Yersinia enterocolitica isolates of differing biotypes from humans and animals are adherent, invasive and persist in macrophages, but differ in cytokine secretion profiles *in vitro*

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Previous epidemiological studies have demonstrated a potential link between the serotypes of *Yersinia enterocolitica* recovered from cattle, sheep and pigs and those isolated from human disease cases. Further studies utilizing amplified fragment length polymorphisms have shown a relationship at the genetic level between strains of biotypes 3 and 4 from humans and livestock, and also suggested that some biotype 1A isolates, classically defined as non-pathogenic, are closely related to biotype 3 and 4 isolates. This study sought to understand further the pathogenic potential of *Y. enterocolitica* isolates from livestock in Great Britain. A range of surrogate *in vitro* models, such as invasion of epithelial tissue cultures, survival in cultured macrophages and cytokine secretion response, was employed to assess the pathogenicity of 88 strains. The results suggested that all isolates examined were capable of adhering to and invading epithelial cells and of surviving within macrophages. However, the inflammatory response of the infected macrophages differed with the infecting *Y. enterocolitica* subtype, with the response to pathogenic biotype 3 and 4 isolates different to that observed with biotype 1A isolates, and with the biotype 3 O:5,27 isolates recovered exclusively from animals. Infections of porcine tissue also suggested the possibility of host-tissue tropism within *Y. enterocolitica* subtypes.

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INTRODUCTION

Yersinia enterocolitica is a human pathogen that can cause a range of diseases from mild diarrhoea to the severe complication of mesenteric lymphadenitis (Bottone, 1999). Disease is generally self-limiting, although severe disease can occur, especially in immunocompromised individuals. *Y. enterocolitica* infection involves invasion of the gastrointestinal epithelium, primarily at the follicle-associated epithelium (Grassl *et al.*, 2003; Hamzaoui *et al.*, 2004). The organisms then escape from the host cell via the basolateral membrane, allowing the bacteria to propagate in the lamina propria, where the production of a variety of virulence factors aids subversion of the host immune response (Cornelis & Wolf-Watz, 1997).

The virulence factors and mechanisms of pathogenesis of *Y. enterocolitica* are well documented, primarily in several well-studied biotype 1b O:8 strains. The organism has been shown to adhere to and invade a wide selection of eukaryotic cells *in vitro* (Finlay & Falkow, 1988; Miller & Falkow, 1988), with the genes encoding the adhesins and invasin protein identified and characterized (Grassl *et al.*, 2003; Pepe & Miller, 1993). The interaction of *Y. enterocolitica* with macrophages *in vitro* has also been studied in detail. However, most of the emphasis to date has been on how the organism avoids uptake by macrophages (Grosdent *et al.*, 2002), rather than how it survives within macrophages (Grant *et al.*, 1999; Yamamoto *et al.*, 1996). The genetic basis of resistance to macrophages has been well characterized, especially the role of the pYV virulence plasmid and the encoded Yop proteins (Grosdent *et al.*, 2002; Neyt & Cornelis, 1999; Tardy *et al.*, 1999; Trülsch *et al.*, 2004). In particular, the ability of *Y. enterocolitica* to modulate the host immune response has been highlighted, with various Yop proteins and other virulence factors shown to play a role in down-regulating the secretion of various pro-inflammatory cytokines, such as tumour necrosis factor (TNF)- α .

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Abbreviations: AFLP, amplified fragment length polymorphism; GFP, green fluorescent protein; IL, interleukin; IVOC, *in vitro* organ culture; TNF, tumour necrosis factor.

(Boland & Cornelis, 1998; Carlos *et al.*, 2004; Ruckdeschel *et al.*, 1997), interleukin (IL)-6 (Denecker *et al.*, 2002; Dube *et al.*, 2004), and IL-8 (Schulte & Autenrieth, 1998). Increased synthesis of the anti-inflammatory cytokine IL-10 has also been demonstrated *in vitro* (Sing *et al.*, 2002a).

Previous studies have highlighted *Y. enterocolitica* as ubiquitous in livestock. The organism has been isolated from 26.1% of pigs, 10.7% of sheep and 6.3% of cattle at slaughter in Great Britain (McNally *et al.*, 2004). Of those isolates recovered, 58% belonged to biotype 1A, which is classically defined as non-pathogenic due to its lack of lethality in the mouse infection model (Tennant *et al.*, 2003). Biotype 1A isolates also lack the majority of the defined virulence determinants of *Y. enterocolitica* found in the presumptively pathogenic biotypes 1b and 2–5 (Cornelis & Wolf-Watz, 1997; Tennant *et al.*, 2003). Despite this, recent investigations using *in vitro* and *in vivo* models suggest that biotype 1A isolates may still be capable of causing disease in humans (Grant *et al.*, 1998, 1999; Morris *et al.*, 1991; Tennant *et al.*, 2003). Such a hypothesis is consistent with the evidence that 53% of *Y. enterocolitica* strains isolated from humans in Great Britain between 1999 and 2000 were biotype 1A (McNally *et al.*, 2004).

Amplified fragment length polymorphisms (AFLPs) have been used to genotype selected *Y. enterocolitica* isolates from humans and animals (Fearnley *et al.*, 2005). Two main clusters have been identified, one comprising solely biotype 1A isolates and the other comprising primarily, but not exclusively, biotype 2–5 isolates. Within the two main clusters, isolates were further subdivided according to serotype. There was no obvious relationship with source, with the exception of biotype 4 O:3 human strains, which formed a distinct group from biotype 4 O:3 isolates from pigs.

This study has elaborated on the relationship between human and animal *Y. enterocolitica* strains by means of phenotypic comparisons. A variety of virulence mechanisms was examined in selected strains of human and animal origin, using defined *in vitro* assays. These included association with and invasion of eukaryotic cells (Miller & Falkow, 1988), resistance to intra-macrophage killing (Yamamoto *et al.*, 1996, 1997) and modulation of host-cell cytokine secretion (Denecker *et al.*, 2002; Dube *et al.*, 2004; Meyer-Bahlburg *et al.*, 2004; Schulte & Autenrieth, 1998). In addition, some preliminary work was undertaken on the interaction with animal intestinal tissue through *in vitro* organ culture (IVOC) (Fitzhenry *et al.*, 2002a, b; Phillips *et al.*, 2000).

METHODS

Bacterial strains and growth conditions. The sources and bio- serotyping of strains has been described previously (McNally *et al.*, 2004). Strains were selected to represent a cross-section of biotypes and serotypes present in humans and animals in Great Britain (Table 1), with all biotype 3 and 4 strains confirmed as pYV-positive by growth on CMO agar and microarray comparison in a linked

Table 1. Representation of the biotype, serotype and source of the 88 strains selected for characterization by pathogenesis potential phenotypes

Biotype	Serotype	Human	Pig	Sheep	Cattle	Total
1A	O:??*	2	2	2	2	8
	O:5	4	3	2		9
	O:19,8	2	2	2	2	8
	O:6,30	4	3	4	3	14
1B	O:8	1				1
2	O:9		1	1		2
3	O:5	2	2			4
	O:9	5	5			10
	O:5,27		5	5	2	12
	O:?	2	3			5
4	O:15		2			2
	O:3	5	5	1		11
	O:5,27		2			2
Total		27	35	17	9	88

*Serotype O:? refers to isolates that were non-typable by the modified Wauters scheme classically used to type *Y. enterocolitica*.

study (Howard *et al.*, 2006). The *Y. enterocolitica* sequenced strain 8081 (biotype 1B) and *Escherichia coli* DH5- α were used as controls in all phenotypic assays. All *Y. enterocolitica* strains were grown routinely at 28 °C for 18 h on 10% (v/v) sheep blood agar or in Luria–Bertani (LB) broth with shaking, unless otherwise stated. *E. coli* was grown at 37 °C for 18 h on 10% sheep blood agar or in LB broth with shaking, unless otherwise stated.

Cell cultures. HEp-2 human epithelial cells were cultured in minimal essential medium (MEM; Life Technologies) supplemented with 10% (v/v) heat-inactivated fetal calf serum (Life Technologies), 1% (v/v) L-glutamine and 1% (v/v) non-essential amino acids (Sigma). Cells were grown at 37 °C in a humidified 5% CO₂ atmosphere and subcultured every 4 days. Two days prior to infection, cells were seeded into a 24-well tissue culture plate (Nunc) at a concentration of approximately 2×10^5 cells ml⁻¹ (as determined by cell cytometer counts). U937 human monocytic cells were maintained in RPMI 1640 (Sigma) supplemented as above. Seventy-two hours prior to infection, cells were seeded into a 24-well tissue culture plate (Nunc) at a concentration of approximately 2×10^5 cells ml⁻¹ and treated with 10 nM phorbol myristate acetate (Sigma) upon seeding to trigger differentiation into active macrophages (Song *et al.*, 2003).

Association and invasion of epithelial cells by *Y. enterocolitica* isolates. Bacterial association with and invasion of HEp-2 cells was assayed as described previously (Dibb-Fuller *et al.*, 1999; Finlay & Falkow, 1988; Miller & Falkow, 1988). Bacteria were grown overnight in LB broth, harvested by centrifugation and resuspended in MEM plus 1% amino acids and 1% L-glutamine, to give approximately 2×10^7 c.f.u. ml⁻¹ (as determined by plate counts). Bacterial suspension (1 ml) was added in triplicate to wells of a pre-seeded 24-well plate (m.o.i. of 100). The infected cells were incubated for 3 h at 37 °C. Cells were washed three times with 0.1 M PBS, followed by the addition of MEM plus 1% amino acids, 1% L-glutamine and 100 µg gentamicin ml⁻¹. The cells were further incubated for 2 h. Cells were then washed twice in PBS and lysed with a 1% Triton X-100 solution for 5 min. Dilutions of the lysed cells were plated onto LB agar in triplicate and incubated overnight. Colonies were counted to give the total number of invasive bacteria

per millilitre. In a parallel series of wells, cells were lysed after the initial 3 h infection period to ascertain the total number of associated bacteria per millilitre.

Persistence of *Y. enterocolitica* isolates within U937 cells.

Bacteria were grown, harvested and resuspended as for the HEp-2 invasion assay. Differentiated U937 cells were infected at the same m.o.i. as for the HEp-2 invasion assay and incubated for 1 h at 37 °C. Cells were washed three times with 0.1 M PBS, and fresh medium containing 100 µg gentamicin ml⁻¹ was added, before a further incubation for 5 h. Cells were then washed twice in PBS and lysed by addition of a 1% Triton X-100 solution for 10 min. Dilutions of the lysed cells were plated onto LBG agar in triplicate and incubated at 28 °C overnight. Colonies were counted to give the total number of invasive bacteria per millilitre. In a parallel series of wells, the cells were lysed 15 min after the initial addition of gentamicin in order to ascertain the number of organisms internalized by the cells at 1 h post-challenge.

Trypan blue exclusion assay. The integrity of infected U937 cells was examined by their ability to exclude trypan blue dye. Cells were exposed to bacteria as above and then overlaid with 0.5% trypan blue for 5 min. At least 1000 cells were examined per well and the proportion of cells with dye uptake was recorded. Experiments were repeated in triplicate.

Cytokine production in infected U937 cells. To examine the effect of different *Y. enterocolitica* isolates on cytokine secretion, differentiated U937 cells were challenged as described above and incubated for 3 h. Supernatants were collected and centrifuged at

13 000 r.p.m. for 5 min in a bench-top microcentrifuge, and 200 µl of the supernatant was collected and stored at -20 °C until analysed. IL-6, IL-8, IL-10 and TNF-α levels were assessed using Quantikine sandwich ELISA kits (R&D systems) according to the manufacturer's instructions, with quantities presented as levels of cytokine normalized against levels present in cells unstimulated by bacteria.

Construction of a green fluorescent protein (GFP)::*gyrA* fusion in *Y. enterocolitica*.

In order to visualize the interaction of *Y. enterocolitica* strains with intestinal tissue, fusions constitutively expressing GFP were constructed in seven *Y. enterocolitica* isolates, which were selected to represent each biotype from each host. The list of strains, plasmids and primers used is shown in Table 2. *Y. enterocolitica* isolates were made electrocompetent by repeated washing of a mid-exponential broth culture in ice-cold 10% glycerol solution. Cells were then stored at -80 °C until use (Sambrook *et al.*, 1989). All molecular biology techniques were performed according to standard protocols (Sambrook *et al.*, 1989). The *gfp*⁺ gene was amplified from pMN402 (Scholz *et al.*, 2000) using primers GFP5 and GFP3. Plasmid pAM4 was constructed by digesting pACYC184 with *Clal*, blunt-ended by treatment with the large Klenow fragment of DNA polymerase I and religated using T4 DNA ligase. Plasmid pAM4 was digested with *EcoRV*, treated with calf intestinal phosphatase and ligated to the amplified *gfp*⁺ gene to create pAM6. Clones were selected by loss of tetracycline resistance. The *gyrA* promoter was amplified from *Y. enterocolitica* 8081 using primers GYR5 and GYR3. The amplified product and pAM6 were digested with *EcoRV* and *Clal*, the linearized plasmid was treated with phosphatase, and

Table 2. List of specific strains used in this study for IVOC, and the plasmids and primers used in molecular biology techniques

Strain, plasmid or primer	Details	Source
Strains		
<i>Y. enterocolitica</i> 8081	Biotype 1B O:8 sequenced strain	Professor Brendan Wren (London School of Hygiene and Tropical Medicine)
<i>E. coli</i> DH5α	<i>endA1</i> , <i>r⁻m⁺</i> , <i>thi-1</i> , <i>recA1</i> , <i>gyrA</i> (<i>NaF</i>), Δ (<i>lacIZYA</i>)	General cloning background strain
YE149/02	Biotype 3 O:5,27; pYV-positive	Sheep abattoir isolate
YE201/02	Biotype 4 O:3; pYV-positive	Pig abattoir isolate
YE208/02	Biotype 1A O:5; pYV-negative	Pig abattoir isolate
YE237/02	Biotype 3 O:9; pYV-positive	Pig abattoir isolate
YE12/03	Biotype 4 O:3; pYV-positive	Human faecal isolate
YE53/03	Biotype 1A O:5; pYV-negative	Human faecal isolate
YE56/03	Biotype 3 O:9; pYV-positive	Human faecal isolate
Plasmids		
pMN402	Hyg ^r , <i>ColE1</i> /PAL5000 <i>ori</i> , <i>gfp</i> ⁺	Dr Michael Niederweis (University of Nurnberg)
pACYC184	Cm ^r , Tet ^r ; <i>ColE1 ori</i>	General cloning vector
pAM4	Cm ^r , Tet ^r ; pACYC 184 Δ <i>Clal</i>	This study
pAM6	Cm ^r ; pAM4 with <i>gfp</i> ⁺ inserted via <i>EcoRV</i>	This study
pAM19	CM ^r ; pAM6 with <i>gyrA</i> promoter inserted via <i>EcoRV/Clal</i>	This study
Primers (5'→3')*		
GFP5	GATATCGATGAAGGAGATATACATATGGCTAG	This study
GFP3	GCTCGAATTCATTATTGTAGAG	
GYR5	GATATCCGCGCCATGCTTTCAGCT	This study
GYR3	ATCGATGAGCCGCTATCCCTCTAC	

*Engineered restriction enzyme recognition sites are indicated in bold.

the fragments were ligated to create pAM19. Electrocompetent *Y. enterocolitica* strains were electroporated with 1 µl pAM19 at 1.5 kV, 2.5 µF, 200 Ω.

IVOC. Two 3-month-old male pigs were euthanized on separate days. Immediately after euthanasia, the terminal ileum and spiral colon sections were harvested. The contents of the tissues were removed and 2 cm² tissue sections prepared, washed in pre-warmed 0.1 M PBS (pH 7.2) and placed on sterile gauze. The tissue sections were placed individually in a square Petri dish and partially submerged in pre-warmed DMEM containing 1% L-glutamine and 1% non-essential amino acids. Bacterial overnight cultures were harvested by centrifugation and resuspended in PBS to give approximately 10⁸ c.f.u. ml⁻¹. One hundred microlitres of culture was pipetted directly onto the apical surface of the tissue and incubated at 37 °C in 5% CO₂ for 6 h, with media changed after 3 h. Tissues were washed vigorously in PBS and fixed in ice-cold 4% paraformaldehyde for 24 h.

Tissue preparation and confocal fluorescent microscopy. Fixed tissue samples were snap frozen in isopentane pre-cooled in liquid nitrogen to between -40 and -60 °C. Tissue sections were cut using a cryostat (-20 °C) to a thickness of 4–6 µm and collected directly onto slides. The slides were mounted with Vectashield with DAPI mounting medium and stored in the dark at room temperature. Confocal fluorescent microscopy was performed on a Leica microscope with an excitation wavelength of 488 nm and an emission filter of 507 nm.

Statistical analysis. For each of the four sets of cytokine data, an analysis of variance was done to compare the mean cytokine responses to the strain types, with the results averaged into groups of biotype 3 O:5,27, biotype 1A, biotype 4 O:3 and biotype 3 O:9. The control results were all zero due to normalization and hence omitted, as were all other zero or missing results. As the variance of the data tended to increase with the mean, the data were transformed to their log₁₀ values for the analysis. When the overall *F* test for the effects of strains was significant (*P*<0.05), all pairs of strain were compared using Tukey's HSD test based on the Studentized range.

RESULTS

Adherence to and invasion of epithelial cells *in vitro* by *Y. enterocolitica* of various biotypes and sources

Association and invasion assays on cultured HEp-2 cells were performed on all 88 isolates to ascertain the ability of *Y.*

enterocolitica of different biotypes and from various hosts to adhere to and invade epithelial cells *in vitro*. HEp-2 cells were chosen over intestinal cell lines as *Y. enterocolitica* has previously been shown to invade these at higher levels than INT-407 and Caco-2 cells (Finlay & Falkow, 1988). The arithmetic mean association and invasion of each source/biotype combination is presented in Fig. 1. All of the *Y. enterocolitica* biotypes were capable of adhering to epithelial cells in high numbers, with approximately 10% of the organisms added associating with the HEp-2 cells (~10⁶ c.f.u. ml⁻¹ from 10⁷ c.f.u. ml⁻¹ added for infection). The data confirmed that isolates from biotypes 2–4 are more invasive than biotype 1A strains (Grant *et al.*, 1999), with the pathogenic biotype isolates invading at 10⁴–10⁵ c.f.u. ml⁻¹, compared with 10²–10³ c.f.u. ml⁻¹ for biotype 1A strains. Nevertheless, it was clear that biotype 1A isolates were invasive. Averaged association with and invasion of epithelial cells of biotypes 3 O:5,27, 3 O:9 and 4 O:3 showed that all bio/serotype strains invaded at statistically similar levels. Invasiveness appeared to be independent of the source of the isolate, although anomalies were observed; in particular, the sheep biotype 4 O:3 isolates displayed reduced invasion compared with other biotype 3 and 4 strain types.

Persistence of *Y. enterocolitica* isolates within U937 macrophages

The ability of isolates of different biotypes and sources to survive within macrophages was assessed in differentiated U937 cells. In order to determine the optimal times for the experiment, a time-course assay was performed using a single biotype 1A isolate and the sequenced 8081 pathogenic strain (Fig. 2a). The growth kinetics mirrored those previously reported for intra-macrophage survival of various *Y. enterocolitica* strains (Grant *et al.*, 1999; Yamamoto *et al.*, 1996, 1997) and those for other bio/serotypes tested (data not shown), suggesting that these phenotypes are representative of the phenotypes likely to be observed for test strains. The results suggested that, at 5 h post-challenge, the bacterial numbers began to increase.

Fig. 1. Mean levels of association and invasion of cultured HEp-2 epithelial cells by biotype 1A (all serotypes), biotype 4 O:3, biotype 3 O:9, biotype 3 O:5,27 and 'other' bio/serotype (BT1b; BT2; BT3 O:5, O:15, O:?, BT4 O:5,27) isolates of human, porcine, ovine and bovine origin. Results are displayed as c.f.u. bacteria ml⁻¹ recovered from an infected well and are means ± SEM of individual duplicate experiments performed in triplicate. White portions of bars, mean association; black portions of bars, mean invasion.

Fig. 2. Persistence of YE53/03 (BT1A isolate) and *Y. enterocolitica* 8081 within cultured U937 human macrophages over a 24 h time course (a), and the mean levels of persistence of biotype 1A, 4 O:3, 3 O:9 and 3 O:5,27 isolates of human, porcine, ovine and bovine origin (b). Results are displayed as c.f.u. bacteria ml⁻¹ recovered from an infected well after 5 h (T5) compared with bacteria recovered after an initial 1 h infection (T0) and are means $\pm$ SEM of duplicative experiments performed in triplicate.

This was selected as the time point for examination rather than, for example, the time at which Yop expression was maximal, as it allowed a fairer comparison between pYV⁺ and pYV⁻ strains based solely on growth kinetics within the macrophages. The results obtained for the persistence of *Y. enterocolitica* isolates within U937 cells (Fig. 2b) suggested that *Y. enterocolitica* is capable of survival within macrophages irrespective of biotype or source. The mean number of organisms recovered after 5 h was similar to the number recovered after the initial 1 h infection period among biotypes 1A, 3 and 4; however, there was some variation within individual strain types, with sheep and pig biotype 1A isolates persisting to higher levels than the other strain types tested. The number of biotype 4 O:3 isolates recovered after 5 h was slightly lower than that for the other strain types; however, trypan blue exclusion assays indicated that this was due to a concomitant increase in the number of non-viable macrophages in infections carried out with biotype 4 O:3 isolates.

U937 responses to *Y. enterocolitica*

The pYV virulence plasmid, encoding the Yop proteins known to modulate cytokine secretion during *Y. enterocolitica* infection, is absent from biotype 1A strains. In order to assess the secretion of cytokines following infection with

different strain types, supernatants from infected U937 cells were harvested and assayed using commercial ELISA kits (Fig. 3). Infections were carried out with four selected isolates from each pathotype/host combination, namely: YE111/02, YE149/02, YE203/02 and YE238/02 (animal BT3 O:5,27); YE201/02, YE204/02, YE220/02 and YE227/02 (pig BT4 O:3); YE13/03, YE35/03, YE38/03 and YE53/03 (human BT1A); YE214/02, YE218/02, YE236/02 and YE237/02 (pig BT3 O:9); YE03/03, YE07/03, YE08/03 and YE12/03 (human BT4 O:3); YE02/02, YE13/02, YE208/02 and YE221/02 (animal BT1A); and YE39/03, YE56/03, YE58/03 and YE74/03 (human BT3 O:9).

Modulation of IL-6 secretion. Secretion of IL-6 was increased with biotype 3 O:5,27 isolates compared with the other strain types tested ($P=0.061$), with levels of IL-6 secretion slightly elevated for biotype 1A strains ($P=0.2$) (Fig. 3a). The biotype 4 O:3 and 3 O:9 strains exhibited decreased levels of IL-6 secretion, particularly the pig biotype 4 O:3 and human biotype 3 O:9.

Modulation of IL-8 secretion. Secretion of IL-8 by infected U937 cells was also slightly, although significantly, increased with biotype 3 O:5,27 isolates compared

Fig. 3. Mean levels of secretion of IL-6 (a), IL-8 (b), IL-10 (c) and TNF- α (d) by U937 human macrophages after infection with biotype 1A, 4 O:3, 3 O:9 and 3 O:5,27 isolates of human, porcine, ovine and bovine origin. Data are presented as fold difference in the level of cytokine production relative to the unstimulated control. Cells were infected with four randomly selected isolates from each biotype/source combination. Duplicative experiments were performed in duplicate and results are shown as means $\pm$ SEM.

with the other strain types ($P=0.045$) (Fig. 3b). IL-8 secretion with biotype 1A strains of both human and animal origin was also elevated compared with biotype 3 O:9 and 4 O:3 strains ($P=0.05$), although secretion levels were slightly higher in the environmental biotype 1A isolates than in the human strains.

Modulation of IL-10 secretion. Levels of secreted IL-10 were similar for all of the isolates tested and did not differ significantly from the levels secreted by uninfected cells, although they were greatly reduced compared with an LPS control (Fig. 3c).

Modulation of TNF- α secretion. Infected U937 cells secreted large amounts of TNF- α upon infection with human or animal biotype 1A strains. Secretion induced by animal biotype 1A strains was on average 3.9-fold greater than that observed upon infection with pathogenic strains ($P=0.022$). Secretion induced by human biotype 1A isolates was approximately a further twofold greater

than that observed after infection with animal biotype 1A strains ($P=0.061$).

Colonization of pig terminal ileum by IVOC

The seven isolates selected for IVOC analysis were successfully transformed with the plasmid containing the constitutive *gfp::gyrA* fusion. Fluorescence from the fusion was confirmed using a confocal fluorescent microscope and compared with an untransformed strain as a control (data not shown). All seven isolates successfully colonized porcine terminal ileum and spiral colon tissues during IVOC (Fig. 4). There was little evidence of bacteria closely adhered to intestinal tissue or within tissues, with the majority of organisms appearing to be more closely associated with mucus and cellular debris. The strains of animal origin tested associated with the porcine tissue in greater numbers than the strains of human origin (Table 3) in both the terminal ileum and spiral colon. All of the strains tested displayed a preference towards colonization of the

Fig. 4. Colonization of porcine intestinal tissue by GFP-tagged strains of *Y. enterocolitica* in IVOC experiments. The panels show spiral colon tissue colonized by GFP-tagged strains YE149/02 (a), YE201/02 (b), YE208/02 (c), YE237/02 (d), YE12/03 (e), YE53/03 (f) and YE56/03 (g). Images were captured using a Leica confocal fluorescent microscope and a $\times 63$ objective and were then magnified a further threefold using the Leica confocal microscope capture software. Large arrows indicate cell nuclei counterstained with DAPI; small arrows indicate GFP-tagged bacteria.

follicle-associated, epithelium-rich spiral colon tissue compared with the terminal ileum.

DISCUSSION

The aim of this study was to investigate the human pathogenic potential of *Y. enterocolitica* isolates of different biotypes and from various veterinary sources by comparing their phenotypes with isolates from human disease cases. A total of 88 isolates was selected for characterization to represent the various biotypes and serotypes present in pigs,

cattle, sheep and humans collected contemporaneously within England and Wales (McNally *et al.*, 2004). No statistical differences between biotypes could be detected in the ability of isolates to adhere to and invade cultured epithelial cells or to persist in macrophages other than those already reported between biotype 1A strains and pathogenic biotypes. However, differences were observed in the response of cultured human macrophages to infection with some isolates. Biotype 3 O:5,27 infection resulted in slightly increased levels of IL-6 ($P=0.061$) and significantly increased ($P=0.045$) IL-8 secretion when compared with other biotypes. Infection with biotype 1A isolates resulted in greatly increased levels of TNF- α secretion ($P=0.022$). These results suggested that, whilst all *Y. enterocolitica* biotypes may possess pathogenic potential, the duration and severity of infection or disease pathology may differ due to differences in host response to some biotypes.

The levels of invasion observed for the pathogenic biotype isolates were higher than those observed for the biotype 1A strains, which is consistent with previous data (Grant *et al.*, 1998, 1999). There was no significant difference in the ability of strain types of bovine, porcine, ovine or human origin to invade HEP-2 cells.

All isolates were capable of persisting in macrophages. Previous research has focused on the ability of *Y. enterocolitica* to avoid host macrophage ingestion via the Yop type III secreted effector proteins (Cornelis & Wolf-Watz, 1997). Increasingly, the importance of studying *Y. enterocolitica*

Table 3. Enumeration of bacteria colonizing IVOC porcine intestinal tissue

The results represent the mean number of bacteria enumerated from five randomly selected fields of view. Strains were scored according to the number of organisms per field (+, 0–20; ++, 20–100; +++, 100–500; +++, >500).

Strain	Terminal ileum	Spiral colon
YE149/02	++	+++
YE201/02	++	++++
YE208/02	++	++++
YE237/02	++	++++
YE12/03	+	++
YE53/03	+++	++
YE56/03	+	++

inside macrophages has been raised (Pujol & Bliska, 2005) and in this study it was decided to examine bacteria actively persisting within intact macrophages. Using U937 macrophage-like cells, no statistical differences in levels of intramacrophage survival were found between biotypes or source. The levels of bacteria recovered were, on average, slightly lower for the pathogenic biotype 4 O:3 and 3 O:9 strains tested than for the biotype 1A isolates, but this phenomenon was explained by the increased number of non-viable macrophages, as determined by trypan blue staining, which was almost certainly due to the production of Yop effector proteins upon contact with the U937 cells (Cornelis & Wolf-Watz, 1997). The levels of persistence for the biotype 1A isolates were surprising and differed slightly from those published previously (Grant *et al.*, 1999). However, our study utilized human macrophage-like cell lines, whilst previous work has focused on the mouse RAW macrophage cell line. The relevance of data with RAW cells is questionable, as biotype 1A isolates are non-pathogenic in a mouse infection model (Bottone, 1997).

IVOC has been developed as a powerful tool for investigating host-pathogen relationships (Fitzhenry *et al.*, 2002a, b; Phillips *et al.*, 2000). In order to investigate differences in association with whole intestinal tissue, one isolate from each biotype/source combination was selected and transformed with a plasmid containing a constitutive *gyrA::gfp*⁺ fusion. These strains were then used to conduct IVOC studies on porcine tissue. The GFP⁺ derivative was chosen, as it has been shown to be a more stable version of GFP and has been used successfully *in vivo* (Hautefort *et al.*, 2003; Scholz *et al.*, 2000). The porcine IVOC study showed that all of the strains were capable of colonizing pig intestinal tissue *in vitro*. The animal strains appeared to colonize porcine tissues at higher numbers than the human isolates, suggesting that there may be differences in tissue tropism between strains. This was in contradiction to the association observed in the *in vitro* cell line-based assays; however, there were numerous other factors present in the IVOC assay, including the presence of a mucous layer, that may influence the way that the organism interacts with the surface. The biotype 1A strains were equally as capable of colonizing the IVOC tissue as the other biotypes tested, although there was a slight reduction in the numbers of biotype 1A strains present in intestinal tissues compared with the pathogenic biotypes. The possibility of tissue tropism in *Y. enterocolitica* isolates requires further work, in particular the interaction of *Y. enterocolitica* biotypes with human intestinal tissue.

The host-cell response to infection with different *Y. enterocolitica* biotypes was investigated. This was undertaken using commercial sandwich ELISA kits to measure the levels of secretion of several cytokines by infected U937 cells. This method has been used extensively in previous *Y. enterocolitica* cytokine studies (Denecker *et al.*, 2002; Dube *et al.*, 2004; Kampik *et al.*, 2000). The pro-inflammatory cytokine TNF- α was selected for analysis, as modulation of

its secretion by *Y. enterocolitica* Yop effectors has been reported (Boland & Cornelis, 1998; Carlos *et al.*, 2004; Ruckdeschel *et al.*, 1997). Similarly, secretion of IL-6 (Denecker *et al.*, 2002; Dube *et al.*, 2004) and IL-8 (Denecker *et al.*, 2002; Kampik *et al.*, 2000; Schmid *et al.*, 2004; Schulte & Autenrieth, 1998; Schulte *et al.*, 1996, 2000) was examined, as both have been shown to play a role in *Y. enterocolitica* infection. Lastly, the anti-inflammatory cytokine IL-10 was studied, as *Y. enterocolitica* has been shown to increase production of IL-10 to avoid the immune response (Erfurth *et al.*, 2004; Sing *et al.*, 2002a, b, 2003).

The data showed that infection with biotype 3 O:5,27 isolates, which are ubiquitous in animals in Great Britain but not in humans (McNally *et al.*, 2004), resulted in increased secretion when compared with other biotypes of IL-6 and IL-8, two cytokines that play key roles in clearing *Y. enterocolitica* infection. It is therefore tempting to speculate that the absence of biotype 3 O:5,27 strains in humans is a consequence of an elevated IL-6/IL-8-mediated immune response. Future work, including responses to infection in a variety of host tissue types, is currently being pursued to address this. The biotype 1A strains tested displayed IL-6 and IL-8 secretion to levels equivalent to those observed in biotype 4 O:3 and 3 O:9 strains and also displayed IL-10 secretion to similar levels. However, the biotype 1A isolates displayed large-scale secretion of TNF- α by human macrophages, with greatly elevated levels observed for both human and animal isolates. Indeed, infection with human biotype 1A isolates induced an approximately twofold increase in TNF- α secretion compared with animal-source biotype 1A isolates. It may therefore be hypothesized that infection of humans with biotype 1A strains leads to a different pathology than infection with biotype 3 O:9 or 4 O:3, supported by statistically similar levels of IL-6 and IL-8 secretion but massively increased TNF- α secretion. This would be consistent with macrophages being a preferred environment for biotype 1A organisms, with TNF- α release leading to migration of more macrophages to the site of infection. The relevance of these findings requires more in-depth analysis, including analysis of the active role played by the bacteria in the differences in host response.

In conclusion, this study has highlighted that all of the *Y. enterocolitica* isolates tested in this study from livestock had the ability to adhere to and invade cultured epithelial cells, as well as to persist in cultured human macrophages. However, *Y. enterocolitica* biotype 3 O:5,27, which was isolated from animals and not humans, led to elevated IL-6 and IL-8 release by infected macrophages compared with other biotype 3 and 4 isolates. In contrast, *Y. enterocolitica* biotype 1A isolates showed no significant differences in IL-6 and IL-8 secretion compared with biotype 3 and 4 isolates, but infection of human macrophages led to a significant release of TNF- α . These findings indicate that the differences in biotype-disease association in human infection are a reflection of host responses to the various biotypes. The data suggest that the differences may reside in the ability of

the organisms to interact with the host intestinal surface or to control the host immune response.

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