) and each unit contains 3-5 different sugars (Hancock *et al.*, 1994). In the present study the *S.ealing* (NTU) and *S.arizonae* (NTU) produced LPS banding profiles with 21-30 bands (O-chain repeat units). Therefore in LPS isolated from wild type bacteria, the core structure represents a tiny fraction of the total amount of LPS. Hence elucidation studies of core oligosaccharide requires its isolation from LPS. This could be achieved in two ways. The first option, that would produce large quantities of relevant material was to cleave the O-chain from S-type LPS. However successful cleavage of the O-chain depends on the type of linkage involved. The GLC-MS results indicated that the bonds in the backbone of the repeating O-chain units of both strains were stable glycosidic bonds (Dr Otto Holst, personal communication). Thus in order to attain cleavage of the O-chain from *S.ealing* and *S.arizonae* S-type LPS a harsh chemical treatment would be required. Unfortunately since this would also destroy some of the core oligosaccharide the S-type LPS could not be used to isolate core oligosaccharide. An alternative method of isolating

smaller quantities of core oligosaccharide is gel permeation chromatography. If successful this size exclusion technique would separate the LPS population into two fractions. The high molecular weight fraction would be eluted first. This fraction would be regarded as S-type LPS (long chain). The second fraction peak of lower molecular weight would be regarded as R-type LPS probably with some contaminating short chain LPS. Two methods of gel permeation chromatography were assessed. These are termed 'the isolation of core oligosaccharide' (Section 3.10.3.1) and 'the isolation of R-type LPS' (Section 3.10.3.2).

3.10.3.1 *The isolation of core oligosaccharide*

The LPS of *S.ealing* and *S.arizonae* was treated by mild acid hydrolysis and centrifuged to remove the lipid A. The lipid A from *S.ealing* LPS was recovered, lyophilised and weighed. It represented 12.5% of the freeze dried weight of *S.ealing* LPS. The core oligosaccharide suspension was separated into high and low molecular weight fractions by gel permeation chromatography (Section 2.16.2). The elution profile of *S.ealing* showed three peaks (Figure 3.30). Eluate samples were combined into four fractions (F1, F2, F3, F4), dialysed and lyophilised. Fractions F1 (corresponding to the first elution peak) and F2 (corresponding to the second elution peak) which were considered to be O-chain attached to core oligosaccharide and predominantly core oligosaccharide respectively were analysed by structural elucidation methods. Fractions F3 (corresponding to samples between peaks two and three) and F4 (corresponding to peak three) were not analysed. The elution profile of *S.arizonae* showed two peaks termed F1 and F2 (Figure 3.31). The eluate samples representing these peaks were combined, dialysed and lyophilised for further analysis by structural elucidation methods.

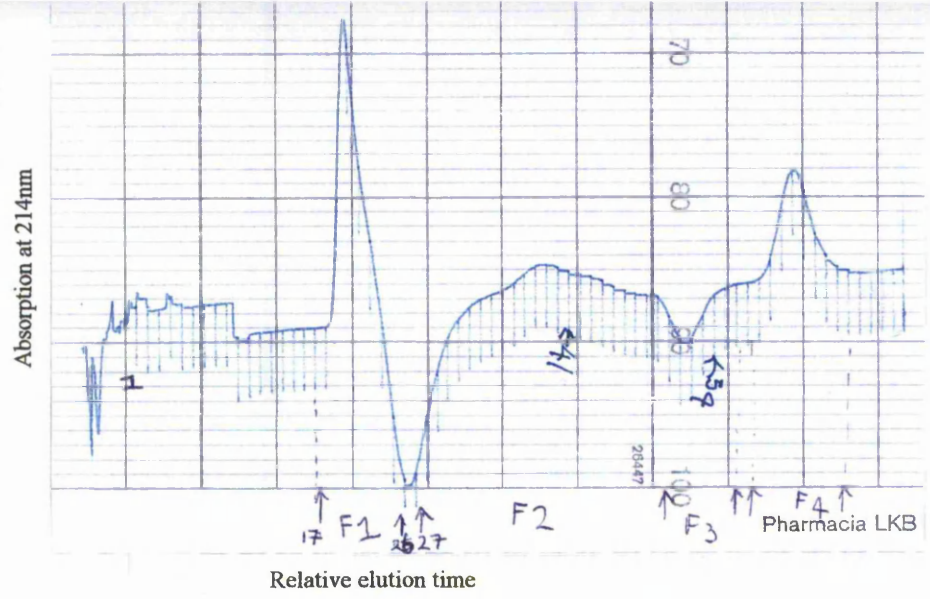


Figure 3.30: Gel permeation chromatographic elution profile for the lipid A deficient LPS from *S.ealing* (NTU)

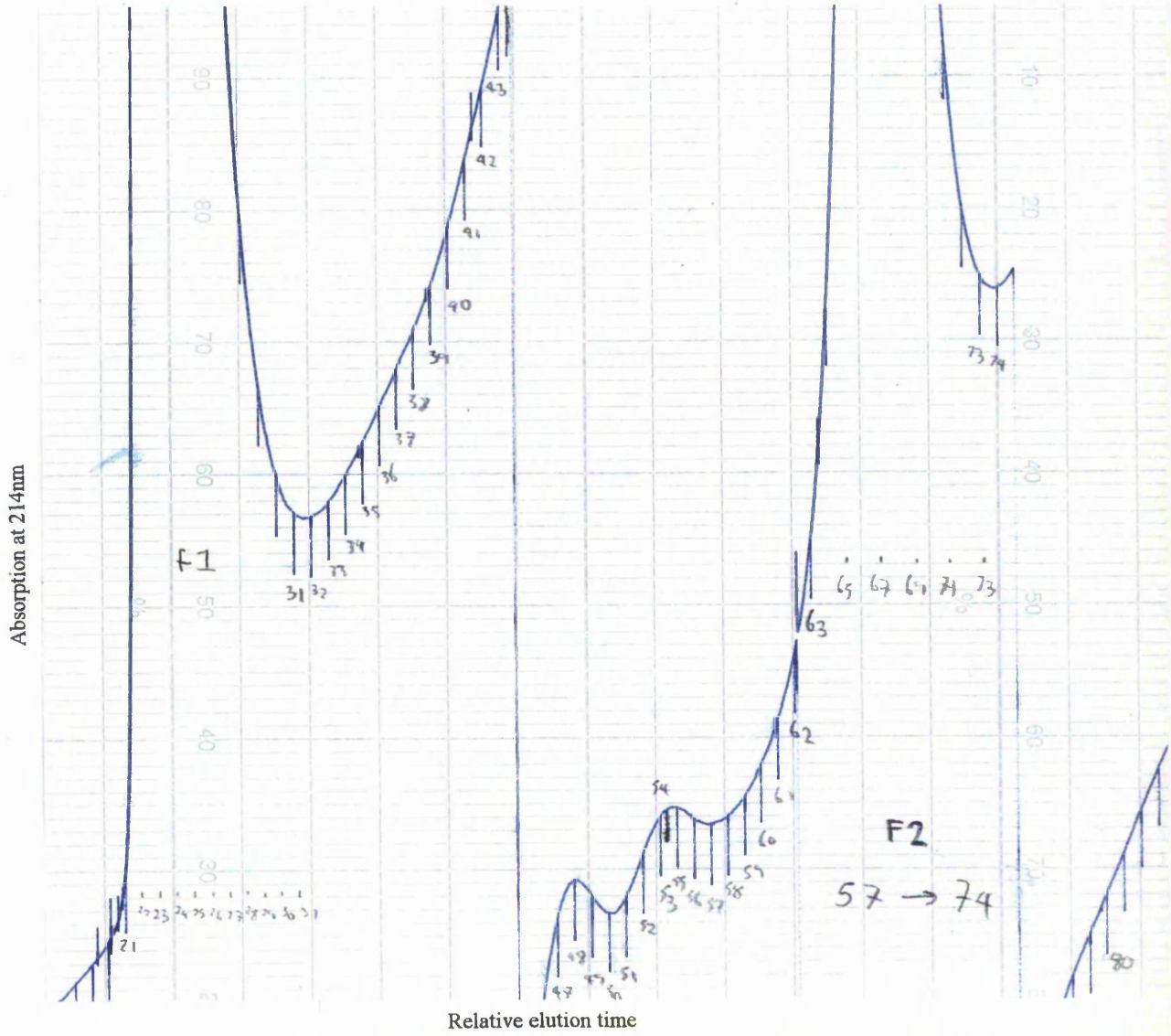


Figure 3.31: Gel permeation chromatographic elution profile for the lipid A deficient LPS from *S.arizonae* (NTU)

3.10.3.2 *The isolation of R-type LPS*

The isolation of intact R-type LPS was achieved by a modified Peterson and McGroarty (1985) gel permeation chromatography method (Section 2.16.3). Total carbohydrate content was determined by the anthrone-sulphuric acid method (Section 2.17). Sample fractions were analysed by SDS-PAGE (Section 2.12.2) and immunoblot assay with M105 antibody detection (Section 2.9).

The elution profile produced by *S.ealing* LPS formed a peak with a broad shoulder (Figure 3.32). Neutral sugar analysis of alternate fractions produced a similar profile (Figure 3.33). From these results it could be inferred that the LPS of *S.ealing* was made up of a broad range of similar O-chain lengths. Elution samples were combined into three fractions termed F1 (corresponding to the first elution peak), F3 (corresponding to the second peak/ shoulder) and F2 which was collected so that fractions F1 and F3 were quite separate. The elution samples representing fractions F1, F2 and F3 were combined, dialysed and lyophilised for further analysis by SDS-PAGE and immunoblot assay and structural elucidation studies. The SDS-PAGE analysis of fractions are shown in Figure 3.34 with unfractionated LPS included for comparative purposes. Fraction F1 was made up of long chain LPS of ≥ 18 O-chain repeat units. Bands of ≤ 17 O-chain repeat units were present. However these were stained less intensely and the banding pattern did not mirror the unfractionated LPS. Fraction F2 was made up of S- and SR-type LPS i.e. ≥ 2 O-chain repeat units. Moreover doublet bands were clearly visible in the lower half of the gel. Fraction F3 was made up of predominantly medium to short chain LPS including R-type LPS i.e. ≤ 8 repeat units. From the corresponding immunoblots it was confirmed that unfractionated LPS and fraction F3 contained M105 reactive R-type LPS (Figure 3.34). From these results it could be concluded that the modified gel permeation chromatography method of Peterson and McGroarty (1985) was effective at isolating predominantly medium to short chain LPS including R-type LPS from populations of wild type *S.ealing* LPS. However short chain and R-type LPS fractions were not separated.

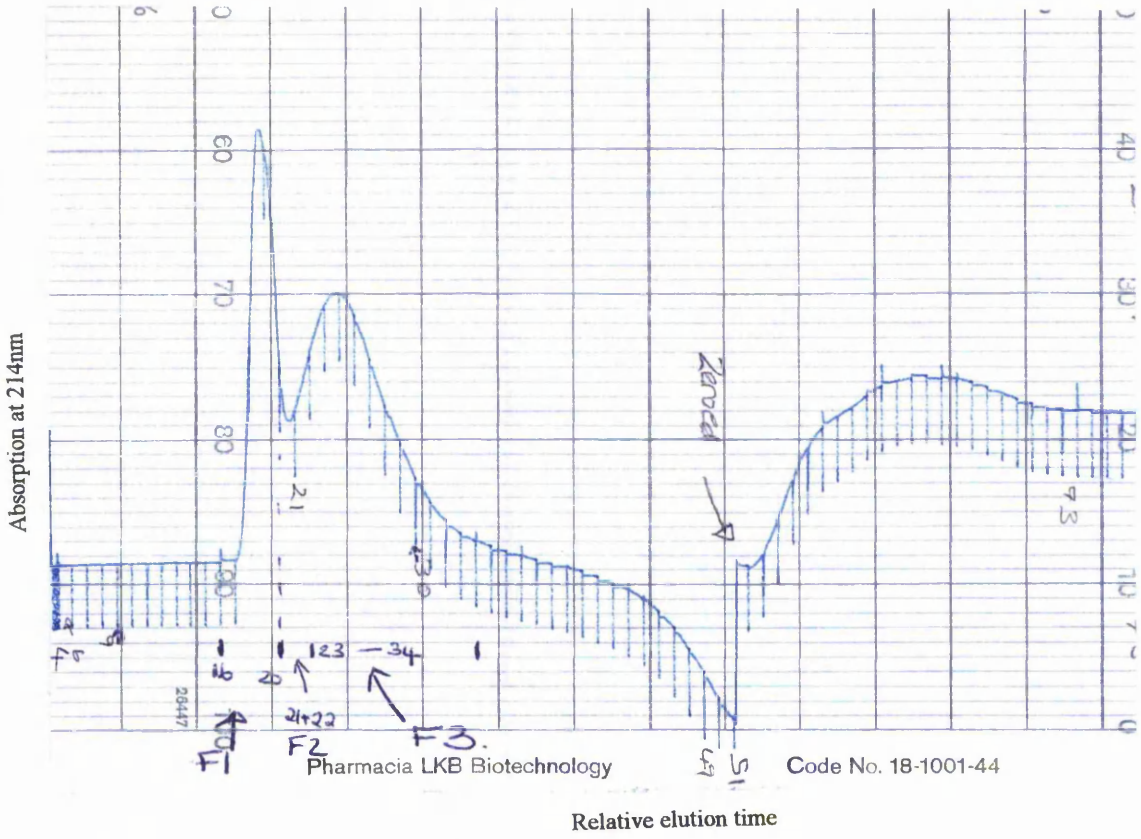


Figure 3.32: Elution profile for the LPS from *S.ealing* (NTU)

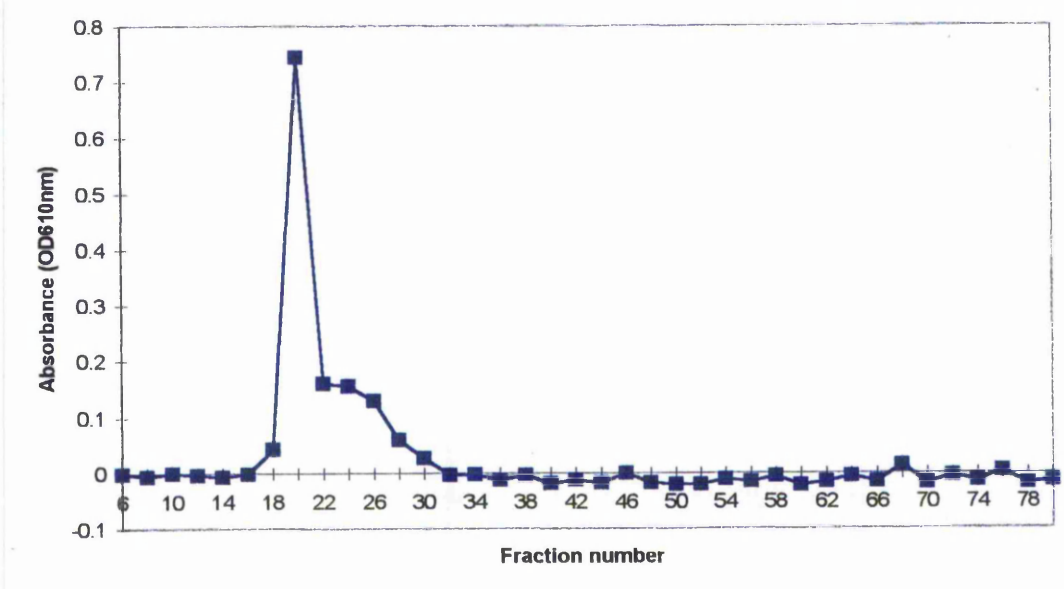


Figure 3.33: Fractionation of LPS from *S.ealing* (NTU) with Sephadex G50 monitored for neutral sugars

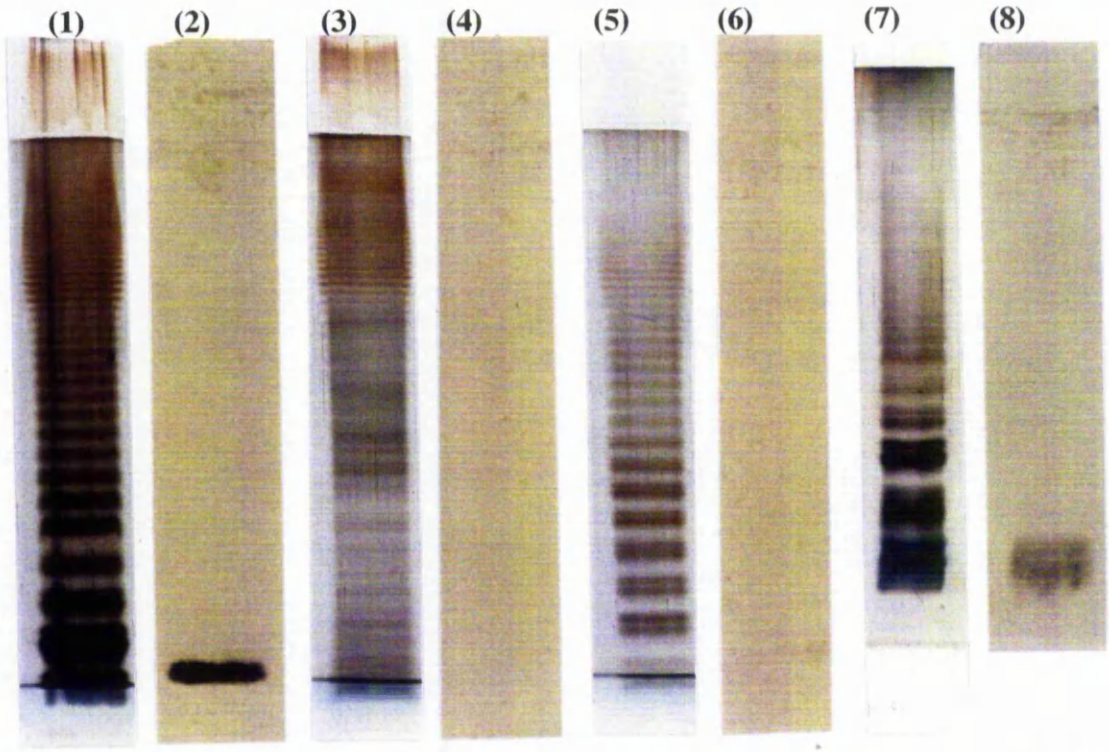


Figure 3.34: SDS-PAGE and immunoblot assay profiles for *S.ealing* (NTU) LPS (Lanes 1 and 2) and fractionated LPS samples F1 (Lanes 3 and 4), F2 (Lanes 5 and 6) and F3 (Lanes 7 and 8)

The elution profile of *S.arizonae* LPS showed a tall narrow peak with a second smaller broader peak (Figure 3.35). The neutral sugar analysis of alternate fractions revealed a tall narrow peak (Figure 3.36). However the second smaller broader peak (of the elution profile) was not obvious. From these results it could be infered that the LPS of *S.arizonae* was predominantly made up of LPS of similar medium to long O-chain length. The eluate samples were combined into three fractions termed F1 (corresponding to the first peak), F2 (corresponding to the second peak) and F3 (corresponding to the eluate samples collected after fraction F2). Elution samples representing fractions F1, F2 and F3 were combined, dialysed and lyophilised for further analysis by SDS-PAGE and immunoblot assay and structural elucidation studies. The SDS-PAGE analysis of fractions are shown in Figure 3.37 with unfractionated LPS included for comparative purposes. Fraction F1 was made up of mainly medium to long chain LPS of ≥ 10 O-chain repeat units. In addition bands of ≤ 9 O-chain repeat units were present. However these were only feintly stained. Fraction F2 contained predominanly short chain LPS of ≤ 2 O-chain repeat units. Fraction F3 was not stained and therefore did not contain LPS. The corresponding immunoblots showed that unfractionated LPS and fraction F2 contained M105 antibody reactive R-type LPS (Figure 3.37). From these results it could be concluded that gel

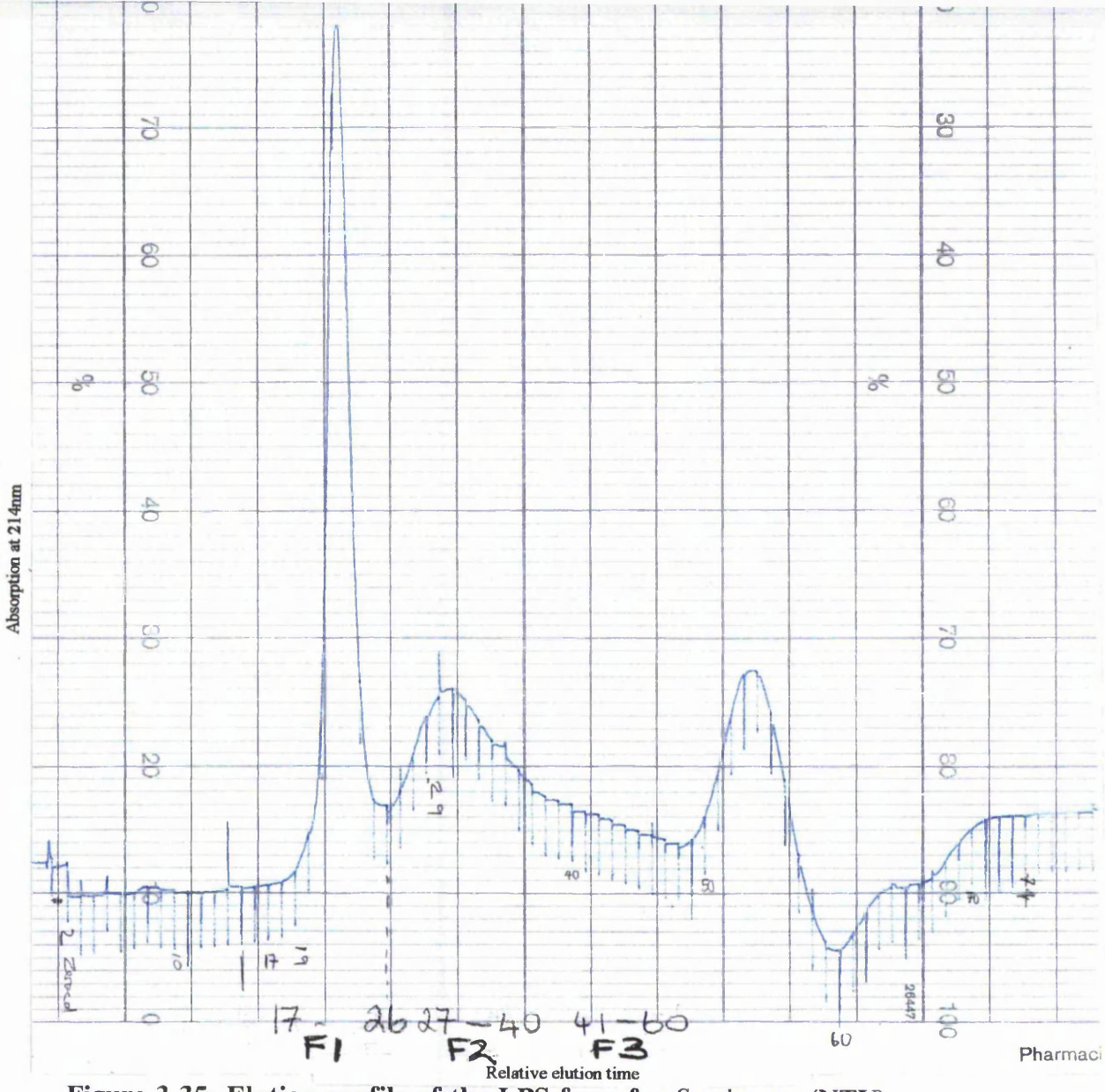


Figure 3.35: Elution profile of the LPS from for *S.arizonae* (NTU)

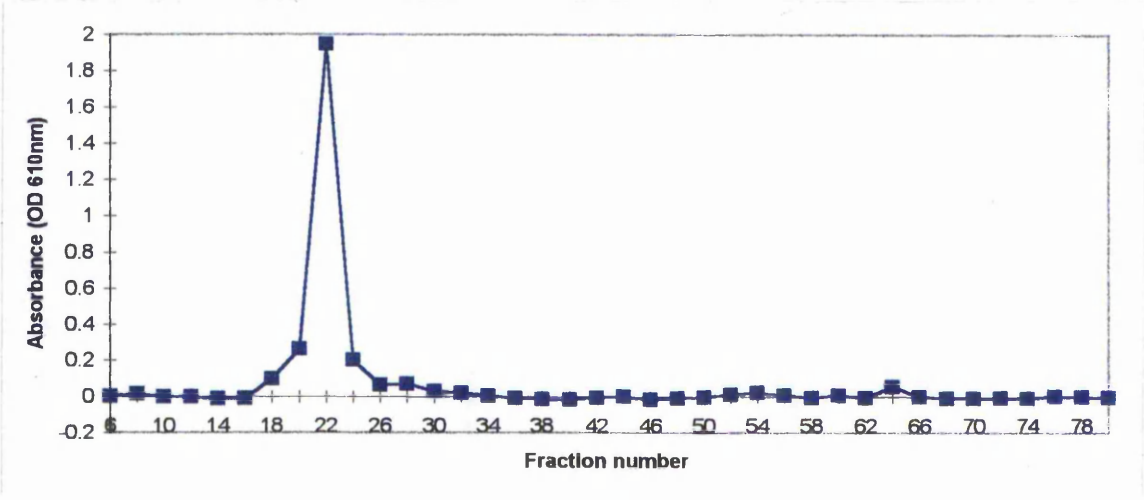


Figure 3.36: Fractionation of LPS from *S.arizonae* (NTU) with Sephadex G50 monitored for neutral sugars

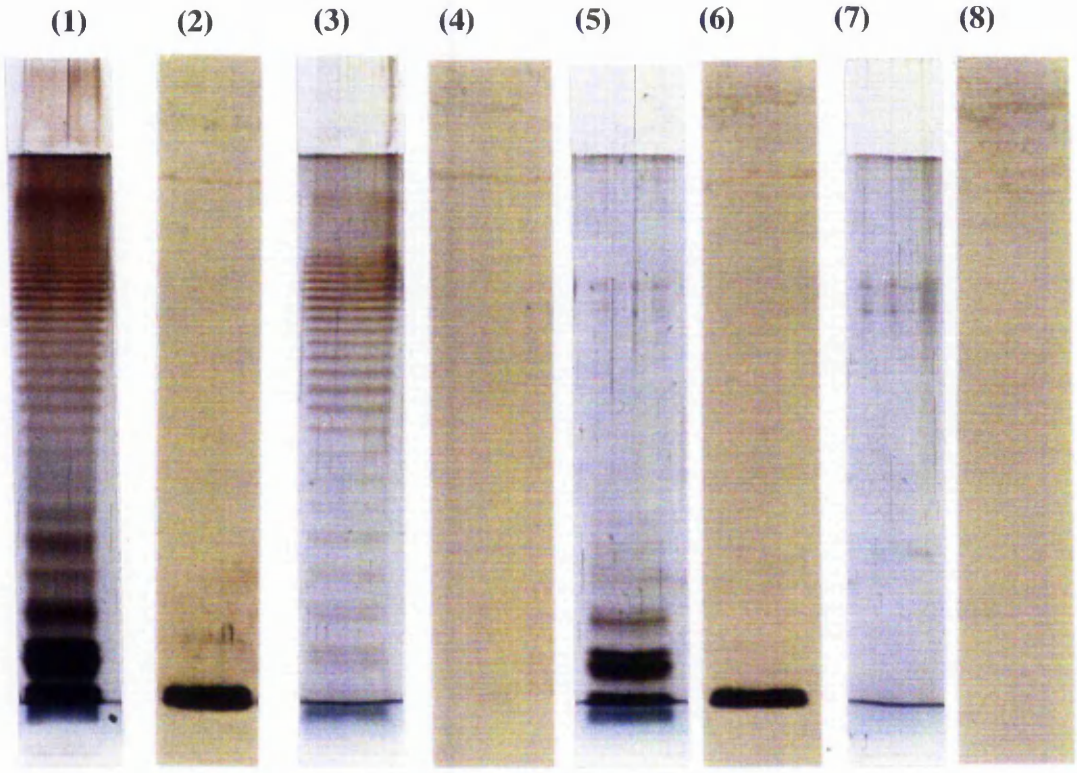


Figure 3.37: SDS-PAGE and immunoblot assay profiles for *S.arizonae* (NTU) LPS (Lanes 1 and 2) and fractionated LPS samples F1 (Lanes 3 and 4), F2 (Lanes 5 and 6) and F3 (Lanes 7 and 8)

permeation chromatography was effective at isolating predominantly R-type LPS from wild type populations of *S.arizonae* LPS.

The carbohydrate structure of the eluted fractions, representing short chain and R-type LPS and long chain LPS with and without lipid A, were analysed by methylation analysis and selective degradation by methods described by Hakamori (1964), Haishima *et al.* (1992), Hase and Rietschel (1976) and Holst *et al.* (1994). However the carbohydrate structure of core oligosaccharide and O-chain could not be accurately determined because of insufficient material. Due to time restrictions more material was not isolated.

The major aim of the present study has been achieved. An IMS-ELISA salmonella detection method, using the M105 antibody, has been developed. The results from these investigations suggested that non Ra type core structures existed in some wild type *Salmonella* strains. Further serological analysis of the LPS from salmonella and non-salmonella bacteria was carried out. The findings of this serological study led to the chemical analysis of LPS isolated from two *Salmonella* strains, *S.ealing* (NTU) and *S.arizonae* (NTU).

CHAPTER FOUR

DISCUSSION

4 DISCUSSION

Salmonellosis caused by ingestion of foods contaminated with *Salmonella* spp. is a major burden on society, causing considerable suffering and loss of productivity, adding to the cost of food production and health care. Therefore the presence of any *Salmonella* spp. in ready-to-eat foods is significant. This is reflected in current legislation which requires the total absence of Salmonella in 25g of processed food (Roberts *et al.*, 1995).

The 'ideal' salmonella isolation and detection technique would

(i) have the ability to detect the common, if not all the 2,400 *Salmonella* serotypes

(ii) fail to isolate non-*Salmonella* spp.

(iii) have the ability to detect sublethally injured salmonellas

(iv) detect low levels of salmonella from a range of food types and hence a multitude of background flora

(v) would produce results within a day and

(vi) would be low cost, with low technology and would be easy to use.

These requirements are in part met by traditional cultural techniques. However a crucial shortcoming is the total test time taken to detect these pathogenic bacteria. Presumptive results are only available after 72-96h. This delay has prompted a considerable effort to develop alternative methods to reduce the time of detection of presumptive *Salmonella* spp.. A variety of strategies have been developed which reduce this time of detection by 24-45h (Tables 1.9 and 1.11). However as yet none of the alternative methods meet all the requirements of the 'ideal' salmonella isolation and detection technique. The limitations of the currently available alternative salmonella isolation and detection methods (Section 1.9) emphasises the need for further research into more rapid but also reliable alternative techniques.

A possible means of achieving a reliable rapid method of salmonella detection and the subject of this report is an adaption of the Dynabeads® anti-Salmonella immunomagnetic separation (IMS) technique. It is a microtitre plate based method termed the immunomagnetic separation enzyme linked immunosorbent assay (IMS-ELISA). In brief salmonella cells are captured by specific antibodies bound to paramagnetic beads. The subsequent salmonella-bead complex is detected by a double antibody detection/ visualisation system. Using the IMS-ELISA technique means that a next day result is

plausible and in addition affords multiple sample analysis.

For the sake of clarity the discussion has been divided into sections which correspond to the aims of the research project presented in Figure 1.16.

4.1 THE IMS-ELISA SALMONELLA ISOLATION AND DETECTION SYSTEM

4.1.1 *CONFIRMATION OF THE M105 EPITOPE SITE*

Tsang *et al.* (1991a) demonstrated that the M105 antibody reacted strongly with Ra type lipopolysaccharide (LPS) and in addition reacted with Rb_1 and Rb_2 type LPS with decreasing reactivity. Under the experimental conditions of the present study the M105 antibody reacted strongly with LPS from rough mutant Ra strains of *S.typhimurium* and *S.minnesota* (Table 3.3). In addition the antibody reacted to a lesser degree with a rough mutant Rb_1 strain of *S.typhimurium*. The M105 antibody did not react with the LPS from deep rough mutant Rb_2, Rc, Rd_1, Rd_2 and Re strains. Since it is generally accepted that all *Salmonella* spp. share a common Ra type core structure (Kato 1993), it follows that the M105 antibody should react with all salmonellas. In addition if an atypical core structure was present which corresponded to the Rb_1 type core structure, the M105 antibody would exhibit a reduced reactivity.

4.1.2 *PRESENCE OF THE M105 EPITOPE*

Todd *et al.* (1991) reported that the M105 antibody did not react with untreated cells of 508/566 *Salmonella* serotypes tested. This general lack of M105 reactivity with untreated *Salmonella* strains were in stark contrast to those reported by other researchers or indeed the present study. Although the present study tested a small range of *Salmonella* strains, a study by Tsang *et al.* (1991a) showed that the M105 antibody reacted with representatives of the *Salmonella* serogroups A, B, C_1, C_2, D_1, E_1, E_4, F, G, H, I, J, K, L, M, N, O, P, Q, R, S, T, U, V, W, X, Y, Z, 51, 52, 53, 55, 56, 57, 58, 59, 60, 61, 62, 63, 65, 66 and 67. Only one strain which belonged to the monophasic *Arizona* group of subspecies IIIa was reported to be non M105 reactive (Tsang *et al.*, 1991a). Differences in the reactivities, reported by these studies, could have been due to strain variation. An alternative and more likely explanation is method variation. Todd *et al.* (1991) used horseradish peroxidase (HRP) labelled M105 antibody. A later study by Nielsen and Tsang (1992) reported that M105 formed an unstable conjugate when labelled with HRP or alkaline phosphatase (AP) resulting in severe loss of antibody reactivity. Hence reduced M105 reactivity of the *Salmonella* strains analysed by Todd *et al.* (1991)

could have been a direct result of the enzyme labelling method.

In the present study the dot blot assay technique was used to screen a range of *Salmonella* spp. for the presence of the M105 epitope. Since the numbers of *Salmonella* spp. tested were limited with respect to the total number of *Salmonella* serotypes (2,400), representative strains were analysed. These included strains from the most commonly isolated *Salmonella* serogroup B-E and examples from the higher serogroups L and O. The *Arizona* group until relatively recently was a separate genus. Since the O-antigens of the *Arizona* group are often difficult to determine (Rowe and Hall 1989) a strain belonging to the *Arizona* group was also tested.

Salmonella serotypes of the most frequently human isolated serogroups B, C_1, C_2, C_3, D, E_1 and E_4 reacted with the M105 antibody (Table 3.9). Amongst which were the top three most commonly isolated serotypes, *S.enteritidis*, *S.typhimurium* and *S.virchow*. In addition strains belonging to the higher serogroups of L (*S.minnesota*) and O (*S.adelaide*; *S.ealing*, NTU; *S.ealing*, Canada) were also positive, as was a strain belonging to serogroup 51 (*S.arizonae*, NTU). Hence the results of the present dot blot assay study indicated that the M105 antibody had the potential to detect all *Salmonella* spp..

The M105 antibody exhibited a wide range of reactivity with the *Salmonella* strains assayed using the standard dot blot assay protocol with 10^9 cfu/ml of untreated cells (Table 3.9). The weakest reactions were produced by *S.gallinarum* (mean relative colour intensity K/S 0.49), *S.kentucky* (K/S 0.44), *S.minnesota* (K/S 0.34), *S.arizonae* (K/S 0.27), *S.livingstone* (K/S 0.25), *S.adelaide* (K/S 0.17), the Canada strain of *S.ealing* (K/S 0.17) and the NTU strain of *S.ealing* (K/S 0.15). The strongest reactivities of K/S values >2.00 were produced by *S.senftenberg* (K/S 2.10), *S.enteritidis* (K/S 2.55) and *S.agona* (K/S 2.94). The wide range of M105 antibody reactivity suggested that variable amounts of antigen were available for antibody binding. This could be explained by variations in the total amount of LPS present in each serotype. However previous investigations have reported that the variation in the total amount of LPS per Gram-negative cell is only negligible (3-8%; Rietschel *et al.*, 1984) and therefore alone cannot explain the diversity between serotypes. The sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) technique and size exclusion chromatography have shown that isolated LPS is made up of a heterogenous population of varying O-chain length (Peterson and McGroarty 1985). Kastowski *et al.* (1992) suggested that the long chain LPS of whole cells, produce

cross-overs which create a barrier against harmful substances such as antibiotics. This physical barrier may also mask M105 reactive core structures so that areas of short chain and R-type LPS may represent 'holes' through which the M105 antibody can bind. The number of 'holes' may vary significantly between serotypes and could account for the range in M105 antibody reactivity. *Salmonella* spp. are generally motile with peritrichous flagella. It is therefore plausible that other structures such as flagellar or indeed fimbriae on the cell surface may cause a steric hindrance to antibody binding. Moreover the presence of partial Rb_1 type (atypical) core structures could account for the weakest reactions.

Previous studies have shown that the M105 did not cross-react with over 300 strains of non-salmonella bacteria (Tsang *et al.*, 1997). These included a range of closely related Enterobacteriaceae: *Citrobacter freundii*, *C.diversus*, *Enterobacter aerogenes*, *Ent.cloacae*, *Edwardsiella tarda*, *Klebsiella pneumoniae*, *K. oxytoca*, *Proteus mirabilis*, *P.vulgaris*, *Providencia stuarti*, *Morganella morganii*, *Serratia marcescens*, *Shigella sonnei*, *Sh.flexneri* and *Yersinia enterocolitica*. Only three non-*Salmonella* strains were found to cross-react with the M105 antibody. These were two strains of *Ent.agglomerans* (Tsang *et al.*, 1997) and a strain of *Sh.flexneri* (Tsang *et al.*, 1991a).

In the present study a small range of non-*Salmonella* spp. were tested for the presence of the M105 epitope by dot blot assay. Non-*Salmonella* strains tested included *Ent.agglomerans*, *Sh.flexneri*, *E.coli* and eight strains of *C.freundii*. Proportionally more *Citrobacter* sp. were tested because these strains commonly cross-react with anti-salmonella antibodies (Jay and Comar 1988; Eckner 1992; Bobbit and Betts 1993; Blackburn *et al.*, 1994; Jones and Betts 1994). In the present study untreated *Ent.agglomerans*, *E.coli* and *Sh.flexneri* and eight strains of *C.freundii* expressed a weak cross-reaction with the M105 antibody. Mean relative colour intensity readings of between K/S 0.02 (*Sh.flexneri*) and 0.27 (*Ent.agglomerans*) were recorded (Table 3.10). The weak M105 reactivity could have indicated the presence of a common epitope, since the outer core structures of *E.coli* and *Shigella* spp. are similar to *Salmonella* spp. (Tsang *et al.*, 1987). However a more likely explanation for the cross-reaction expressed by the non-*Salmonella* strains was given by Tsang *et al.* (1991a). These researchers suggested that the M105 epitope may be present on other cell surface structures such as carbohydrate residues in the capsule or glycoproteins.

Ng *et al.* (1996) reported that the T6 antibody is capable of detecting an estimated 98% of all salmonellas known to naturally affect humans. Since the M105 and T6 antibodies share a common core epitope (Figure 1.14), it follows that M105 will do the same. Hence the dot blot assay results of the present study highlights the potential usefulness of the M105 antibody in the serological detection of salmonella.

4.1.3 *AMPLIFICATION OF ANTIBODY-ANTIGEN REACTION*

Since salmonella naturally contaminate foods at low levels cultural enrichment steps are required to increase salmonella numbers to detectable levels. Although some attempts to reduce pre-enrichment time has been successful (van Schothorst and van Leusden 1975), in general extended incubation periods are required to provide sufficient time for repair of stressed or injured salmonellas (D'Aoust *et al.*, 1992). With respect to the immunoassay technique any decrease in detection time is dependent on the sensitivity of the immunological reaction. The more sensitive the system the shorter the required enrichment time. Results of the dot blot assay experiment showed that untreated *Salmonella* serogroup O and *S.arizonae* (NTU) expressed a weak reaction with the M105 antibody (Table 3.9). If this weak reactivity was reflected by the IMS-ELISA system, sensitivity would be compromised. Hence prolonged media incubation stages would be necessary and would result in an extended total test time. Therefore any method to enhance the immunological reaction would be beneficial.

A number of reports have described that heat and chemical treatment exposes previously unavailable LPS epitopes for antibody binding by releasing outer membrane (OM) fragments (Leive *et al.*, 1968; Tsuchido *et al.*, 1985; Tsang *et al.*, 1987; Blais and Yamazaki 1989a). Hence *S.arizonae* (NTU) and a representative of the serogroup O *S.ealing* (NTU), which exhibited a weak M105 reactivity, were exposed to a variety of heat, chemical and physical treatments in an attempt to increase M105 antibody reactivity and therefore IMS-ELISA sensitivity. In addition representatives of the more commonly isolated serogroups B, C, D and E were assayed (*S.typhimurium*, *S.virchow*, *S.enteritidis*, *S.give*).

Todd *et al.* (1991) described a method based on hydrophobic grid membrane filters to evaluate the M105 antibody against a range of *Salmonella* and non-*Salmonella* strains. Cells were pre-treated by various chemical agents in an attempt to expose the M105 antigen. The M105 antibody failed to react with 508/566 untreated *Salmonella* serotypes.

However after harsh pre-treatment by boiling at pH 13.0 for 10s, the M105 antibody reacted with all serotypes (with the exception of a strain of *S.ealing*) including serogroups B, C, D, E, G, K, L, N, O, R, Z, *Arizonae* strains, rough and untypable strains.

Similarly the present study showed that chemical, heat and physical pre-treatment could be used to increase M105 antigen exposure (activity) in all *Salmonella* spp. tested with the exception of *S.typhimurium*. Combinations of tris, ethylene diamine tetra acetic acid (EDTA), sodium dodecyl sulphate (SDS), Triton X-100, tris buffered saline (TBS), distilled water, heat and physical treatment had a differential affect on strains of *S.typhimurium*, *S.virchow*, *S.enteritidis*, *S.give*, *S.ealing* (NTU) and *S.arizonae* (NTU) tested (Tables 3.4-3.7). Previous investigations by Tsang *et al.* (1987) have shown that treatment of salmonella cells with tris-EDTA buffer made cryptic core LPS epitopes more accessible for detection by the core specific T6 antibody. In the present study the same treatment conditions (Table 3.4) resulted in a significant decrease in M105 reactivity of strains of *S.typhimurium* ($P<0.001$), *S.virchow* ($P<0.01$) and *S.arizonae* (NTU; $P<0.01$). The antigenicity of *S.give* and *S.ealing* (NTU) were apparently unaffected. Only *S.enteritidis* produced a significant increase in M105 reactivity ($P<0.001$). Another study by Blais and Yamazaki (1989a) reported that boiling salmonella cells in 50mM EDTA for 10min increased the reactivity of anti-salmonella LPS antibodies fourfold compared with untreated cells. When these conditions were repeated in the present study (Table 3.5) the M105 reactivity with *S.enteritidis*, *S.ealing* (NTU) and *S.arizonae* (NTU) was significantly increased ($P<0.01$), *S.virchow* was apparently unaffected and *S.typhimurium* significantly reduced ($P<0.001$).

Kastowski *et al.* (1992) suggested that long chain LPS of wild type bacteria formed a barrier against harmful substances. *Salmonella* serogroups are distinguished by differences in the O-chains of LPS with respect to sugar type, sequence arrangement and linkage (Figure 1.5). Moreover the size and distribution of LPS molecules in the OM, which can be observed by SDS-PAGE analysis, is dependent on serotype. Hence it is plausible that differences in the reactivity of *S.typhimurium* (serogroup B), *S.virchow* (serogroup C), *S.enteritidis* (serogroup D), *S.give* (serogroup E), *S.ealing* (NTU; serogroup O) and *S.arizonae* (NTU; serogroup 51) could be explained by differences in the effectiveness of the LPS barrier.

LPS is an amphiphilic molecule with hydrophilic (carbohydrate) and hydrophobic (lipid)

fragments. As such isolated pure LPS spontaneously associates to form aggregates. This was seen in the dot blot assay experiments, when purified *S.typhimurium* Ra mutant LPS appeared as a precipitate rather than a smooth even coloured dot (Figure 3.7). When dissolved in different chemicals LPS forms various complexes (Kato 1993). Hence the physical shape of LPS polymers are dependent on diluent. For example in aqueous form isolated LPS creates flat ribbon-like and spherical structures (Kato 1993). These can be broken down into smaller units by the addition of surfactants. The binding of surfactants to LPS is reversible - upon removal the LPS reaggregates (Shands 1971). Using the M105 antibody in a dot blot assay Tsang *et al.* (1993) noted that LPS diluted in sterile distilled water produced a dot of twice the colour intensity of LPS diluted in tris-Tween buffer. In the present study LPS preparations were dissolved in TBS. Therefore the question of antigen accessibility arises. The LPS released from pre-treated whole cells is not pure. It forms complexes which in addition to LPS contains proteins, phospholipids and other components of the bacterial cell. However it is possible that the OM fragments and LPS released or exposed by the treatment conditions of the present study could have formed complexes with themselves or with the treatment chemicals in much the same way as isolated LPS, thereby covering the M105 reactive site and resulting in a decrease in M105 antibody reactivity. Another example of the possibility of chemicals covering reactive sites was shown when the M105 antibody was diluted in 1% BSA in TBS containing Tween 20 (Section 3.1.1). The addition of Tween 20 markedly reduced M105 reactivity to purified LPS from *S.typhimurium*.

The wide range of M105 reactivity produced by treatment conditions could also be explained by the poor binding capacity of nitrocellulose for LPS. Differences in the binding abilities of the cell, OM fragments and LPS to the nitrocellulose membrane, caused by the different sugar residues that make up the O-chain of each *Salmonella* serotype, could have resulted in a loss of weakly bound antigen by the washing process or masking of the epitope by treatment released antigen. This theory could be supported by the results of preliminary dot blot investigations. Purified LPS from *S.typhimurium* (L-6511) was easily removed from nitrocellulose by increased washing times in buffer containing Tween 20. While the addition of SDS led to feinter dots being produced (Section 3.1.1). Furthermore studies by other researchers have shown that inclusion of SDS in gel buffers reduces the transfer efficiency of SDS-PAGE differentiated LPS from gel to nitrocellulose membrane (Pyle and Schill 1985). The binding capabilities of LPS to a number of membranes has been investigated by researchers at the Forschungs Zentrum,

Borstel, Germany. Nitrocellulose produced the poorest readings although it was the most straight forward method to use (Dr Otto Holst, personal communication).

Although the interpretation of the results was complex due to the characteristics of the nitrocellulose membrane, they should be kept in perspective. The aim of this part of the study was to increase the M105 antibody reactivity and this aim was successfully carried out. With respect to the IMS-ELISA, a standard treatment was required which would not only enhance the M105 antibody-antigen reaction of (in particular) weakly reactive strains, but could also be carried out in the industrial food testing laboratory. With these criteria in mind, a standard treatment of boiling cells in TBS at 100°C for 30min was chosen.

A number of reports have suggested that heat and chemical treatment of Gram-negative bacteria leads to a release of OM fragments (including LPS) and the disruption of barrier function (Leive *et al.*, 1968; Tsuchido *et al.*, 1985; Blais and Yamazaki 1989a). The results of standard heat treatment (100°C, 30min) experiments of the present study appear to confirm this. Heat treatment was shown to significantly ($P < 0.05$) increase the M105 reactivity of medium to weakly reactive strains (Table 3.9). These results indicated that previously unavailable cryptic core epitopes were made accessible for M105 antibody binding by heat treament.

Blais and Yamazaki (1989a) examined the effect of combined heat and EDTA treatment on the reactivity of anti-salmonella LPS antibodies. These researchers found that 85% of the total activity was released into the supernatant. In the present study the supernatant fraction accounted for approximately 50% of the total antigenic activity of heat treated *S.typhimurium* cells (Figure 3.5). In comparison the M105 reactivity of untreated cells was confined to the cell pellet.

Ten of the 11 non-*Salmonella* strains treated by heat produced significantly ($P < 0.05$) weaker reactions with the M105 antibody after heat treatment (Table 3.10). These results suggested that, as a result of the heat treatment process, the M105 antigen was removed or obstructed. LPS molecules are heat stable (Raetz 1993). Therefore it is also possible that the weak reactivity of untreated non-*Salmonella* strains was caused by other carbohydrate residues sited on the cell surface. If these were heat labile the heat treatment would have destroyed their structure and hence antigenicity. With respect to the IMS-ELISA this result was very promising. Even if non-salmonella organisms were captured

by Dynabeads® anti-Salmonella, heat treatment of cells would mean that cross-reaction to the M105 antibody would be negligable, so that false positive results would be avoided.

4.1.4 *DEVELOPMENT AND EVALUATION OF AN ELISA BASED IMS SYSTEM*

To alay confusion the reader is referred to Figures 3.16, 3.20 and 3.22 for a reminder of the terms IMS-ELISA detection method, the single bead and double bead (without enrichment) protocols and the double bead (with enrichment) protocol respectively.

4.1.4.1 *Purification and labelling of the M105 antibody*

Dynabeads® anti-Salmonella are coated with anti-salmonella antibodies raised in mouse. Hence the enzyme labelled species specific (anti-mouse) detection antibody used in the dot blot and immunoblot assays could not be used in the IMS-ELISA. Therefore attempts were made to label the M105 antibody. The advantage of using a single enzyme labelled salmonella specific antibody was that the detection time of the IMS-ELISA would be reduced by omitting a second antibody incubation stage and washing steps.

At the request of Dynal, the M105 antibody was conjugated with HRP. The Jackson and Zymed methods were unsuccessful due to severe loss of antibody activity (Section 3.6.1). In contrast the Amdex method produced HRP labelled M105 antibody which retained its activity (Dr Berit Grūn, Dynal A/S, Oslo, Norway; personal communication). The HRP labelled M105 antibody was stored at 4°C according to the Amdex Laboratory recommendations. However storage (one month) at 4°C resulted in a complete loss of specificity (Section 3.6.1). This result was not surprising since Nielsen *et al.* (1993) reported that prolonged storage at 4°C of unlabelled M105 antibody resulted in a loss of activity. Researchers suggested that this was due to the fragility of isotype IgG_{2b} antibodies and their propensity to spotaneous degradation (Nielsen *et al.*, 1993). Further investigations into the effect of diluent and lyophilisation on M105 antibody reactivity after storage at 4°C has been carried out by Dynal Research and Development Laboratories, Oslo, Norway. However these results were not available for comment.

4.1.4.2 *Evaluation of the IMS-ELISA salmonella detection technique*

During the course of the present research project a paper was published which reported the use of pre-commercial Dynabeads® anti-Salmonella in an ELISA based system (Cudjoe *et al.*, 1995). This so called 'immunomagnetic particle based ELISA (IMP-ELISA)' used the same technique as the IMS-ELISA. Therefore the IMP-ELISA was compared with the IMS-ELISA in the following sections. The IMS-ELISA used the salmonella core specific monoclonal M105 antibody in an AP labelled double antibody system. In contrast Cudjoe's IMP-ELISA used a single HRP labelled CSA-1 polyclonal antibody (Kirkegaard and Perry Laboratories, Gaithersburg, USA). The CSA-1 antibody recognises numerous common structural antigens (CSA) widely shared by salmonellas. In addition the IMS-ELISA is compared with commercially available immunoassays.

The detection limit of commercially available immunoassays including the TECRA™ visual immunoassay (VIA), Salmonella-Tek™, Transia-*Salmonella*, Path-Stik and EiaFoss Salmonella isolation and detection system is in general reported to be about 10^5-10^7 cfu/ml (Table 1.9). The detection limit of Cudjoe's IMP-ELISA was 10^5-10^6 cfu/ml (Cudjoe *et al.*, 1995). Similarly the IMS-ELISA detected method detected heat treated *S.typhimurium*, *S.virchow*, *S.enteritidis* and *S.give* at 10^5-10^6 cfu/ml (Figure 3.17). Thus the IMS-ELISA was comparable with the IMP-ELISA and the majority of commercially available immunoassays in terms of sensitivity.

The IMS-ELISA detection limit of untreated cells was 10^6-10^7 cfu/ml. These results indicated that boiling cells did not adversely affect the binding of cells to Prototype Dynabeads®. In fact untreated cells gave lower absorbance values than heat treated cells at all cell densities tested (Figures 3.17 and 3.19). From these results it can be concluded that heat treatment (100°C, 30min) was necessary to increase the sensitivity of the IMS-ELISA. Similarly the IMP-ELISA required samples to be boiled for 10min to optimise CSA-1 antibody reactivity (Cudjoe *et al.*, 1995). The majority of commercially available immunoassays also require samples to be heat treated (100°C, 15-20min) before analysis (Table 1.9). Although the reasons behind these treatments may simply be to kill the pathogen in order to help reduce the risk of cross-contamination.

Due to the significant ($P < 0.05$) variation in the absorbance readings produced by the no antigen control (OD_{405nm} 0.16-0.82; Section 3.6.3.3), a general threshold value was not set. Instead the threshold value was re-calculated from the no antigen controls run in each

assay. Samples which produced readings above this threshold were considered as presumptive salmonella positive. In theory, this would avoid false positive results and in addition reduce the risk of falsely identifying samples as negative. By incorporating a threshold value the specificity of the IMS-ELISA, to 23 *Salmonella* strains, was determined. All *Salmonella* strains tested, with the exception of *S.ealing* (Canada) exhibited a presumptive salmonella positive IMS-ELISA reaction (Table 3.13). This suggested that heat released antigen from serotypes B-E, L, O and 51 bound readily to the beads. The Canada strain of *S.ealing* (serogroup O) produced readings marginally lower (OD_{405nm} 0.50 ± 0.16) than the threshold value (OD_{405nm} 0.53) and was therefore incorrectly reported as salmonella negative. Results of an IMS-ELISA using serogroup O specific antisera confirmed that heat treated *S.ealing* (Canada) bound to the beads (Table 3.15). Moreover dot blot assay results had already confirmed that the M105 antibody reacted with *S.ealing* (Canada), albiet weakly (Table 3.9). Therefore it is plausible that the relatively high background readings masked a weak positive signal. It is also possible that not enough antigen was bound to the bead to produce a signal.

The specificity of the IMS-ELISA was compared with Cudjoe's IMP-ELISA. Although tenfold more cells were analysed by the IMS-ELISA (10^9 cfu/ml) this was not considered to be significant. This was because the absorbance readings recorded for 10^8 and 10^9 cfu/ml of *S.typhimurium* (serogroup B), *S.virchow* (serogroup C), *S.enteritidis* (serogroup D) and *S.give* (serogroup E) using the IMS-ELISA were not significantly different ($P > 0.05$; Figure 3.17). The absorbance values of the *Salmonella* serogroup B-E analysed by the IMS-ELISA ranged from OD_{405nm} 1.45-2.66 (Table 3.13). Similarly the IMP-ELISA produced a range of readings (OD_{405nm} 1.10-3.31; Cudjoe *et al.*, 1995). Strains of *S.agona*, *S.bredeney*, *S.typhimurium*, *S.infantis*, *S.virchow* and *S.enteritidis* analysed by Cudjoe *et al.* (1995) were tested by the IMS-ELISA so that direct comparisons could be made (Table 3.13). In general the IMP-ELISA produced higher values compared with the IMS-ELISA. The higher readings may have resulted from differences in the detection systems used (CSA antigen specific HRP-labelled CSA-1 antibody; core specific AP-labelled double antibody system). Cudjoe's IMP-ELISA did not include a blocking stage prior to bead incubation with antigen. According to the results of the present study antigen readily adhered to the microtitre plate wells (Scetion 3.6.3.1). Mean absorbance readings of OD_{405nm}2.05 were recorded for unblocked wells. In contrast wells which were blocked produced a significantly ($P < 0.05$) lower mean absorbance value of OD_{405nm} 0.39. Hence the higher absorbance values produced by the IMP-ELISA may have resulted from

microtitre plate bound antigen as well as bead bound antigen.

Bacterial cells used for the IMP-ELISA were grown in brain heart infusion broth (Cudjoe *et al.*, 1995). However, since buffered peptone water (BPW) broth is recommended for use by the British Standards Institution (BSI) for the isolation of salmonella from foods (Roberts *et al.*, 1995), cultures used in the IMS-ELISA were grown in BPW. The amount of S- and R-type LPS isolated from wild strains of bacteria is reportedly affected by growth conditions (Schlecht 1986; Nelson *et al.*, 1991; Schlecht and Galanos 1994; Schlecht and Mayer 1994). Already mentioned is the possibility that differences in the production and arrangement of S- and R-type LPS affects the LPS barrier. Gigliotti and Shenep (1985) suggested that increases in the amount of O-chain reduces the accessibility to core LPS. Thus differences in the growth conditions of the IMP-ELISA and IMS-ELISA protocols could have caused variation in the amount of S- and R-type LPS produced by *S.agona*, *S.bredeney*, *S.typhimurium*, *S.infantis*, *S.virchow* and *S.enteritidis*. Hence differences in the OD values of the IMP-ELISA and IMS-ELISA may have resulted from differences in the amount of accessible antigen.

The absorbance readings produced by non-*Salmonella* spp. recorded by Cudjoe's IMP-ELISA ranged from OD_{405nm} 0.19 to 0.67 (Cudjoe *et al.*, 1995). Similarly a range of M105 reactivities was produced by the IMS-ELISA (OD_{405nm} 0.25 for *Ent.agglomerans* to OD_{405nm}0.47 for *E.coli*). Dot blot assay results had shown that heat treated non-*Salmonella* strains exhibited a weak cross-reaction with the M105 antibody (Table 3.10). Moreover untreated *C.freundii* (NTU) and *E.coli* bound non-specifically to Prototype Dynabeads® (Table 3.11). Therefore it was possible that the non-*Salmonella* strains would be IMS-ELISA positive. However all 11 non-*Salmonella* strains tested by IMS-ELISA were salmonella negative producing readings lower than the calculated threshold value (OD_{405nm}0.52). Thus although the calculated threshold value was at times relatively high it did prevent some false positive results.

Commercially available immunoassays commonly exhibit cross-reactivity to non-*Salmonella* strains. For example cross-reactions have been reported for the TECRA™ VIA (*Citrobacter* spp.; Jay and Comar 1988), Locate *Salmonella* Immunoassay (*C.freundii*, *C.amalonaticus*, *E.coli*; Eckner 1992; Bobbit and Betts 1993), EiaFoss (*C.diversus*, *Serratia fonticola*; Jones and Betts 1994), Mastazyme™-Salmonella (*E.coli*, *Enterobacter* spp., *Proteus* spp., *Hafnia* spp., *Citrobacter* spp.; Bolton and Franklin 1996)

and the VIDAS Salmonella Assay (*Ent.cloacae*, *C.freundii*; Blackburn *et al.*, 1994). Moreover some *Salmonella* strains may not be detected. Jones and Betts (1994) reported that the EiaFoss Salmonella isolation and detection system failed to detect two non-motile strains including *S.gallinarum*. Hence the IMS-ELISA was no different from other immunoassays by failing to detect the Canada strain of *S.ealing*.

4.1.5 *PRELIMINARY FOOD TRIAL EXPERIMENTS*

A food trial was undertaken to assess the feasibility of the IMS-ELISA under 'real' conditions. Although the IMS-ELISA was effective at isolating *Salmonella* serogroups B, C_1, C_2, C_3, D, E_1, E_4 and L from pure culture (Table 3.13), pure culture experiments do not reflect the situation of food samples. The intrinsic flora of foods is diverse and can adversely affect the growth of salmonellas (van Schothorst and Renauld 1985). Moreover the antimicrobial properties and fatty nature of some foods can impede the recovery of salmonella (Section 1.5.1). In the present study samples of skimmed milk powder (SMP) were analysed which represented a processed food likely to contain low numbers of background flora. Although the specifications for the absence of one salmonella cell relates to 25g of processed foods, salmonella isolation and detection methods are also used for raw meat products and animal feeds. Therefore it is usual practice to test methods against a variety of food types. Cudjoe *et al.* (1994b) employed the IMS method to detect salmonella in raw chicken samples. *Proteus* spp. and mucoid coliforms including *E.coli*, *Klebsiella aerogenes* and *Enterobacter* spp. cross-reacted with and bound non-specifically to the immunomagnetic beads. Therefore, in addition to SMP, the present study tested samples of raw chicken. These represented a fatty food likely to contain high numbers of cross-reactive non-salmonella organisms.

Processing practices designed to kill bacteria frequently generate sublethally injured cells which under the correct conditions are capable of repair and growth and therefore food poisoning. Thus inoculating food trial samples with healthy 18h old cells (such as in the pure culture experiments) would be incorrect. Instead aged (48h) *Salmonella* cells were used to observe the effect of the food matrix and background flora on the recovery of *Salmonella*. It was planned to use sublethally injured cells such as freeze dried cultures in the next step of the evaluation study.

Almost any of the 2,400 *Salmonella* serotypes have the potential to cause food poisoning. Additionally salmonella may contaminate foods at low levels. Thus to simulate this, food

samples were artificially inoculated with representatives of the higher serogroups (*S.ealing*, NTU; *S.arizonae*, NTU) as well as the more commonly isolated serogroups B-E (*S.typhimurium*, *S.virchow*, *S.enteritidis*, *S.give*) at low inoculum levels (approximately 1, 10, 100 cells).

The single bead salmonella isolation and detection protocol (Figure 3.20) was successfully used to detect *S.typhimurium*, *S.virchow*, *S.enteritidis*, *S.give*, *S.ealing* (NTU) and *S.arizonae* (NTU) in artificially inoculated BPW-SMP samples (Table 3.16). Similarly Cudjoe's IMP-ELISA was used to isolate and detect *Salmonella* spp. from artificially inoculated BPW-liquid egg white, whole egg powder, egg white powder and egg yolk powder samples (Cudjoe *et al.*, 1995). Beckers *et al.* (1986b) reported that when the number of competing bacteria present in a food sample was high, the chance of isolating a *Salmonella* sp. was significantly reduced. Moreover van Schothorst and Renauld (1983) reported that the growth of salmonellas was affected by the number of competing organisms. Hence the successful application of the IMS-ELISA and IMP-ELISA could be explained: heat processed samples such as analysed in the present study and by Cudjoe *et al.* (1995) were likely to harbour only low levels of competitive flora, so that the growth of *Salmonella* spp. was not impaired and therefore their numbers reached the detection limit of the IMS-ELISA and IMP-ELISA.

The direct use of the IMP-ELISA technique on boiled BPW-raw chicken samples resulted in false negative samples and was a direct result of the heavy growth of competing flora (Cudjoe *et al.*, 1995). Researchers suggested that high levels of intrinsic competing *Enterobacteriaceae* inhibited the growth of artificially inoculated *Salmonella* spp. or blocked the immunological binding between released salmonella antigen and the immunomagnetic particles (Cudjoe *et al.*, 1995). Similarly in the present study attempts to isolate heat released salmonella antigen from BPW-raw chicken samples was unsuccessful. Ten out of 18 false negatives were recorded (Table 3.17). However this unacceptably high false negative rate was not attributed to the overgrowth of competitive flora. Instead it was due to loss of magnetic particles. Heat treatment of aliquoted BPW produced a highly viscous, almost semi-solid sample. It was visually apparent that beads could not move through this medium to be concentrated by the magnet and so were discarded with supernatant. Particle loss due to the influence of certain types of sample matrices have been previously reported with the standard IMS-selective agar technique (Cudjoe *et al.*, 1994b; Coleman *et al.*, 1995). For example Cudjoe *et al.* (1994b)

recorded a 78% loss of beads from samples of undiluted raw blended eggs. Hence results of the present study indicated that the single bead IMS-ELISA protocol would not be ideal for foods of a high fat content or viscous nature.

Since the single bead IMS-ELISA protocol failed to detect artificially inoculated salmonella from BPW-raw chicken samples an additional bead step was included. In this double bead (without enrichment) protocol (Figure 3.20) a primary batch of beads was introduced to isolate and concentrate salmonella from the pre-enrichment medium. The salmonella-bead complex was then boiled to release antigen. Heat released soluble antigen was then detected by the IMS-ELISA method (Figure 3.16). However the double bead (without enrichment) protocol failed to detect $<10^8$ cfu/ml of *S.typhimurium*, *S.virchow*, *S.enteritidis* and *S.give* (Table 3.18). This poor result was unexpected. The sensitivity limit of the IMS-ELISA was reported as 10^5-10^6 cfu/ml (Figure 3.17). Dynabeads® anti-Salmonella have a minimum recovery rate of 10% depending on *Salmonella* serotype and cell number (Dr Egil Olsen, Dynal A/S, Oslo, Norway; personal communication). Based on the assumption that all IMS captured antigen is released into solution by boiling, it can be predicted that at the very minimum the detection limit for the double bead (without enrichment) protocol would be 10^6-10^7 cfu/ml. Hence the double bead (without enrichment) protocol was up to 100-fold less sensitive than anticipated.

Additional studies confirmed that boiling did not in fact release all bead bound antigen of *S.typhimurium* and *S.give* (Section 3.8.1.1). It was determined that approximately half the antigenicity remained attached to the boiled beads (Table 3.19). For *S.typhimurium* absorbance readings of OD_{405nm} 1.72 and 1.67 were produced for bead bound and heat released antigen respectively. Likewise for *S.give* the bead bound and heat released antigen expressed similar absorbance readings (OD_{405nm} 1.88 and 1.96). In the light of these findings, the absorbance readings produced by tenfold dilutions of *S.typhimurium*, *S.virchow*, *S.enteritidis* and *S.give* (Table 3.18) were re-examined. If half of the antigenicity remained attached to the primary batch of beads, one would expect 10^6 cfu/ml of *S.typhimurium* and *S.virchow* and 10^7 cfu/ml of *S.enteritidis* and *S.give* to be positive by the double bead (without enrichment) IMS-ELISA technique. Since the detection limit was in fact up to 100-fold less sensitive, one can infer that the second batch of beads only captured a fraction of the total amount of heat released antigen. The sensitivity limit of the double bead (without enrichment) protocol was unacceptably high at 10^8 cfu/ml (Table 3.18). This number of salmonella organisms is not commonly found in BPW pre-enriched

food samples. It was therefore necessary to introduce a post enrichment stage. The primary batch of beads would capture and concentrate salmonella by IMS from a mixed background flora. Post-enrichment in GN broth containing novobiocin (nGN) would increase the selection pressure for salmonellas by promoting their growth at the expense of competing flora, particularly *Citrobacter* spp.. Salmonella antigen would be released by boiling and detection would be by IMS-ELISA. In general the double bead (with enrichment) protocol (Figure 3.22) increased the absorbance values produced by presumptive salmonella positive samples. However *Salmonella* spp. were still not detected in approximately half (8/17) of the artificially inoculated BPW-raw chicken samples (Table 3.20). Growth on loop inoculated XLD plates confirmed the presence of *Salmonella* spp. in the nGN broths of all the false negative samples. It could therefore be concluded that in the false negative samples, salmonella numbers failed to reach the detection limit of the IMS-ELISA. Preliminary growth studies had confirmed that a 6h incubation in nGN broth was sufficient time for 100 *S.typhimurium* cells to multiply to 10^6 cells (Figure 3.21), which is within the 10^5-10^6 cfu/ml detection limit of the IMS-ELISA (Figure 3.17). Therefore in the double bead (with enrichment) protocol, the false negative samples indicated that less than 100 salmonella cells were captured by IMS from the pre-enrichment medium. Between 1 and 100 cells were inoculated into the pre-enrichment medium prior to an 18h incubation. Hence the results suggested that flora of other competing *Enterobacteriaceae* prevented salmonella numbers from reaching high enough levels to be detected by the IMS-ELISA. Alternatively non-salmonella organisms could have blocked the immunological binding reaction between salmonella and the primary batch of Dynabeads® (Cudjoe *et al.*, 1995).

The time required for commercially available immunoassays to detect presumptive positive *Salmonella* spp. varies considerably from 24 to 56h (Table 1.9). The single bead protocol was used to detect *Salmonella* spp. in artificially inoculated SMP samples within 21h. However the IMS-ELISA cross-reacted with an unidentified natural isolate (Table 3.16). Such false positive samples could be largely overcome by introducing an additional step in which salmonellas were selected for. Hence for all food types, pre-enrichment followed by IMS and post-enrichment is recommended before detection by IMS-ELISA. Even though this double bead (with enrichment) protocol would extend the total test time to 27h, it was still faster than the majority of commercially available immunoassay protocols and was comparable with the EiaFoss Salmonella detection system (24-33h). In addition the IMS-ELISA could co-analyse three times the number of samples that the

EiaFoss analyser could (96 well per microtitre plate compared with 27 tubes). Furthermore Dynabeads® and microtitre plates do not take up as much incubator space as tests such as the TECRA™ Unique (Section 1.5.3.6).

4.1.6 *ADDITIONAL COMMENTS*

In the present study the master cultures of bacterial strains were stored at -20°C for up to three years. During this period some *Salmonella* strains were analysed yearly by dot blot assay. The results indicated that prolonged storage of *S.agona*, *S.bredeney*, *S.saint paul*, *S.infantis*, *S.livingstone*, *S.virchow*, *S.hadar*, *S.newport*, *S.dublin*, *S.enteritidis*, *S.panama* and *S.senftenberg* led to reduced M105 antibody reactivity (Figure 3.6). This change in M105 reactivity due to long term storage in the laboratory of *Salmonella* strains emphasised the need to test fresh, natural isolates from food and other sources. Unfortunately due to time constraints the analysis of fresh isolates were not undertaken.

4.1.7 *IMS-ELISA SUMMARY AND CONCLUSIONS*

Dot blot assay, PAGE (denaturing SDS and non-denaturing) analysis and immunoblot assay techniques were used to

(1) determine the M105 epitope site

(2) study the M105 antibody reactivity with *Salmonella* and non-*Salmonella* strains

(3) visualise the affects of chemical, heat and physical treatment on M105 antibody reactivity and

(4) monitor the reactivity and specificity of the enzyme labelled M105 antibody.

In the present study the M105 reactive epitope was confirmed as LPS. The M105 antibody reacted with Ra and to a lesser extent Rb_1 type salmonella core structures. Screening studies showed that all *Salmonella* strains analysed including the most commonly isolated serogroup B-E, as well as representatives of the higher serogroups (L and O), including a member of the *Arizona* group possessed the M105 epitope. Pre-treatment of salmonella cells was shown to affect M105 reactivity. Heat treatment by boiling (100°C, 30min) was shown to significantly ($P < 0.001$) increase the reactivity of the M105 weakly reactive *Salmonella* strains (*S.ealing*, NTU; *S.arizonae*, NTU). A very weak M105 reactivity was exhibited by non-*Salmonella* strains which was reduced by boiling. This suggested that the reactive epitope in non-salmonella was not heat stable LPS.

An IMS-ELISA technique was developed based on Prototype Dynabeads® and the M105

antibody. Attempts to label the M105 antibody were unsuccessful resulting in a severe loss of reactivity. Evaluation studies showed that all *Salmonella* strains tested, with the exception of *S.ealing* (Canada), were detected by the IMS-ELISA method. Although non-*Salmonella* strains were not detected in pure culture, an unidentified natural isolate produced absorbance values above the threshold and was therefore incorrectly reported as salmonella positive. A sensitivity limit of 10^5-10^6 cfu/ml was reported. Preliminary food trial experiments showed that the IMS-ELISA technique could be used directly on pre-enriched SMP samples. Results were therefore available after 21h. A 27h double bead (with enrichment) protocol successfully isolated salmonella cells from BPW-raw chicken samples. However the numbers of salmonella did not always reach the detection limit of the IMS-ELISA and were therefore inaccurately reported as salmonella negative.

In conclusion the present study has shown that the M105 antibody has the potential to react with all commonly isolated *Salmonella* serogroups and in addition strains from the higher serogroups. The IMS-ELISA has been shown to be comparable to commercially available immunoassays in terms of sensitivity and specificity. However the double bead (with enrichment) protocol, as it stands, could not be used to isolate low levels of salmonella from BPW-raw chicken samples.

4.1.8 *IMS-ELISA: THE WAY FORWARD*

For the IMS-ELISA technique to be a feasible alternative to commercially available immunoassay systems, it has to be comparable if not better in terms of sensitivity, specificity or time of detection. The present study has shown that the IMS-ELISA is comparable in terms of specificity and sensitivity with respect to pure culture experiments. Unfortunately the productivity of the IMS-ELISA was adversely affected by the intrinsic flora of some food samples tested. All false negative results for the raw chicken samples in the double bead (with enrichment) protocol were caused by low numbers rather than an absence of salmonella. One way to possibly overcome this problem would be to follow the pre-, selective and post-enrichment protocol of commercially available immunoassays. This would decrease the number of false negative samples but would increase the time of detection. Moreover the Dynabeads® would be employed to act less like a separation and concentration technique and more like a solid phase matrix. The resultant ELISA would be no better than commercially available ELISAs and would be worse than the standard Dynabeads® anti-Salmonella IMS-selective plating method at least in terms of time of detection.

An alternative method would be to enhance the sensitivity of the IMS-ELISA. If successful the number of false negative samples would be reduced and the post-enrichment culture time of the double bead protocol could theoretically be reduced to under 6h and the aim of having an assay performable within one working day may be realistic. It is for this reason that attempts should be made to increase the sensitivity of the IMS-ELISA. This might be possible by incorporating chemiluminescence reactions as these have been reported to be able to detect femto- or even attomolar quantities of analyte (Tsang and Nielsen 1992).

Alternatively increased Dynabead® incubation times may increase sensitivity. Luk and Lindberg (1991) reported a *Salmonella* serogroup C_1 specific isolation and detection system based on *Salmonella* serogroup C_1 specific antibodies coated onto Dynabeads® in an EIA system. These researchers incubated beads and samples for 45min to achieve a detection limit of 10^3 cfu/ml. Prolonged incubation periods have been reported to increase the number of cross-reactions (Safarík *et al.*, 1995), which is a problem only when attempting to isolate small numbers of salmonellas from a large number of background flora such as found in pre-enrichment broths. However the double bead (with enrichment) protocol involved the isolation of *Salmonella* from pre-enrichment media by IMS, followed by a short 6h post-enrichment in GN broth plus novobiocin. Under these circumstances where the ratio of *Salmonella* to non-*Salmonella* strains was altered in favour of *Salmonella* strains, prolonged incubation with the second batch of Dynabeads® may not be a problem.

The addition of another salmonella specific antibody, for example against a flagellar antigen, could increase the specificity of the IMS-ELISA to include the M105 non-reactive strains such as *S.ealing* (Canada). Moreover an additional antibody could enhance the detection of weakly reactive strains such as found in the higher *Salmonella* serogroups (*S.adelaide*; *S.ealing*, NTU; *S.arizonae*, NTU).

Under the current IMS-ELISA protocol the ELISA tray wells were blocked with 3% SMP in TBS-T. The purpose of which was to stop non-salmonella antigen from interfering with the M105-salmonella antigen reaction. Since a post-enrichment stage was introduced, this step is no longer required. If the blocking stage was omitted from the protocol salmonella antigen would passively adhere to the ELISA tray wells (as with the Locate ELISA) which may increase sensitivity of the IMS-ELISA system.

At present it is unknown how many salmonella cells bind to the Dynabeads® during post-enrichment. Since the results of the present study have shown that boiling does not release all bead bound antigen, it follows that the sensitivity of the double bead (with enrichment) protocol may be compromised by analysing the supernatant alone. A significant portion (50%) of the antigenicity may remain attached to the primary batch of beads (primary beads) which according to the current double bead (with enrichment) protocol were discarded and therefore wasted. The post-enriched primary beads could be concentrated and boiled together with an aliquot of supernatant. The boiled primary beads and supernatant could then be mixed with a fresh batch of Dynabeads® and the assay carried out. This would theoretically increase the sensitivity of the double bead protocol. In the VIDAS immunocapture salmonella (ICS) antibody captured salmonella are released by an enzymatic cleavage method and post-enriched before analysis by ELISA. If this approach could be incorporated into the double bead IMS-ELISA protocol this would ensure that all antigen was detected.

4.2 INVESTIGATION OF SALMONELLA LPS CORE STRUCTURE

To aid in the understanding of the next section the reader is reminded that the term 'wild type bacteria' is synonymous with the term 'smooth type bacteria'. The lipopolysaccharide (LPS) isolated from smooth type bacteria is termed 'smooth form LPS'. Smooth form LPS is made up of R-, SR- and S-type LPS. R-type LPS is made up of lipid A plus core oligosaccharide. Whereas SR-type LPS contains lipid A, core oligosaccharide plus one O-chain repeat unit. S-type LPS is composed of lipid A, core oligosaccharide plus two or more O-chain repeat units. The LPS isolated from rough mutant strains of bacteria is termed 'rough form LPS'. Rough form LPS is made up of R-type LPS with either complete or partial core structures linked to lipid A (Figure 1.3).

The primary aim of this project was to develop and test an ELISA system based on IMS capture and M105 antibody detection. The key to its success as a *Salmonella* genus specific detection method was largely based on the general belief that all *Salmonella* spp. share a common LPS or Ra type core structure (Kato 1993; Figure 1.3). However a small number of reports proposed that non-Ra type or atypical core structures exist (Tsang and Schlecht 1990; Tsang *et al.*, 1991a; 1991b; 1992; Hoofdvak and Olsthoorn 1995). Extensive investigations were therefore carried out to determine the presence of the M105 epitope in LPS based on serological and chemical analysis.

4.2.1 *LOCALISATION OF THE M105 EPITOPE SITE WITHIN THE LPS MOLECULE*

Although similar numbers of cells were assayed by dot blot analysis (10^9 cfu/ml) the results of the present study showed that the M105 antibody expressed a wide range of reactivity with the *Salmonella* strains tested (Table 3.9). Rietschel *et al.* (1984) reported that LPS represented 3-8% of the dry weight of the Gram-negative bacterial cell which could not alone explain the wide range of reactivity. Based on the assumption that all *Salmonella* spp. share a common core structure (Kato 1993), the range of M105 reactivity could be attributed to the differential binding of the M105 antibody to LPS molecules of varying O-chain length. Hence the SDS-PAGE technique was used to observe the heterogeneity of LPS isolated from wild type bacteria with respect to O-chain length and amount of S- and R-type LPS.

Pyle and Schill (1985) reported that the addition of 0.1% SDS in acrylamide gels resulted in a tenfold decrease in the transfer efficiency of LPS to nitrocellulose membrane. Moreover they reported that the omission of SDS did not affect LPS mobilities. In another report Peterson and McGroarty (1985) suggested that the slowest migrating bands of SDS-PAGE differentiated LPS from *E.coli* were in fact LPS multimers of long O-chains and not LPS with very long chains. These researchers recommended that the concentration of SDS in the separating gel should be increased from 0.1% to 0.5% to prevent multimer formation. For the purposes of the present study the silver stained SDS-PAGE separated LPS profiles were not as significant as the immunoblot reaction of the M105 antibody to the electrophoretically transferred LPS; hence SDS was omitted from all gel buffers.

Rietschel and Brade (1992) reported that the LPS of Gram-negative bacteria typically consist of 20-40 repeating O-chain units. The present study confirmed this since the majority of strains analysed by the QuantiScan method produced 20 to >30 bands (Table 3.21). The number of O-chain repeat units described for the SDS-PAGE separated *S.typhimurium* varies from 30-40 (Tsai and Frasch 1982), >40 (Goldman and Leive 1980), to >80 (Peterson and McGrorty 1985). In the present study 21-30 and >30 bands were reported for *S.typhimurium* (L-6511) and *S.typhimurium* respectively (Table 3.21). The reasons for the differences in the number of bands could be due to strain variation.

There appears to be contradictions in the literature concerning the presence of so called 'doublet bands'. For example in the serotype *S.typhimurium*, doublet bands have been reported as both present (Goldman and Leive 1980; Hitchcock and Brown 1983) and

absent (Munford *et al.*, 1980; Tsai and Frasch 1982). The presence of doublet bands has been attributed to phosphate heterogeneity in the lipid A-core (Hitchcock and Brown 1983) or due to the occurrence of intermolecular interactions between O-chains of LPS molecules of approximately the same length (Peterson and McGroarty 1985; Solovjeva and Ovodov 1992). In the present study doublet bands were restricted to strains of *S.arizonae* (NTU), *S.ealing* (NTU), *S.ealing* (Canada), *S.hadar*, *S.infantis*, *S.virchow*, *S.gallinarum*, *S.kentucky*, *S.livingstone* and *S.manchester*. Moreover the appearance of doublet bands was dependent on the amount of LPS applied to the gel. Overloading meant that doublet bands although presumably present were seen as a single thick band (Figure 3.25). Hitchcock and Brown (1983) reported that the uppermost band of the doublet was stained grey and the lower band was stained red. Other investigations have described orange and dark brown staining for the top and bottom bands respectively (Darveau and Hancock 1983). In the present study both bands were stained a red/brown colour. However the lower band was thicker and darker. Preliminary results had shown that the more LPS applied to the gel, the thicker and darker the bands (results not included). Hence differences in the colour of the doublet were interpreted as differences in the number of LPS molecules present in each band.

According to Goldman and Leive (1980) and Palva and Mäkelä (1980) the lowest molecular weight band represents R-type LPS devoid of O-chain. This was confirmed by the reaction of M105 salmonella core specific antibody and the lack of reaction of anti-serogroup O O-chain specific antisera with the fastest moving band of the LPS profile of *S.ealing* (Figure 3.29).

Protein molecular weight markers are not suitable for estimating the size of LPS molecules (Tsai and Frasch 1982). Moreover LPS molecular weight markers are not commercially available. However, in theory, the molecular weights of LPS molecules could be calculated. This could be achieved in two ways:

(1) Based on known chemical structures

The chemical structures of lipid A and the Ra core are well defined (Section 1.4.1). In addition the O-chain repeat units of a limited number of *Salmonella* strains have been elucidated (Knirel and Kochetkov 1994). Hence the molecular weight of each LPS component could be calculated. In an SDS-PAGE LPS banding profile the fastest moving band is R-type LPS. Each band above this represents an addition of a single O-chain

repeat unit (i.e. the second band represents lipid A-core with one O-chain repeat unit, the third represents lipid A-core plus two O-chain repeat units and so forth). Hence the molecular weight of each band in the LPS profile could be calculated.

(2) By comparing the migration pattern of a standard

The LPS from a smooth strain of known O-chain structure could be used as a standard to be run in every gel. By using method (1) the molecular weight of each band in the LPS profile of the standard could be calculated. Then by comparing the location of the bands from the standard with the unknown, the molecular weight of the bands from the unknown strain could be estimated (Tsai and Frasch 1982).

Immunoblotting of LPS profiles enables the examination of the antibody reaction with LPS molecules of varying O-chain length. Hence the electrotransferance technique was used to transfer LPS from polyacrylamide gel to nitrocellulose membrane. Electrophoresis time and conditions vary between wet and dry systems. The transfer of LPS profiles from mini-gels to nitrocellulose membrane by semi-dry systems can be achieved by 15-30min at 10-15V (Towner and Cockayne 1993). These systems often result in the complete transferance of LPS from gel to nitrocellulose membrane. Comparisons between gel and immunoblot are achieved by running duplicate gels. However this can lead to problems of misalignment (Pyle and Schill 1985). In the present study effective partial transfer with a uniform representation of LPS bands was achieved by 2h at 100mA (Figure 3.1). This enabled the direct comparison of LPS profiles on the gel and nitrocellulose membrane. Combinations of high current and extended transfer times may result in some LPS molecules passing straight through the membrane (Towner and Cockayne 1993). This did not appear to be true for the electrotransfer conditions carried out in this study, since no LPS was present on the back up piece of nitrocellulose (Section 3.1.2).

When the LPS of the M105 antibody reactive strains (as detected by dot blot assay) were analysed by immunoblot assay, all were shown to possess the M105 epitope (Table 3.23). This confirmed that when wild type salmonella cells reacted with the M105 antibody, their reactive component was LPS. However three types of immunoblot reaction were produced. These were referred to as reaction types 1, 2 and 3 (Figure 3.28).

In reaction type 1 the M105 antibody reacted with all bands in the LPS profile which confirmed that the M105 epitope was present and accessible in all LPS molecules

regardless of O-chain length and composition. The M105 antibody also reacted with all LPS with the exception of very long O-chain length. This reduced reaction could be explained by a less efficient electrotransfer of antigen from gel to nitrocellulose caused by excess transfer buffer. Alternatively it could imply steric hindrance of M105 antibody binding to very long O-chain LPS (Giglotti and Shenep 1985). Reaction type 1 was expressed by *Salmonella* strains from serogroups B, C_2, C_3, D and E (Table 3.23).

When considering reaction type 2 and 3 the reader must bear in mind that the M105 epitope is present at the distal end (outer core region) of the Ra core (Figure 1.14). In reaction type 2 the M105 antibody reacted with LPS molecules of short O-chain length and R-type LPS only. Kastowsky *et al.* (1992) used molecular modeling techniques to calculate the possible 3D architecture and conformational flexibility of complete S-type LPS. Researchers suggested that the O-chain, in contrast to the well defined cylindrical shape of the core, would bend at specific sites. The O-chain repeat units of LPS from different *Salmonella* serogroups is made up of a variety of sugars, in various configurations and with different side chains (Knirel and Kochetkov 1994; Figure 1.4). This presumably creates 3D structures of varying shape and rigidity. Hence the restricted binding of serogroup C_1, F and L salmonellas could be explained by steric hindrance of the M105 epitope caused by the addition of a few or more O-chain units.

Although they share common O-antigens (Figure 1.4), the O-chains of subgroup C_2 and C_3 (reaction type 1) and C_1 (reaction type 2) are made up of different sugars. For example, the O-chain of *Salmonella* subgroup C_2 and C_3 have main chains built up of tetrasaccharide repeating units and two lateral monosaccharide substituents (abequose and glucose). While the O-chain of *Salmonella* subgroup C_1 have a main chain with pentasaccharide repeating units, again with side chains (Knirel and Kochetkov 1994). Hence differences in the reactivities of subgroup C_2 and C_3 and C_1 could also be explained by differences in the 3D architecture and rigidity of their O-chains.

The immunoblots of *Salmonella* strains from serogroup G, K, O, U, V, X and 63 revealed that the M105 antibody bound to R-type LPS with reduced reactivity (reaction type 3). Failure of M105 to react with SR- and S-type LPS suggested that the M105 epitope became unavailable once a single O-chain repeat unit was added (Nelson *et al.*, 1992). The M105 antibody reactive R-type LPS could either be complete Ra core or partial Rb_1 type core (with the terminal GlcNAc missing) since the M105 antibody has been shown

to react with Ra and Rb_1 type LPS (Table 3.3).

A number of additional points can be made by comparing dot blot and immunoblot assay results (Tables 3.9 and 3.23). Despite the restricted binding of the M105 antibody to short O-chain and R-type LPS of *S.infantis* and *S.virchow* (serogroup C_1), the M105 antibody showed medium (*S.infantis*; K/S 1.03) to strong (*S.virchow*; K/S 1.86) reactions in dot blot assay with untreated cells. This indicated that there was sufficient exposed SR- and R-type LPS to allow binding by the M105 antibody (Nelson *et al.*, 1992).

Dot blot assay results showed that the M105 antibody reacted with untreated (K/S 0.34) and boiled (K/S 0.62) *S.minnesota* cells; but was unreactive with purified LPS derived from another strain (L-6261; Figure 3.4). Whereas immunoblot assay results confirmed that short O-chain and R-type LPS from both strains reacted with M105 (Table 3.23). This phenomenon could be easily explained. The distribution of LPS in untreated and boiled cells was such that a limited number of reactive cores was available for M105 antibody binding. In contrast the M105 epitope was not accessible in purified non-differentiated LPS. Lüderitz *et al.* (1982) reported that the hydrophobic lipid A regions organise such that they are isolated from aqueous solutions by layer of polar (polysaccharide) heads to form ribbon-like structures and discs. It is therefore plausible that LPS produces complexes where the M105 epitope is inaccessible to antibody binding. However since SDS-PAGE differentiates the LPS molecules into monomers, it follows that the M105 reactive short O-chain and R-type LPS were released and made available for antibody binding.

The M105 antibody expressed a limited cross-reaction with untreated *C.freundii* (NTU, 4/840, 4/841, 4/1155, 4001, 4002, 5/308, 5/785), *Ent.agglomerans*, *E.coli* and *Sh.flexneri* by dot blot assay (Table 3.10). This reactivity was reduced by boiling cells at 100°C for 30min (Table 3.10). No reaction was expressed by SDS-PAGE differentiated LPS derived from these *Enterobacteriaceae* (Section 3.9.2). Hence these results confirmed the earlier theory that the M105 epitope was expressed in other carbohydrate residues of the cell envelope and did not indicate a common LPS core structure.

The antibody T6 was reported to be specific for the outer core region of Ra type LPS, however unlike M105 it does not react with Rb_1 type LPS (Figure 1.14; Tsang *et al.*, 1987). The T6 antibody specificity is not only defined by the presence of the GlcNAc

residue but also how it is linked to the core oligosaccharide (Tsang *et al.*, 1987). Due to this strict monospecificity, T6 has been used to study the outer core structures of salmonella LPS. Typical Ra type LPS core structures have been confirmed in many *Salmonella* serotypes from serogroups A, B, C, D and E (Tsang *et al.*, 1987; 1991b; Choi *et al.*, 1992; Ng *et al.*, 1996). In contrast a large proportion of the *Salmonella* strains from higher serogroups analysed lacked the T6 epitope. For example Tsang and Schlecht (1990) noted that T6 did not react with the LPS of *S.adelaide*, a *Salmonella* serogroup O strain. Furthermore, Tsang *et al.* (1991b) reported that ten serotypes of the subspecies IIIa *S.arizonae*, four serotypes of the subspecies *S.houtenae*, three out of five serogroup N strains, four out of five serogroup O strains, one out of four serogroup U strains and three out of three serogroup V strains also failed to react with the T6 antibody. In their investigations Ng *et al.* (1996) studied 232 *Salmonella* strains for the presence of the T6 epitope. Twenty-nine percent of the higher serogroup F-67 did not react with the T6 antibody. By comparing the reactivities of the M105 and T6 antibodies (Table 3.23) it was possible in the present study to tentatively identify Ra type core structures, reveal some structural diversity in this part of the LPS molecule and in some cases confirm the presence (by immunological means) of atypical Rb_1 type core structure. Strains which reacted with the M105 antibody but were T6 unreactive were considered to possess atypical Rb_1 type cores. These included both strains of *S.godesberg* (1140; 1431), *S.adelaide* (1464), *S.milwaukee* (1139), *S.nirembe* (1660), both strains of *S.bere* (10392; 10427) and *S.arizonae* (10463). Whereas the atypical core structures of M105 and T6 unreactive strains of *S.djakarta* and *S.arizonae* (1803) could not be determined by serology alone. These results indicated the potential diversity of salmonella LPS outer core structure.

4.2.2 *THE CHEMICAL ANALYSIS OF LPS AND CORE OLIGOSACCHARIDE ISOLATED FROM S.EALING (NTU) AND S.ARIZONAE (NTU)*

The R-type LPS from wild type strains of *Salmonella* serogroup O strains (*S.adelaide*; *S.ealing*, NTU; *S.ealing*, Canada) and *S.arizonae* (NTU) produced a very weak reaction with the M105 antibody (reaction type 3; Figure 3.28). This could be explained by a limited amount of R-type LPS. However the silver stained LPS profiles showed that R-type LPS was not limited (e.g. *S.ealing* Figure 3.28). In addition to Ra type core the M105 antibody reacted with Rb_1 type LPS with reduced reactivity (Table 3.3). Therefore an alternative explanation, of weakly reactive *Salmonella* serogroup O strains and *S.arizonae* (NTU), was that their R-type LPS was made up of atypical Rb_1 type core

structures. This theory was investigated by means of chemical analysis (compositional and ratio of components) of the LPS from a representative serogroup O *S.ealing* (NTU) and *S.arizonae* (NTU).

At this point the reader is reminded that complete Ra type LPS is composed of N-acetyl glucosamine (GlcNAc), glucose (Glc), galactose (Gal), heptose (Hep), 3-deoxy-D-mann-octo-2-ulopyranosonic acid (Kdo) and phosphate (P) residues. The standard salmonella Ra core shows a GlcNAc:Glc:Gal ratio of 1:2:2 (Tsang *et al.*, 1992). Moreover, to date, there is no published data on the O-chain repeat units of *S.ealing* (serogroup O) and serogroup 51 *S.arizonae*.

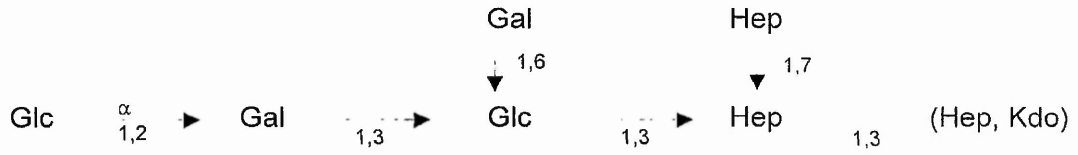
In the present study the results from the spectrophotometric and GLC analyses showed that the LPS from *S.ealing* (NTU) was composed of Glc, Gal and GlcN (representing both glucosamine and N-acetyl glucosamine) levels higher than expected for an Ra type core (15.4:11.1:10.9; Table 3.26). Moreover the MS analysis showed that 3,6-dideoxyhexose was also present (Dr Simon Haseley, the Forschungs Zentrum, Borstel, Germany; personal communication). These results indicated that Glc, Gal, GlcN and 3,6-dideoxyhexose were present in the O-chain repeat unit of *S.ealing*. Kenne *et al.* (1983) elucidated the structure of *S.adelaide*. Researchers reported that the O-chain repeat unit was composed of Glc, Gal, GlcNAc and a 3,6-dideoxyhexose called colitose (Figure 1.4). Since *S.adelaide* and *S.ealing* are serogroup O salmonellas, it is likely that the composition of their O-chains are identical. In contrast the O-chain repeat unit of *S.arizonae* primarily appears to be made up of glucose (17591nmolmg^{-1}; Table 3.26). It is plausible that glucose is the only sugar present. Although this theory can only be confirmed by compositional and structural analysis of isolated O-chain.

In the present study preliminary LPS compositional analysis results for *S.arizonae* (NTU) indicated that the amount of GlcNAc was lower than expected for LPS with an Ra type core (Dr Otto Holst, personal communication). This suggested that the LPS of *S.arizonae* (NTU) possessed an atypical core structure without terminal GlcNAc. Similar findings have been published for rough mutant *Salmonella* strains. For example the terminal GlcNAc residue of a rough mutant strain of *S.djakarta* (48:z_4,z_{24}:-) was reportedly absent, thus resulting in an Rb_1 type core structure (Tsang *et al.*, 1992; Figure 4.1). In the same study Tsang *et al.* (1992) also reported that the terminal GlcNAc residue of the rough mutant strain *Salmonella* IV (18:z_{36},z_{38}:-) had been replaced by Glc (Figure 4.1).

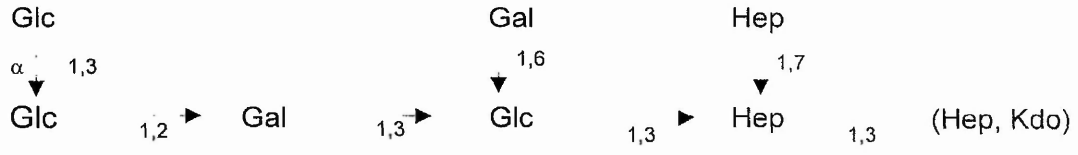
Furthermore Hoofdvak and Olsthoorn (1995) reported that the LPS isolated from rough mutant strains of *S.arizonae* IIIa were heterogeneous. Researchers proposed that the core structure of the major species lacked terminal GlcNAc and Glc (Figure 4.1). The minor species was also identified and in addition to the loss of GlcNAc, lacked the 3-linked Gal residue. Although not specified by the researchers, these core structures resemble Rb_2 type (major species) and Rb_3 type (minor species) core structures (Figure 1.3). A study by Schlecht *et al.* (1992) reacted R-antisera (anti-Ra, Rb_1,Rb_2, Rc) against SDS-PAGE differentiated LPS profiles of a range of wild type salmonella. In this report Schlecht discovered that R-type LPS was not entirely composed of Ra type LPS. Instead R-type LPS was made up of a heterogeneous mixture of complete and partial core structures. Moreover the composition of the R-type LPS was different among the different S-form LPS preparations. Researchers stated that this partial substitution of the core with side branched GlcNAc residues was quite common in the R-type LPS of wild type *Salmonella* strains.

Therefore a question arises: can the structure of core oligosaccharide isolated from rough mutant strains accurately reflect the structure of wild type strains? Hoofdvak and Olsthoorn (1995) in addition to rough mutant strains of *S.arizonae* IIIa also analysed a wild type strain. The chemical analysis results suggested that the LPS isolated from the wild type strain possessed an Ra type core without the GlcNAc residue but bearing an extra Glc residue. This result was in contradiction to the rough mutant strains which possessed the Rb_2 or Rb_3 type core structures. Another study by Tsang *et al.* (1991a) reported that a wild type strain of *S.djakarta* failed to react with the core specific T6 antibody. In the present study the same strain did not react with the M105 antibody in a immunoblot assay (Table 3.23). From these serological analyses it could be concluded that wild type *S.djakarta* did not possess either an Ra or Rb_1 type core structure. Contrary to these findings chemical analysis of LPS showed that a rough mutant strain of *S.djakarta* had lost the terminal GlcNAc without replacement, resulting in an Rb_1 type core structure (Tsang *et al.*, 1992). Clearly serological analysis of the wild type strain did not comply with the chemical analysis results of the rough mutant strain. Thus in answer to the earlier question, these results suggest that rough mutant strains should not be used, as advocated by Tsang *et al.* (1992) and Hoofdvak and Olsthoorn (1995), to confirm the presence of atypical core structures in wild type strains.

S.djakarta



Salmonella IV



S.arizonae III

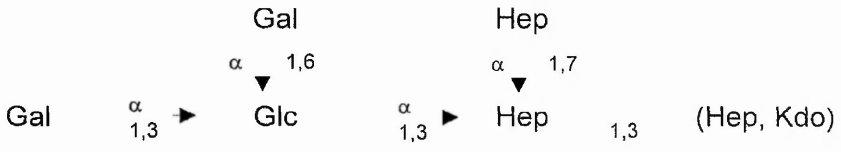


Figure 4.1: The proposed core structures of LPS from rough mutant strains of *S.djakarta*[a], *Salmonella* IV[a] and *S.arizonae IIIa*[b] (from [a], Tsang *et al*., 1992; [b], Hoofdvak and Olsthoorn 1995)

Glc, glucose; Gal, galactose; Hep, heptose; Kdo, 3-deoxy-D-manno-octo-2-ulopyranosonic acid

While a detailed knowledge of the biosynthesis of salmonella LPS is beyond the scope of this thesis, the conclusions of previous reseachers warrents comment. Hoofdvak and Olsthoorn (1995) suggested that the presence of partial core structures in rough mutant strains reflected incomplete LPS core biosynthesis or transfer of distal residues. The idea of 'incomplete biosynthesis' is not new. It is reportedly the cause of the intrinsic heterogeneity of lipid A (Rietschel *et al.*, 1991). With respect to the variations in the composition of R-type LPS from wild type strains, researchers proposed that this reflected the variability in the outfit of the biosynthetic enzymes of the individual *Salmonella* strains (Schlecht *et al.*, 1992). Hence it is plausible that in R-type LPS, whether isolated from rough mutant or wild type strains of salmonella, biosynthesis is to varying degrees incomplete. This adds weight to the argument that rough mutant strains should not be used to confirm the existence of non-Ra type core structures in wild type bacteria.

The lack of reactivity of core specific antibodies to S-type LPS has, in the serology section of this report (Section 4.2.1), been explained by the addition of one or more O-chain repeat units. However it is also possible that non-Ra type core structures, which vary in their distal residues, exist in S-type LPS. One such structure has been reported in a strain of *S.arizonae* IIIa (Hoofdvak and Olsthoorn 1995). It is generally believed that, in LPS biosynthesis, core oligosaccharide needs to be complete (i.e. Ra type) before the O-chain sugars can be attached (Whitfield 1995). This system is based on information gathered from studies of a limited number of rough mutant *Salmonella* strains. However to date 2,400 serotypes of wild type salmonella have been identified. Moreover the genus *Salmonella* is a genetically diverse group of bacteria. It is plausible that differences in LPS biosynthesis occur not only in R-type LPS as suggested by Schlecht *et al.* (1992) but in addition occur, with respect to the loss of distal residues, following the biosynthesis of complete S-type LPS (Tsang *et al.*, 1992). It is possible that, for *S.arizonae* IIIa described by Hoofdvak and Olsthoorn (1995), the GlcNAc was removed and replaced by an additional Glc residue.

LPS isolated from wild type strains of *S.ealing* (NTU) and *S.arizonae* (NTU) were subjected to mild acid hydrolysis to remove the lipid A. The lipid A from *S.ealing* (NTU) was shown to represent 12.5% of the freeze dried weight of LPS. This was within the expected 10-25% yield suggested by Mayer *et al.* (1985). The remaining carbohydrate moieties were fractionated by gel permeation chromatography on Sephadex G50 (Section 3.10.3.1). The elution profile of *S.arizonae* (NTU) showed two peaks. These were

interpreted to represent long chain (Peak 1) and core oligosaccharide (Peak 2). In contrast, the elution profile of *S.ealing* (NTU) showed three peaks. Kenne *et al.* (1983) used the same technique to separate the carbohydrate moiety of *S.adelaide* LPS. Similarly three peaks were produced. Subsequent methylation analysis of the fractions representing the peaks showed that each possessed products which were typical of the methylation of a LPS having salmonella core (Kenne *et al.*, 1983). Hence the three peaks produced by *S.ealing* (NTU) could be interpreted as three populations of similarly sized O-chain-core oligosaccharide structures.

Peterson and McGroarty (1985) used gel permeation chromatography to separate LPS populations of similar O-chain length. *S.minnesota* and *S.typhimurium* were separated into three major populations with an average of 70, 20 and 1.2 O-chain repeat units in *S.minnesota* and >80, 30 and 1.2 O-chain repeat units in *S.typhimurium*. Using the same technique Parent *et al.* (1992) fractionated *S.abortis equi* LPS into two peaks. Subsequent SDS-PAGE analysis of fractionated samples showed that Peak 1 represented long chain LPS of ≥ 10 O-chain repeat units and Peak 2 represented short chain LPS of ≤ 6 O-chain repeat units. In the present study the elution profiles produced by *S..ealing* (NTU) and *S.arizonae* (NTU) resembled that produced by *S.abortis equi* (Parent *et al.*, 1992) by producing two fraction peaks. SDS-PAGE analysis of *S.arizonae* (NTU) LPS fractions representing the fraction peaks showed that fraction F1 (Peak 1) represented long chain LPS of ≥ 10 O-chain repeat units (Figure 3.37). Whereas fraction F2 (Peak 2) represented short chain LPS of predominantly ≤ 2 O-chain repeat units. The fractionation of LPS from *S.ealing* (NTU) was not as clear cut. Peak 2 appeared as a 'shoulder' (Figure 3.32) rather than as a separate peak, so that an additional fraction F2 was collected to separate the Peak 1 and Peak 2. SDS-PAGE analysis showed that fraction F1 (Peak 1) was made up of long chain LPS of ≥ 18 O-chain repeat units and fraction F3 (shoulder/Peak 2) represented short chain LPS of ≤ 8 O-chain repeat units (Figure 3.34). From these results it is clear that the size exclusion chromatography technique carried out in the present study could be used to separate long and short chain LPS from *S.ealing* (NTU) and *S.arizonae* (NTU). However it could not be used to separate populations of short chain and R-type LPS.

4.2.3 *LPS CORE STRUCTURE SUMMARY AND CONCLUSIONS*

SDS-PAGE and immunoblot assay techniques were used to observe the heterogeneity of LPS in *Salmonella* and non-*Salmonella* strains and examine the M105 antibody reactivity with respect to LPS molecules of varying O-chain length.

The LPS profiles comprised of between 10 to over 30 bands. The number of LPS molecules in each band was reflected by the intensity of silver stain. Both the number and intensity of the LPS bands was dependent on the strain type. Hence the LPS profiles were unique to each bacterial strain.

The LPS isolated from 11 non-*Salmonella* strains failed to react with the M105 antibody. However 40/42 *Salmonella* strains analysed were shown to possess the M105 epitope. Three types of immunoblot reaction were noted. In immunoblot reaction type 1 M105 reacted with all LPS molecules regardless of O-chain length (serogroups B, C_2, C_3, D and E). In reaction type 2 M105 reacted with R-type LPS and LPS bearing a small number of O-chain repeat units (serogroups C_1, F and L). In reaction type 3 M105 reacted weakly with R-type LPS only (serogroups G, K, O, U, V, X and 63). When the results of the present study was used in conjunction with the T6 antibody results of previous reports (Tsang *et al.*, 1991a) salmonella LPS core structures could be tentatively identified. Immunological reactions confirmed that complete Ra type core structures were present in *S.arizonae* (1823) and *S.cerro* (10390). Salmonella Rb_1 type core structures were identified in *S.godesberg* (1140), *S.godesberg* (1431), *S.milwaukee* (1139), *S.nirembe* (1660), *S.bere* (10392), *S.bere* (10427) and *S.arizonae* (10463). Moreover an absence of Ra and Rb_1 type core structures were confirmed in *S.djakarta* (10403) and *S.arizonae* (1803). All other *Salmonella* strains tested expressed either Ra or Rb_1 type core structures.

The hot phenol/water method of Galanos *et al.* (1969) was used to isolate LPS from M105 weakly reactive strains of *S.ealing* (NTU) and *S.arizonae* (NTU). Preliminary chemical analysis of the isolated LPS, indicated that the terminal GlcNAc residue was missing from *S.arizonae* (NTU).

In conclusion, this study has provided serological evidence that Rb_1 and other atypical core structures exist in wild type *Salmonella* strains. Therefore it questions the universal validity of previous claims that all salmonella share a common Ra core structure (Kato

1993). Moreover this study has provided a suitable method for the isolation of short O-chain and core oligosaccharide from wild type strains of *S.ealing* (NTU) and *S.arizonae* (NTU).

4.2.4 *LPS CORE STRUCTURE: THE WAY FORWARD*

As previously discussed, it is questionable whether rough mutant *Salmonella* strains can provide evidence of the existence of atypical core structures in wild type strains. Moreover R-type LPS of wild type strains may be composed of partial core structures which may or may not be the same as S-type LPS. Therefore to provide indisputable proof of atypical core structures SR-type LPS should be isolated from wild types strains. Then the carbohydrate structure could be analysed by methylation analysis and selective degradation by methods described by Hakamori (1964), Haishima *et al.* (1992), Hase and Rietschel (1976) and Holst *et al.* (1994).

4.3 FINAL STATEMENT

Currently the IMS-ELISA double bead (with enrichment) protocol is not a viable alternative to the traditional or indeed commercially avaliable ELISA salmonella isolation and detection techniques. This is because of the high numbers of false negatives produced in the preliminary food trial experiments. Despite this the IMS-ELISA technique does show potential. In its favour the IMS-ELISA method detected all commonly isolated *Salmonella* serogroup B-E, together with the majority of strains from the higher serogroups. The limitations of the IMS-ELISA technique with regard to specificity and sensitivity have been addressed and possible solutions have been proposed. If successful the IMS-ELISA technique could provide a means by which next day results were possible.

Previous reports have claimed that all 2,400 *Salmonella* serotypes share a common Ra type LPS core structure. This study proposes that this claim is incorrect and has provided serological evidence to dispell this claim. Strains belonging to the higher serogroups have been shown to possess structures corresponding to the Rb_1 type core and in addition may possess other atypical core structures. Moreover this study has proposed a gel permeation chromatography method which isolated core oligosaccharide and core oligosaccharide bearing a small number of O-chain repeat units. This method could be used to provide indisputable evidence of atypical core structures in the SR-type LPS of wild type strains.

Towards the end of writing up this thesis a paper was published which reported the

complete characterisation of a novel LPS core from the SR-type LPS of a wild type strain of *S.arizonae* (Olshoorn *et al.*, 1998; Figure 4.2). Therefore the general belief that all salmonella share a common core structure should be revised. In the present study the M105 antibody did not react with the LPS isolated from strains of *S.djakarta* (10403) and *S.arizonae* (1803). In the light of the Olshoorn publication, it seems highly likely that non-Ra/Rb_1 type structures existed in these *Salmonella* strains.

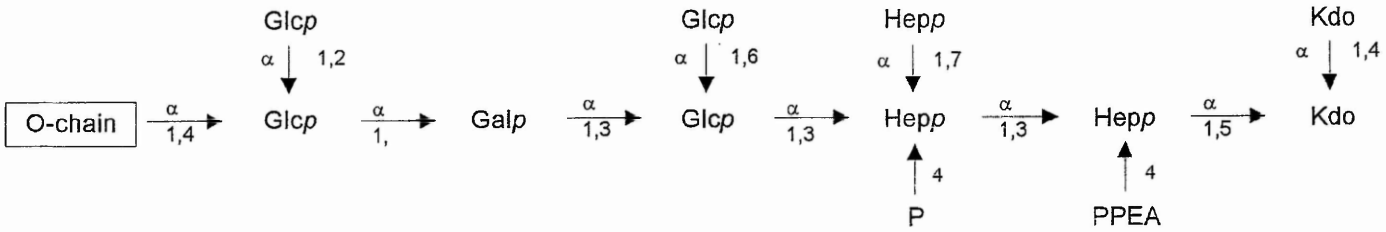


Figure 4.2: Identification of a novel non Ra type LPS core structure from a wild strain of S.arizonae 062[a]
([a], adapted from Olshoorn *et al*., 1998)

Hepp , L-glycero-D-manno-heptopyranose; PPEA, 2-aminethy; diphosphate

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APPENDIX

PUBLICATION ARISING FROM THIS STUDY

Letters in Applied Microbiology 1996, **23**, 104–106

Variation in *Salmonella* core lipopolysaccharide as detected by the monoclonal antibody M105

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L.P. MANSFIELD, E. BILLETT, E. OLSEN AND S.J. FORSYTHE. 1996. The lipopolysaccharide antigenicity of 22 *Salmonella* strains (representing nine serogroups) and four non-salmonellae Enterobacteriaceae to the *Salmonella* genus specific monoclonal antibody M105 was analysed. The monoclonal antibody M105 reacted with all 22 *Salmonella* strains. Probing SDS-PAGE separated LPS molecules with MAb M105 revealed that the antibody reacted with the core region of all *Salmonella* serovars. However, no reaction was obtained to the long-chain LPS of serovars O (*Salm. adelaide* and *Salm. ealing*), C_1 (*Salm. infantis*, *Salm. livingstone* and *Salm. virchow*) or *Salm. arizonae*. It is plausible that the presence of a second core antigenic type results in the lack of reaction between long-chain LPS and the *Salmonella* genus specific monoclonal antibody M105.

INTRODUCTION

Lipopolysaccharides (LPS) are produced by *Salmonella* as part of the outer membrane structure and comprise three regions; lipid A, core-LPS and long-chain LPS. The long-chain LPS is composed of repeating carbohydrate units attached to core-LPS. The variation in carbohydrate sequence of the long-chain LPS is used in the Kauffmann–White scheme for somatic or 'O' antigen *Salmonella* typing (Ewing 1986). Smooth-type *Salmonella* serovars express core-LPS and long-chain LPS. The LPS can be separated by SDS-PAGE into distinct 'ladder' patterns according to the number of repeating carbohydrate units (Goldman and Leive 1980). The *Salmonella* serovar variants identified as phage types 7, 7a, 23 and 30 either express trace long-chain LPS or core-LPS only and have a rough colony appearance (Threlfall *et al.* 1993). Rough *Salmonella* mutants producing truncated LPS have been generated. These are termed Ra to Re mutants according to the length of the remnant core region and are used to study the binding site of antibodies raised against somatic 'O' antigens (Schnaitman and Klena 1993). In contrast to the many long-chain LPS antigenic specificities of the different *Salmonella* serovars (>2000), the core region was believed to be constant (Westpal *et al.* 1983). However, the lack of *N*-acetyl-glucosamine in the core region has been reported in *Salm. adelaide*, *Salm.* IV and *Salm. djakarta* and this would alter the core's antigenicity (Tsang and Schlecht 1990; Tsang *et al.* 1992). This paper demonstrates the variation in core-LPS structure between *Salmonella* serovars as detected by the core specific MAb M105 (Tsang *et al.* 1991).

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MATERIALS AND METHODS

Bacteria

One strain of *Salm. ealing* was donated by Professor K. Nielsen (Animal Diseases Research Institute of Agriculture, Canada). *Salmonella gallinarum* was from Campden and Chorleywood Food Research. Eight *Citrobacter freundii* strains from various food sources were included. All other strains were from the culture collection at The Nottingham Trent University. All cultures were maintained on tryptone soya agar slopes and grown overnight in 10 ml buffered peptone water (Unipath Ltd, UK) at 37°C. Viable counts were determined using the standard plate procedure on tryptone soya agar at 37°C.

Lipopolysaccharides

Purified (phenol extracted) LPS from Ra, Rc, Re and wild type *Salm. typhimurium* were obtained from Sigma Chemical Co. Ltd (Poole, UK). LPS was dissolved (1 mg ml^{-1}) in

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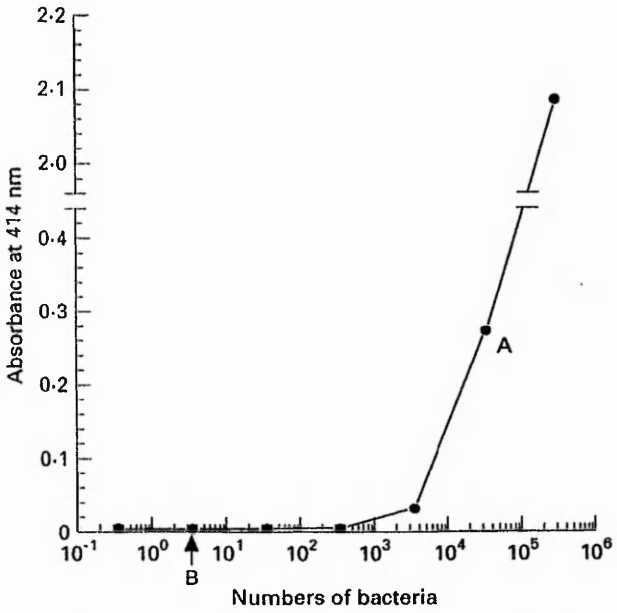


Fig. 3 Detection of *Pasteuralla piscicida* antigens with ELISA. A, Endpoint of ELISA ; B, endpoint of immuno-PCR

template in our system uses the multicloning sites of pBluescript and this sequence is artificially constructed, and thus the primers and template in PCR are unlikely to react with the DNA of the examined host or parasites. The same approach using the multicloning sites of pBluescript vector was also reported by Sanna *et al.* (1995).

Another technical problem is the possibility of DNA amplification in control wells without antigen by insufficient washing or blocking. Thus, it is essential that complete elimination of non-reactive antibody is achieved in the blocking and washing procedures. We examined several concentrations of bovine serum albumin, gelatin or dried milk for blocking material and other washing buffers such as TBS. However, the results were unstable. Although our method reported here is more reliable, a negative control without antigen is necessary to check for insufficient blocking or washing. In diagnosis, specificity is very important. In immuno-PCR, the specificity is dependent on the quality of the first antibody. Because of the high sensitivity of immuno-PCR, a highly specific antibody for first antibody should be used. Our immuno-PCR system showed a high sensitivity and can be used to detect the carrier stage of infection. Immuno-PCR is a powerful method for detection of pathogens in host tissues.

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double distilled water for immunoblot analysis. Whole cell LPS extract was obtained using the proteinase-K procedure of Hitchcock and Brown (1983).

Immunology

Murine M105 ascites (from Professor K. Nielsen) was diluted in 1% bovine serum albumin in Tris buffered saline (TBS). M105 was detected with an alkaline phosphatase labelled rabbit anti-mouse secondary antibody (Dako A/S, Denmark). Antisera against O group *Salmonella* A to S raised in rabbit were obtained from Murex Diagnostics Ltd and detected using an alkaline phosphatase labelled goat anti-mouse secondary antibody (Sigma Chemical Co. Ltd, Poole).

SDS-PAGE and immunoblotting

The SDS-PAGE was as according to Laemmli (1970) except SDS was omitted from gel buffers (Pyle and Schill 1985). LPS profiles were either silver-stained (Tsai and Frasch 1982) or transferred to nitrocellulose (pore size 0·45 μm) for immunoblot analysis. LPS components were transferred at a constant current (100 mA) for 2 h in pH 8·3 transfer buffer using a semi-dry transfer unit. The nitrocellulose was blocked with 3% skimmed milk powder in TBS-0·1% Tween 20 (TBS-T) at 37°C for 30 min and then incubated with primary antibody overnight at 4°C. The nitrocellulose was washed three times with TBS-T and then incubated with the secondary antibody at 37°C for 2 h. After three washes in TBS-T the antibody–antigen complexes were visualized following incubation in Tris buffer containing bromochloro-indolyl-phosphate toluidine salt and nitroblue tetrazolium (Sigma Chemical Co. Ltd).

RESULTS

The characteristic ladder appearance of *Salmonella* LPS was obtained using SDS-PAGE (Fig. 1, lanes 1, 3 and 5). M105 reacted with the long-chain and core-LPS of most *Salmonella* serovars (Table 1). However, M105 only reacted with the core-LPS of serovars C_1, O and *Salm. arizonae* (Table 1 and Fig. 1). Transferance of long-chain LPS from these salmonellae was confirmed by immunoblot analysis with antisera against O groups *Salmonella* A to S. Weaker reactions were observed with increasing chain length of serovars C_2 and C_3. No cross-reaction was observed with proteinase K LPS preparations from *Cit. freundii*, *Escherichia coli*, *Enterobacter agglomerans* or *Shigella flexneri*.

DISCUSSION

M105 has previously been described as a *Salmonella* genus specific antibody due to its binding to the outer core region of *Salmonella* LPS (Tsang *et al.* 1991). It therefore has the potential of being used in rapid, immunological *Salmonella* detection methods such as immunomagnetic separation (Safarik *et al.* 1995). This was partially confirmed using LPS from rough mutants of *Salm. typhimurium* (results not presented). Separation of digested whole cell *Salmonella* LPS using SDS-PAGE demonstrated the characteristic long-chain LPS banding pattern (Fig. 1, lanes 1, 3 and 5). Since long-chain LPS is synthesized from core-LPS by the addition of repeating carbohydrate units it was expected that M105 would react with the complete LPS ladder profile. The monoclonal antibody M105 reacted with the long-chain LPS of all *Salmonella* serovars except serovar C_1 (*Salm. livingstone*, *Salm. virchow* and *Salm. infantis*), serovar O (*Salm. adelaide* and *Salm. ealing*) and *Salm. arizonae* where M105 only reacted with the core-LPS (Table 1, Fig. 1). Immunoblotting with polyclonal anti-*Salmonella* sera confirmed that long-chain LPS of all serovars was transferred to the nitrocellulose membrane using the semi-dry system. Therefore with serovars C_1, O and *Salm. arizonae* there was either steric hindrance between M105 and long-chain LPS due to the folding of the repeating carbohydrate units or alternatively there could be two core sequences; the first core-LPS region sequence being detectable by M105, whereas the second sequence has the repeating carbohydrate unit ('O' antigens) attached and forms the long-chain LPS profile visualized by SDS-PAGE analysis. The second core region could lack *N*-acetyl-glucosamine which has already been reported for some salmonellae (Tsang and Schlecht 1990; Tsang *et al.* 1992).

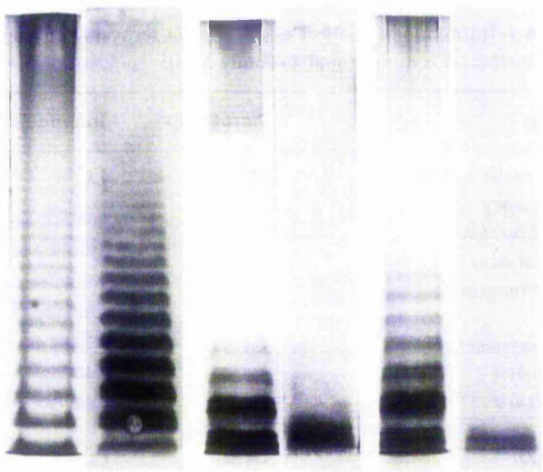


Fig. 1 SDS-PAGE and immunoblotting of lipopolysaccharides from three *Salmonella* serovars. Lanes: 1 and 2, *Salm. typhimurium*; 3 and 4, *Salm. virchow*; 5 and 6, *Salm. ealing*; 1, 3 and 5, silver-stained SDS-PAGE separation of LPS; 2, 4 and 6, immunoblotting with MAb M105 of SDS-PAGE separated LPS

Table 1 Immunoblot analysis of *Salmonella* serovars and non-salmonellae with monoclonal antibody M105

Strain	Serogroup	Immunoblot
Salmonella agona	B	LC
bredeney		
gallinarum		
saint paul		
typhimurium		
livingstone	C_1	C
virchow		
infantis		
hadar	C_2	LC
manchester		
newport		
kentucky	C_3	LC
enteritidis	D_1	LC
panama		
dublin		
give	E_1	LC
senftenberg	E_4	LC
minnesota	L	LC
adelaide	O	C
ealing		
arizonae	NA	C
Citrobacter freundii (8 strains)	NA	—
Shigella flexneri	NA	—
Escherichia coli	NA	—
Enterobacter agglomerans	NA	—

NA, Not appropriate; LC, long-chain and core-LPS reaction; C, core-LPS reaction only.

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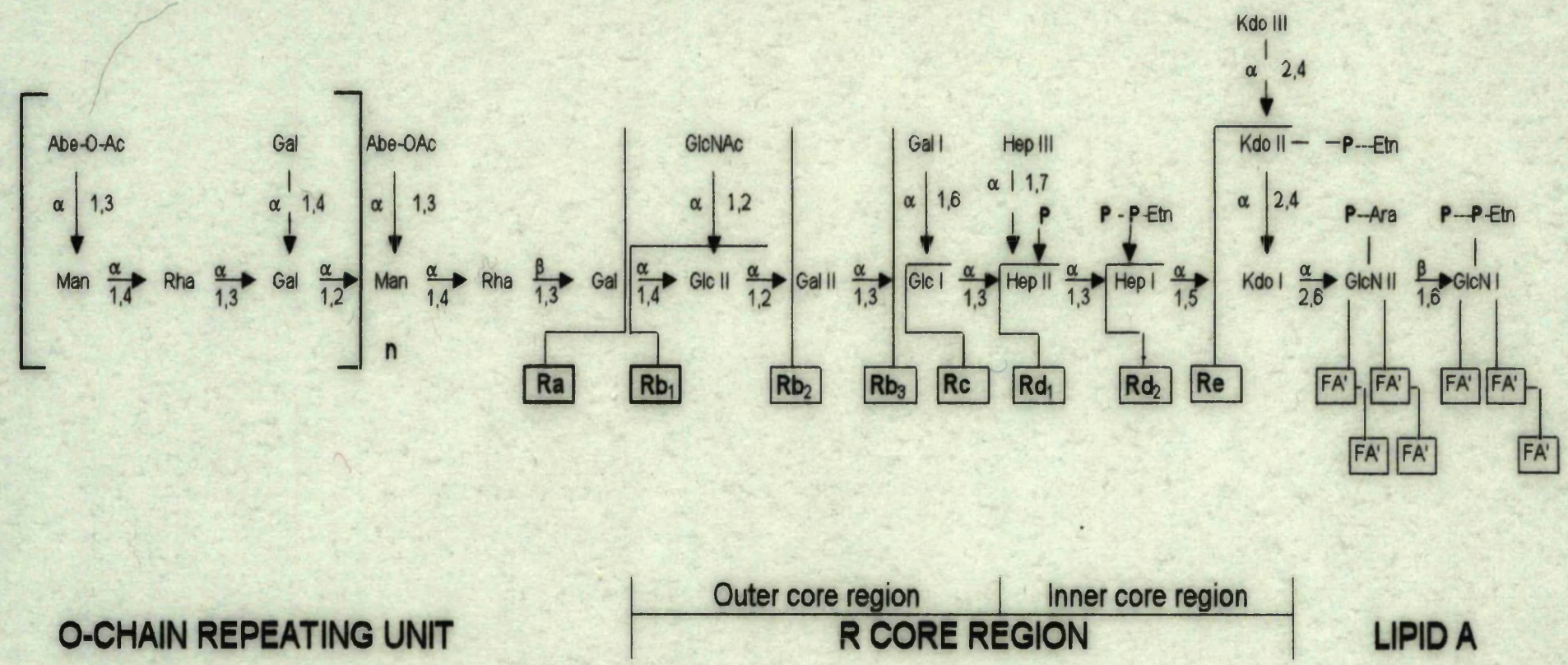


Figure 1.3: The chemical structure of salmonella lipopolysaccharide (Dotted lines indicate incomplete substitutions)

Abe, abequose; Ac, acety; Ara, 4-amino-4-deoxy-L-arabinose; Eth, ethanolamine; FA, fatty acid; FA', hydroxy fatty acid; Gal, D-galactose; Glc, D-glucose; GlcN, D-glucosamine; GlcNAc, N-aceyl-D-glucosamine; Hep, heptose; KDO, 3-deoxy-D-manno-octo-2-ulopyranosonic acid; Man, D-mannose; P, phosphate; Rha, L-rhamnose

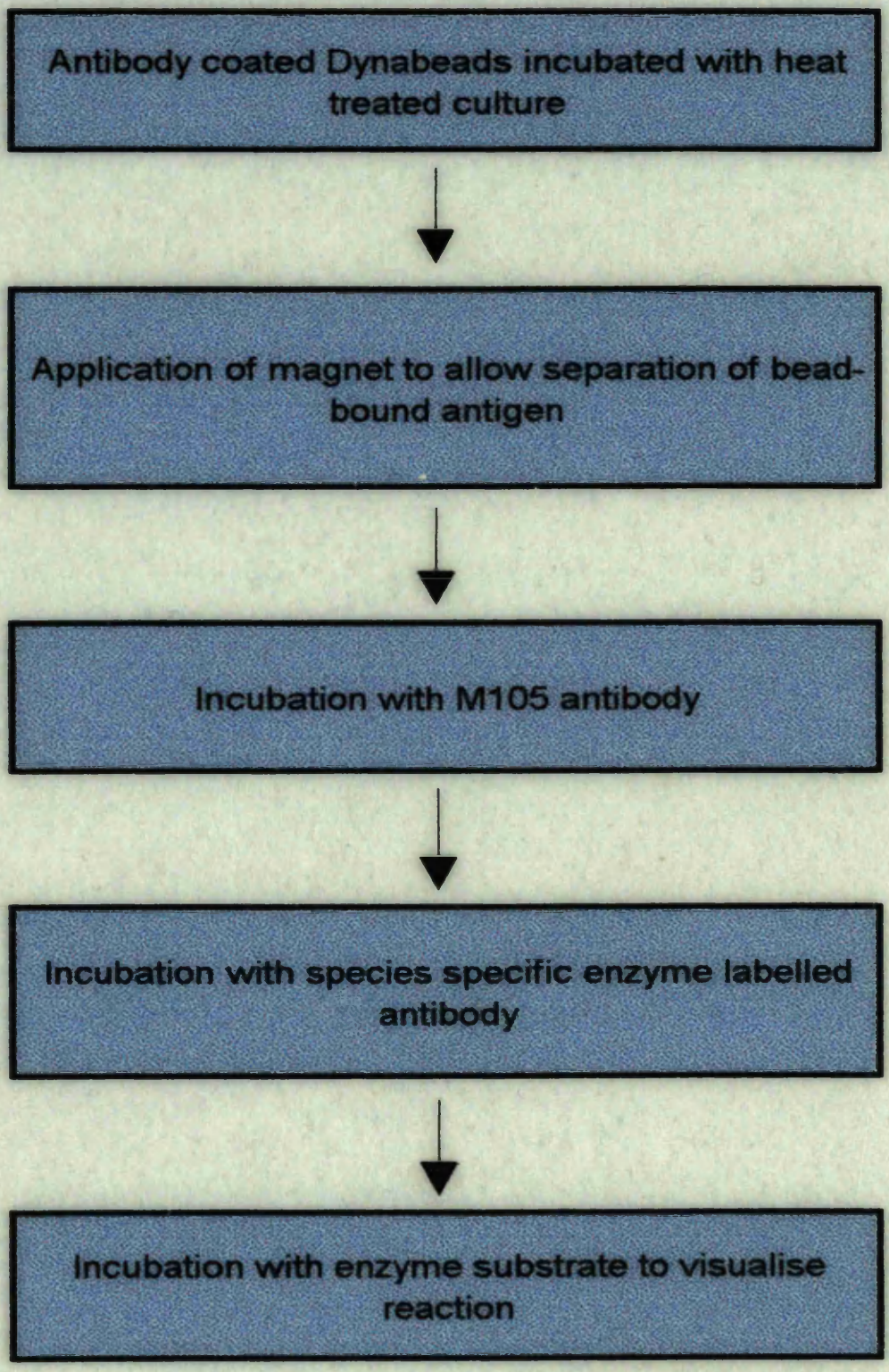


Figure 3.16: Summary of the IMS-ELISA salmonella detection procedure

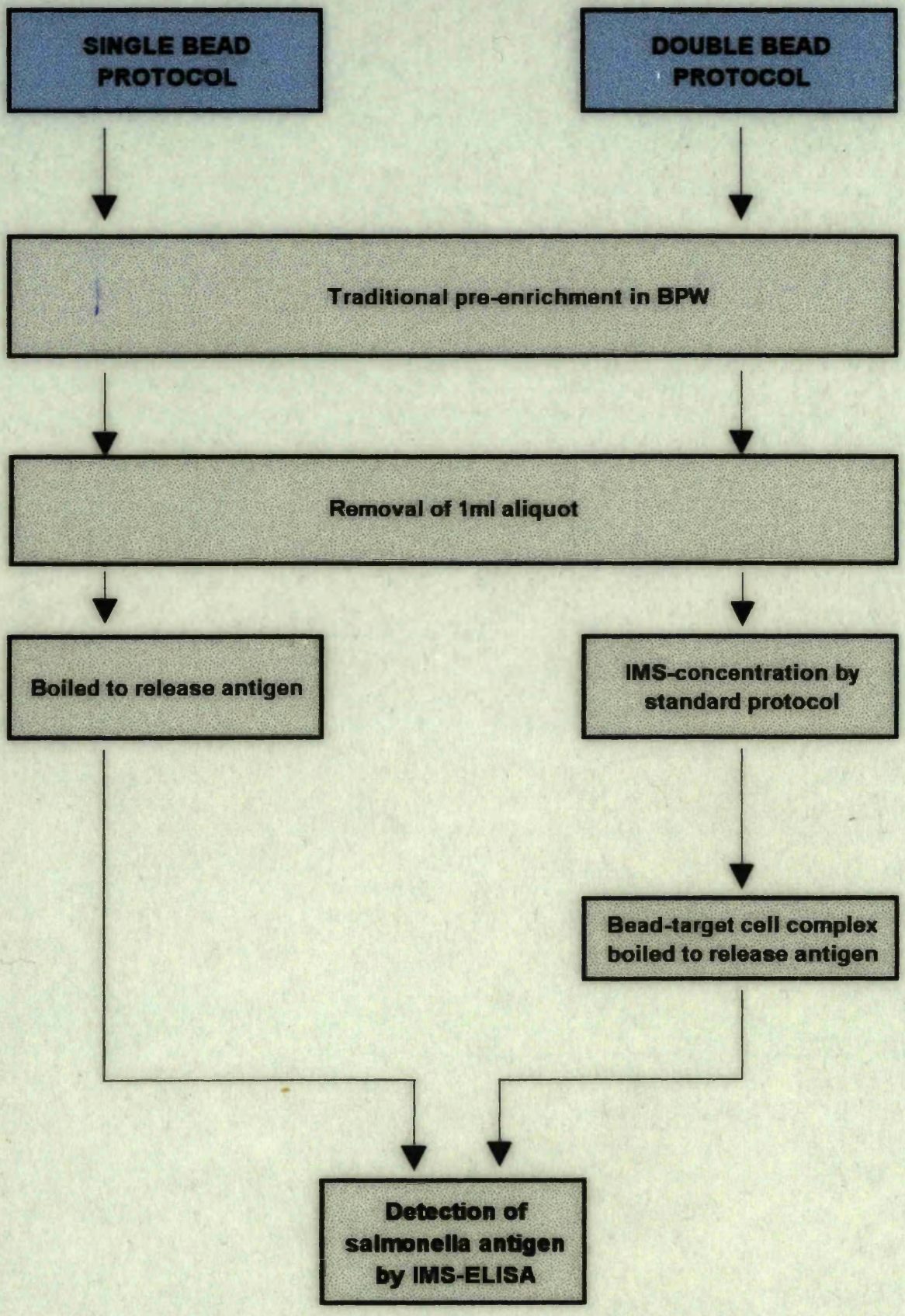


Figure 3.20: Summary of single bead and double bead (without enrichment) salmonella isolation and detection protocols

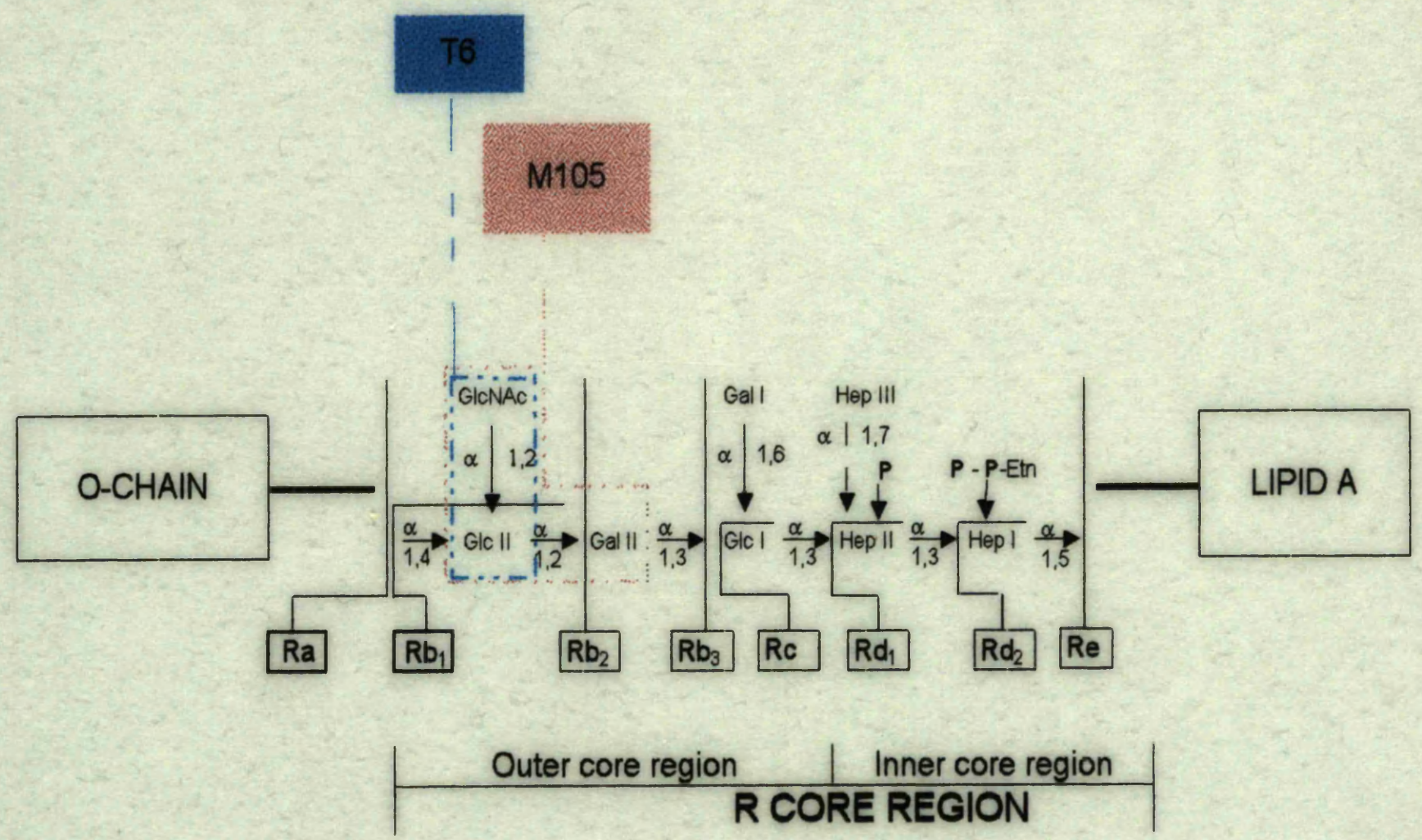


Figure 1.14: The binding sites of the *Salmonella* genus specific monoclonal antibodies T6[a] and M105[b]

([a], Tsang *et al*., 1991b; [b], Tsang *et al*., 1987) (Dotted lines indicate incomplete substitution)

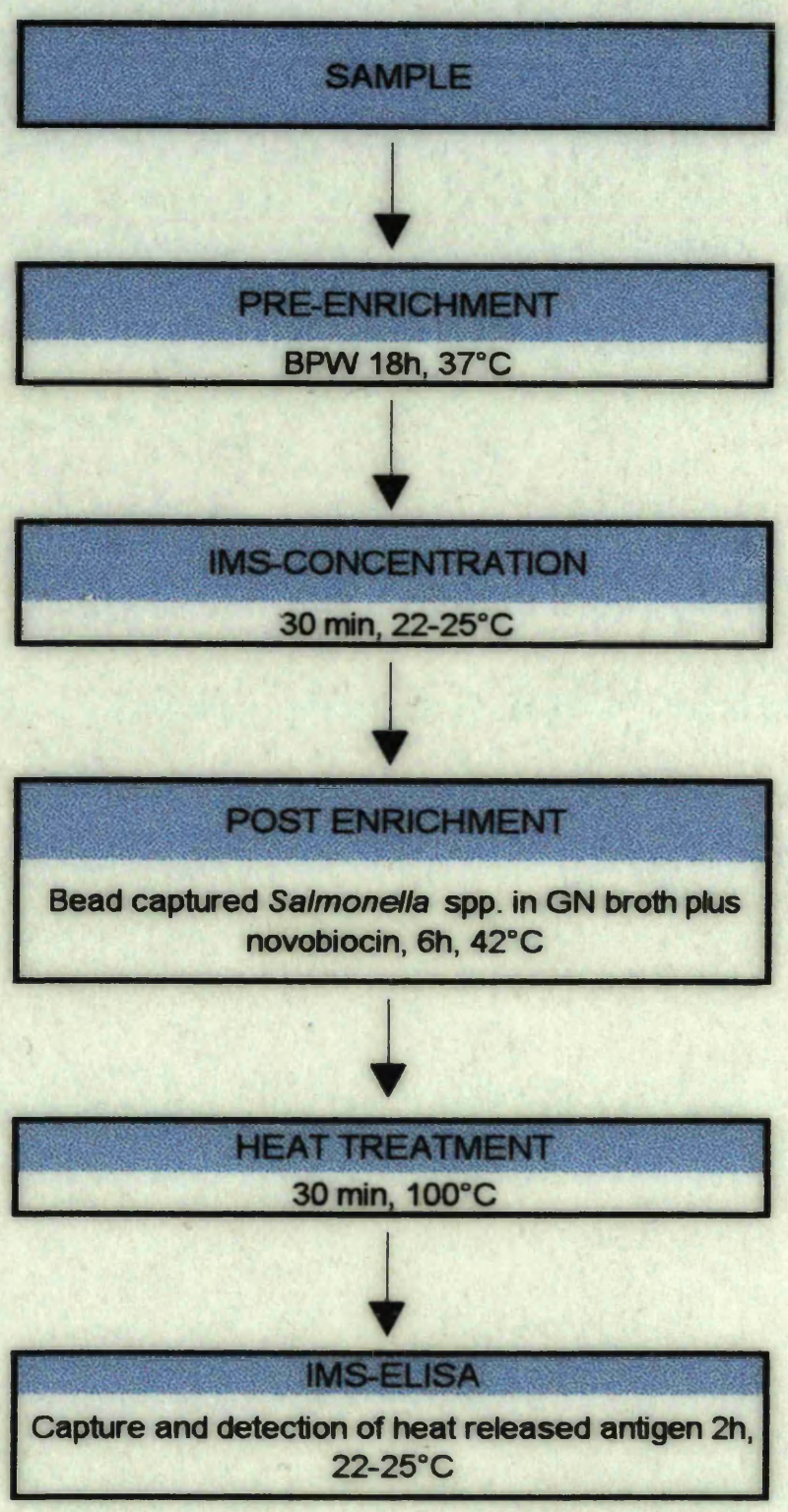


Figure 3.22: Final proposed double bead (with enrichment) protocol for the isolation and detection of salmonella from ready-to-eat foods