Comparison of the biotypes of *Yersinia enterocolitica* isolated from pigs, cattle and sheep at slaughter and from humans with yersiniosis in Great Britain during 1999-2000.

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Running Title: *Y. enterocolitica* in humans and livestock

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ABSTRACT

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Aims: To investigate the relationship between livestock carriage of *Y. enterocolitica* and human disease. The biotypes/serotypes of strains recovered from the faeces of pigs, cattle and sheep at slaughter during a national survey in Great Britain in 1999-2000, were compared to those of strains isolated from human cases of yersiniosis during the same period.

Methods and results: The faecal carriage of *Y. enterocolitica* by cattle, sheep and pigs at slaughter was 6.3%, 10.7% and 26.1% respectively. *Y. enterocolitica* biotype (BT) 1a was the most frequently isolated biotype from livestock (58%) and was the predominant biotype (53%) isolated from human cases over the same period. The main recognised pathogenic *Y. enterocolitica* biotype isolated from livestock was BT3 (O:5,27) (35% of sheep, 22% of pigs and 4% of cattle) but this biotype was not detected in any of the human isolates investigated. The major pathogenic biotypes of strains isolated from humans were BT3 (O:9) (24%) and BT4 (O:3) (19%) whereas of the veterinary isolates investigated, only pigs (11%) carried BT3 (O:9) strains.

Significance and impact of study: Because of significant overlaps in phenotypes of the veterinary and human strains it is not possible to comment on the correlation between host and pathogenicity, especially of biotype 1a. The data suggest that further investigations using methods with greater discriminatory power are required. However the data also suggests that pigs may be the primary reservoir for human pathogenic *Y. enterocolitica* infection.

Keywords: *Y. enterocolitica*, prevalence, pigs, cattle, sheep, humans, biotype.

INTRODUCTION

Yersinia enterocolitica is a Gram negative member of the *Enterobacteriaceae* family. Infection with this organism in humans can lead to a range of diseases from mild diarrhoea to the more severe complication of mesenteric lymphadenitis (Bottone 1999). Although severe disease is rare, septicaemia and death may occur in infected immunocompromised patients and patients transfused with contaminated blood (Bottone 1999). Other post-infection sequelae such as arthropathies are common amongst some patient groups (Bottone 1999). Approximately 300 cases of yersiniosis are reported per annum in England and Wales by the Communicable Disease Surveillance Centre (CDSC), however diarrhoeic stool samples are rarely cultured for presence of *Yersinia* spp. *Y. enterocolitica* is also difficult to isolate from such specimens as it is overgrown by other enteric organisms. Therefore it is likely that the reported incidence is underestimated. In continental Europe, the incidence and importance of yersiniosis as a cause of enteric disease is considerably higher. Belgium, Holland, and Germany report enteropathogenic *Yersinia* as rivalling *Salmonella* spp. as a cause of gastro-enteritis (Doyle 1985; Bottone 1997).

Y. enterocolitica strains are classified on the basis of biochemical characteristics (biotype), and may then be further differentiated by serotype. Biotype 1b strains are presumed to be highly pathogenic due to their lethality in a mouse infection model, while biotypes 2 - 5 are generally considered as having a relatively lower pathogenicity in the same model. Using the same criteria biotype 1a strains are classified as non-pathogenic. Previous surveys of human cases in the UK have implicated strains of biotype 3 (serotype O:9 and O:5,27), biotype 2 (serotype O:9), and biotype 4 (serotype O:3) as major causative agents of yersiniosis (Prentice *et al.* 1991). However, the predominant biotypes may vary with geographical region, with

strains of biotype 1b and biotype 4 (serotype O:3) predominantly isolated in the United States (Bottone 1997).

The primary route of human infection is proposed to be foodborne. Transmission of enteropathogenic *Yersinia* spp. by food, particularly dairy and meat products, is well documented and increasingly prevalent world-wide (Fredriksson-Ahomaa *et al*. 2000; Fredriksson-Ahomaa *et al*. 2001; Falcao *et al*. 2003; Fredriksson-Ahomaa and Korkeala 2003; Fredriksson-Ahomaa and Korkeala 2003; Jones 2003). In particular, porcine products are implicated as the major source of human *Y. enterocolitica* infection, with numerous epidemiological studies linking consumption of uncooked or undercooked porcine reticuloendothelial tissues with yersiniosis (Prentice *et al*. 1991; Bottone 1999; Gourdon *et al*. 1999; Fredriksson-Ahomaa *et al*. 2001; Falcao *et al*. 2003; Fredriksson-Ahomaa and Korkeala 2003; Fredriksson-Ahomaa and Korkeala 2003; Jones 2003). The high prevalence of *Y. enterocolitica* in pigs has also been reported, as well as presence in other livestock, cats and dogs and many wild animals (Skjerve *et al*. 1998; Fredriksson-Ahomaa *et al*. 1999; Floccari *et al*. 2000; Fredriksson-Ahomaa *et al.* 2000; Atanassova *et al*. 2003; Bonardi *et al*. 2003; Watabe *et al*. 2003). As yet, no national structured survey of faecal carriage by livestock has been undertaken in Great Britain.

Although the ubiquitous nature of *Yersinia* spp in livestock is well recognised the relationship between veterinary isolates and human disease remains unclear. The comparative investigation of veterinary and human strains, geographically and temporally related, is therefore required to determine whether all *Y. enterocolitica* strains present in the environment possess the ability to cause disease in humans, or if only a subset of strains are likely to lead to human infection, as is the case with other enteric pathogens (Kim *et al*. 1999; McNally *et al*. 2001).

The faecal carriage of *Y. enterocolitica* in cattle, sheep and pigs at slaughter in Great Britain was last determined in a structured national survey performed in 1999-2000. All *Y. enterocolitica* isolates were then biotyped and serotyped, and the phenotypes compared to strains isolated from human cases of yersiniosis by the Health Protection Agency (HPA) over the same period.

MATERIALS AND METHODS

Abattoir survey and livestock sampling

Cattle, sheep, and pigs were sampled for carriage of *Y. enterocolitica* as part of the first randomised national survey to determine the prevalence of foodborne pathogens in livestock at slaughter in Scotland, England and Wales. The survey was undertaken between March 1999 and February 2000. Ligated caeca were collected from 2509 pigs sampled at 34 abattoirs. Ligated rectal samples were collected from 891 cattle and 973 sheep that were sampled at 118 abattoirs. All ligated samples were transported to VLA testing laboratories for processing within 24 hours of collection. Upon receipt at the testing laboratories, the samples were allocated a sample number, conferring anonymity to the abattoirs and herds from which samples were taken. Sampling was arranged to ensure even seasonal distribution throughout the 12-month period and ensure sample collection was proportionate to the throughput of each of the participating abattoirs. Samples were also taken to ensure an even spread of sampling throughout Great Britain (28.4% Midlands region, 32.8% North and Scotland, 38.8% South). A maximum of 5 samples was collected on any one occasion to prevent the clustering of sampling from the same herds.

Isolation and phenotyping of *Y.enterocolitica* isolates

All samples were processed within 3 days of collection. On receipt at the laboratory the caecal/rectal contents were removed aseptically from the ligated viscera. *Y. enterocolitica* isolation was performed as described according to the method of Schiemann (Schiemann 1979). Briefly, caecal/rectal material (2ml containing approximately 2g) were emulsified in 20ml of 0.066M phosphate buffered saline (PBS - pH 7.3) and refrigerated at 2 - 8°C for 14 days. The broth was sub-cultured onto selective CIN (Cephsulodin-Irgasin-Novobiocin) agar and incubated at 31°C for 18-24 hours. Suspect *Yersinia* colonies were sub-cultured on to 10% sheep's blood agar and MacConkey agar and incubated at 37°C for 18-24 hours. Identification of *Y. enterocolitica* was confirmed by colony morphology, urease test and API 20E biochemical typing strip. All confirmed *Y. enterocolitica* isolates were sub-cultured onto Dorset slopes and despatched to the Laboratory of Enteric Pathogens (LEP), Health Protection Agency (HPA), Colindale for phenotyping. Isolates were biotyped and serotyped according the modified scheme of Wauters (Bottone 1999).

Isolation of human *Y. enterocolitica* isolates

During the corresponding time to the National Abattoir Survey (March 1999 – February 2000), 164 *Y. enterocolitica* strains isolated from patients presenting to their GP with diarrhoea, were confirmed by the LEP. All strains were isolated from patients in England and Wales, and were biotyped and serotyped at the LEP using identical methodology as that employed in the abattoir survey.

Veterinary and human strain comparison

The 814 *Y.enterocolitica* strains isolated from the abattoir survey study were grouped according to source, and then further grouped according to biotype and serotype. The pathotypes of these veterinary strains were then compared to the pathotypes of the 164

Y. enterocolitica isolates sent to LEP from laboratories throughout England and Wales during the same period of 1999/2000. This allowed a temporal comparison of *Y. enterocolitica* isolated in Great Britain during 1999 – 2000.

RESULTS

Prevalence and biotypes of *Y. enterocolitica* in pigs in Great Britain

From a total of 2509 pigs sampled, 742 (29.6%) of the samples tested were positive for *Yersinia* spp. Of the 742 isolates, 654 (26.1% of samples, 88% of total *Yersinia* spp.) were *Y. enterocolitica.* Other species isolated from pigs were *Y. intermediae, Y. rhodei,* and *Y. frederksenii*, the latter being the most common (1.6%, 1.1%, and 8% of *Yersinia* spp. isolates respectively). There was no statistical bias of isolation with respect to geographical region of the UK, though carriage rates were highest during December and January, with carriage rates significantly decreasing during the summer months ($p = 0.00001$, χ^2-test). Of the pig Y. *enterocolitica* isolates, the most common (53.4%) strain type was biotype 1a (Table 1). In addition, 22% were biotype 3 (O:5, 27), 11% biotype 3 (O:9) and 5% biotype 4 (O:3).

Prevalence of *Y. enterocolitica* in sheep and cattle in Great Britain

Only 56 of the 891 cattle sampled in the survey (6.3%) yielded *Y. enterocolitica* by culture. Of these 56 isolates only 2 were recognised pathogenic biotypes, both being biotype 3 (O:5, 27) (Table 2) whilst the remainder were biotype 1a.

Of the 973 sheep sampled in the survey, 104 (10.7%) were positive for *Y.enterocolitica.* Sixty two percent of isolates were biotype 1a but most of the remainder were biotype 3 (O:5, 27) (Table 3).

Characterisation of *Y. enterocolitica* strains from human cases of yersiniosis

During the same period of 1999/2000 that the national abattoir survey was conducted, 164 *Y. enterocolitica* strains were received by the LEP. The majority (53%) were biotype 1a, 24% were biotype 3 (O:9) and 19% biotype 4 (O:3) (Table 4). Only 1.2% were biotype 3 (O:5, 27).

DISCUSSION

In this study, the caecal/faecal carriage of *Y. enterocolitica* in livestock (pigs, cattle and sheep) at slaughter in Great Britain has been defined using a structured national abattoir survey. Over 25% of pigs, 10% of sheep, and 6% of cattle carried *Y. enterocolitica.* Such a high prevalence in pigs is well documented (Skjerve *et al*. 1998; Fredriksson-Ahomaa *et al*. 1999; Fredriksson-Ahomaa *et al*. 2000; Atanassova *et al*. 2003; Bonardi *et al*. 2003; Watabe *et al*. 2003). Although at a lower prevalence, the carriage of *Y. enterocolitica* by sheep, cattle and other domestic animals is also well recognised (Fantasia *et al* 1985; Floccari *et al*. 2000; Falcao *et al*. 2003). More importantly, the role of such veterinary strains in human intestinal infectious disease is unknown. Comparison of strain phenotypes in the various host populations is the classical approach to such a question. However, for *Y. enterocolitica* the population structure, even in humans, appears to be geographically variable. For example, biotype 1B strains are commonly reported in the USA but are almost unreported in Europe, and there is biotype diversity even among studies across Europe (Bottone 1999). Because all *Y. enterocolitica* isolates from human cases from England and Wales are sent to the HPA for characterisation, this national abattoir survey provided a unique opportunity to compare directly, using classical biotyping and serotyping approaches, veterinary and human strains, which are temporally related.

In all three livestock species investigated *Y. enterocolitica* biotype 1a was the major strain type recovered. This biotype is assumed to be non-pathogenic in humans, due to the absence of known virulence factors including the lack of the yersinia virulence plasmid (pYV) (Grant *et al*. 1999). It was interesting that 1a was also the predominant (53%) biotype identified in cases of human yersiniosis. Whether such human isolates were causative of disease or merely secondary colonisers is unclear. In a recent national intestinal infectious disease (IID) study (Food Standards Agency 2000), 85% of *Y. enterocolitica* isolates from patients presented to a General Practitioner (GP), compared with 93% of isolates from matched healthy controls, were biotype 1a, suggesting no association with disease. This was supported by data for community cases and controls (73% and 90% respectively) in the same study. Nevertheless, recent research, based on *in vitro* and *in vivo* models provides support that biotype 1a strains are capable of causing disease in humans (Tennant *et al*. 2003; Tennant *et al*. 2003). One explanation for this anomaly may be that there is a range of virulence potential in biotype 1a strains. This hypothesis is supported by experimental evidence indicating that biotype 1a strains from human disease cases are generally more invasive for, and escape at higher numbers from, eukaryotic cells *in vitro* compared to biotype 1a strains isolated from the environment (Grant *et al*. 1999). From the data presented here and in the IID study (Food Standards Agency 2000) biotyping and serotyping appear to contribute little to this debate. Typing techniques with greater discriminatory power, and preferably linked to pathogenicity markers, are required to address this question. Preliminary evidence suggests that molecular typing techniques such as amplified fragment length polymorphism (AFLP) may be a useful tool in the future (unpublished data).

Previous studies have demonstrated that *Y. enterocolitica* biotype 3 (O:9 and O:5,27), biotype 2 (O:9), and biotype 4 (O:3) are the most common strains of the presumed pathogenic group isolated from humans in the UK (Prentice *et al*. 1991; Bottone 1999). However, during the survey period (1999-2000) the predominant strains received by the LEP were of biotype 3 (O:9) (24% of all isolates) and biotype 4 (O:3) (19% of all isolates), with no biotype 2 observed. Biotype 2 and Biotype 3 strains can often be confused due to the difficulty in interpreting the Indole test, which differentiates the two. All isolates were typed by dedicated staff at the HPA using identical reagents which had successfully identified both BT2 and BT3 strains, suggesting this is a real observation. This is consistent with the IID study (Food Standards Agency 2000) where the only potentially pathogenic strains isolated from human cases were biotype 3 (O:9) (12.7% of all isolates), and biotype 4 (O:3) (2% of all isolates). Evidence for the potential pathogenicity of these biotypes is much stronger as no biotype 4 (O:3) strains and only 1 (2%) biotype 3 (O:9) strain was isolated from healthy humans during this study. In contrast, the prevalence of biotype 4 (O:3) strains in livestock for 1999-2000 was considerably lower than in strains typed by the LEP during the same period. In the abattoir survey prevalence of biotype 4 (O:3) was 5% in pigs, 1% in sheep and none in cattle. Thus, other potential reservoirs of this pathogenic biotype need to be considered, though the possibility may exist that BT4 (O:3) isolates are present in too few numbers to be isolated using these techniques. For example, very low numbers of BT4 (O:3) isolates may be present in faecal contents but may proliferate on refrigerated meat products. Also *Y. enterocolitica* is more prevalent on the tonsils of pigs than in the intestinal contents, leading to the possibility that only heavily colonised animals are detected in this survey (Fredriksson-Ahomaa and Korkeala, 2003). Outside Great Britain, surveys

have shown that other domestic animals, particularly dogs are recognised carriers of biotype 4 (O:3) (Fantasia *et al* 1985; Fenwick *et al* 1994; Fukushima *et al* 1984). Thus, dogs, and potentially other domesticated wildlife, could also be a significant source of infections with this biotype in the UK.

Biotype 3 (O:5,27) was by far the predominant pathogenic biotype carried by sheep and pigs, and was the only one recovered from cattle. As only 1% of human strains during 1999-2000 were biotype 3 (O:5,27), and none from the IID study, it seems likely that this biotype is either poorly transmissible or relatively non-pathogenic to man. In contrast, biotypes 3 (O:9) and 4 (O:3) were not uncommon in pigs (27% and 5 % respectively) and were also recovered from sheep. It seems likely therefore that livestock, and in particular, pigs are potential sources of human infection with these strains. Moreover, strain-specific differences in prevalence among humans and animals suggest some host-associated differences in colonisation potential that warrant further investigation.

Overall it seems from this first national abattoir survey of cattle, sheep and pigs, that *Y.enterocolitica* is a relatively ubiquitous organism in livestock in Great Britain. Unfortunately the relationship between these veterinary strains and human yersiniosis remains unclear as, using biotyping and serotyping as discriminatory tools, there is little correlation between the prevalence of strain carriage in livestock and disease in humans. This is further complicated by the high proportion of strains that belong to the putatively non-pathogenic, 1a, group in both humans and livestock. Clearly further research is required to elucidate the pathogenicity of biotype 1a strains and to determine their role in human infection. Because the only putatively pathogenic biotypes isolated from cattle and sheep were BT3 (O:5,27) strains, which were uncommon in humans, these food producing animals seem unlikely sources of human

infection, however, pigs appear to be the reservoir of human cases caused by biotype 3 (O:9) and possibly biotype 4 (O:3). The use of molecular epidemiological tools, with higher discriminatory power, will be required to further understand these relationships.

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Table 1. Prevalence of biotypes/serotypes of *Y.enterocolitica* isolates from pigs, sampled at abattoirs in Great Britain during 1999-2000.

Biotype	Serotype	No. Isolates	Percentage
1A	06,30	77	11.79
	0,?*	82	12.56
	019,8	46	7.04
	05	42	6.43
	O10, K1	18	2.76
	O41,43	17	2.6
	O7	16	2.45
	O4,32	12	1.84
	O8	12	1.84
	O13,7	7	1.07
	Others †	20	3.05
BT 3	05,27	142	21.75
	0,9	71	10.87
	O,?	26	3.98
	Others	10	1.52
BT4	03	33	5.05
	Others	5	0.77
BT2	Various ‡	3	0.46
BT6	Various	2	0.3

*O? indicates the strain was non-typeable.
†Others refers to uncommon serotypes of which only one strain was identified.
‡Various refers to the presence of more than one serotype within this biotype group.

Table 2. Prevalence of biotypes/serotypes of *Y.enterocolitica* isolates from cattle sampled at abattoirs in great Britain during 1999-2000.

Biotype	Serotype	Number	Percentage
BT1a	O:?*	14	30.44
	O:6, 30	10	21.74
	O:5, 27	4	8.7
	O:19, 8	4	8.7
	O:6, 31	3	6.52
	O:4, 32	2	4.35
	O:47	1	2.17
	O:41, 43	1	2.17
	O:4, 33	1	2.17
	O:37	1	2.17
	O:7	1	2.17
	O:21	1	2.17
	O:14	1	2.17
BT3	O:5, 27	2	4.35

*O? indicates the strain was non-typeable.

Table 3. Prevalence of biotypes/serotypes of *Y.enterocolitica* isolates from sheep sampled at abattoirs in great Britain during 1999-2000.

Biotype	Serotype	Number	Percentage
BT1a	O:6, 30	13	14.94
	O:? *	11	12.64
	O:19, 8	6	6.9
	O:5, 27	6	6.9
	O:4, 32	4	4.6
	O:6, 31	3	3.45
	O:5	2	2.3
	O:47	2	2.3
	O:41, 42	2	2.3
	O:41, 43	1	1.15
	O:7	1	1.15
	O:36	1	1.15
	O:13, 7	1	1.15
	O:10, K1	1	1.15
BT3	O:5, 27	30	34.5
BT4	O:3	1	1.15
BT2	O:9	1	1.15
	O:5, 27	1	1.15

*O? indicates the strain was non-typeable.

Table 4. Prevalence of biotypes/serotypes of human *Y.enterocolitica* isolates received by LEP, HPA during 1999-2000.

Biotype	Serotype	No Isolates	Percentage
1A	O,?*	28	17.1
	05	13	7.93
	O6,30	11	6.71
	O19,8	6	3.66
	O41,43	6	3.66
	O8	5	3.05
	O6,31	4	2.44
	O4,32	3	1.83
	010,K1	2	1.22
	O36	2	1.22
	013,7	1	0.61
	03	1	0.61
	046	1	0.61
	048	1	0.61
	05,27	1	0.61
	07	1	0.61
	O rough	1	0.61
3	O9	39	23.8
	O?	2	1.22
	O5	2	1.22
	O5,27	2	1.22
4	O3	32	19.5

*O? indicates the strain was non-typeable.