Identification of hyperinvasive *Campylobacter jejuni* strains isolated from poultry and human clinical sources

Catherine Fearnley,1 Georgina Manning,2 Mary Bagnall,1 Muhammad Afzal Javed,2 Trudy M. Wassenaar3 and Diane G. Newell1

1Veterinary Laboratories Agency (Weybridge), Surrey KT15 3NB, UK
2School of Science and Technology, Nottingham Trent University, NG11 8NS, UK
3Molecular Microbiology and Genomics Consultants, Zotzenheim, Germany

*Campylobacter jejuni* causes gastroenteritis with a variety of symptoms in humans. In the absence of a suitable animal model, *in vitro* models have been used to study virulence traits such as invasion and toxin production. In this study, 113 *C. jejuni* isolates from poultry and poultry-related (*n* = 74) environments as well as isolates from human cases (*n* = 39) of campylobacteriosis and bacteraemia were tested for invasiveness using INT 407 cells. The method was sufficiently reproducible to observe a spectrum of invasiveness amongst strains. As a result, strains were classified as low, high and hyper-invasive. The majority of strains (poultry and human) were low invaders (82% and 88%, respectively). High invasion was found for 5% of human strains and 11% of poultry-related isolates. However, only 1% of poultry strains were classified as hyperinvasive compared to 13% of human isolates (*P* = 0.0182). Of those isolates derived from the blood of bacteraemic patients, 20% were hyperinvasive, though this correlation was not statistically significant. An attempt was made to correlate invasiveness with the presence of seven genes previously reported to be associated with virulence. Most of these genes did not correlate with invasiveness, but gene *cj0486* was weakly over-represented, and a negative correlation was observed for the gene *ciaB*. This trend was stronger when the two genes were analysed together, thus *ciaB* and *cj0486* were over-represented in high and hyperinvasive strains, with low invaders more commonly found to lack these genes (*P* = 0.0064).

INTRODUCTION

*Campylobacter jejuni* is a common cause of bacterial enteritis in the industrialized world. In 2006, there were over 46,600 cases in England and Wales reported to the Health Protection Agency Centre for Infections (www.hpa.org.uk). Due to under-reporting, this is thought to reflect only about 12% of the true incidence of campylobacteriosis (Tompkins *et al.*, 1999). Although the disease is generally self-limiting, the symptoms can be particularly debilitating, with severe abdominal pain and cramps followed by profuse diarrhoea. In developed countries, disease symptoms indicate an inflammatory infection with blood-containing faeces, even when stools have a more watery appearance (Wassenaar & Blaser, 1999). Transient colonization with few or no symptoms is more common in developing countries where individuals are constantly exposed to campylobacters. Here, individuals are thought to acquire a protective immune response from an early age (Newell, 2002). Asymptomatic infection is rare in the developed world (Food Standards Agency, 2007), but has been reported for people who are frequently exposed to high doses in an occupational setting such as slaughterhouse workers or veterinarians (Cawthraw *et al.*, 2000). This variation in clinical outcome most likely reflects both variations in the pathogenic potential of the infecting strain and the immune status of the host; however, the relative contributions of these two factors are presently unknown.

There have been extensive studies to investigate the disease mechanisms of *C. jejuni*. The accepted mechanisms of pathogenesis are colonization of the mucous layer of the intestine, adhesion to and invasion of the intestinal epithelial cells, and the production of one or more cytotoxins (Wassenaar & Blaser, 1999). The inflammatory nature of the disease, as well as strong evidence from *in vivo* studies (Newell & Pearson, 1984; Ruiz-Palacios *et al.*, 2007; Russell *et al.*, 1993), suggest that invasion is an important virulence trait of this organism. Many *in vitro* invasion assays, largely based upon gentamicin protection (Elsinghorst, 1994; Friis *et al.*, 2005), have been developed.
and used to study the invasiveness of campylobacters using various cell lines including HEP2 (De Melo et al., 1989; Konkel & Joens, 1989), HeLa (Fauchère et al., 1986; Newell & Pearson, 1984), INT 407 (Wassenaa et al., 1991) and Caco-2 (Everest et al., 1992; Russell & Blake, 1994) cells. Several invasion-related genes have been proposed as a consequence of such studies. The flaA gene has been known for some time to be involved with invasion (Wassenaa et al., 1991) and motility of the organism is strongly linked to its invasive capacity. More recently, other genes have been implicated in invasion, notably cadF, a fibronectin-binding protein that may provide a potential binding site for the bacterium (Konkel et al., 1997) with an additional involvement in cell signalling leading to GTPase activation (Krause-Grusczynska et al., 2007); ciaB, which encodes one of eight proteins that are secreted upon contact with the host cell (Konkel et al., 1999); iam (invasion associated marker), identified following fingerprint analysis of invasive strains (Carvalho et al., 2001); and virB8, virB9 and virB11, which are present on the pVir plasmid, first identified in strain 81-176 (Bacon et al., 2000, 2002).

Variation in pathogenicity between strains is a common feature among many enteropathogens, including Salmonella enterica, Escherichia coli and Yersinia enterocolitica. Diversity between C. jejuni strains has been observed in various pathogenicity traits including adherence (Coote et al., 2007; Fauchère et al., 1986; Konkel & Joens, 1989; Zheng et al., 2006) and toxicity (AbuOun et al., 2005; Bang et al., 2001, 2003; Coote et al., 2007; Eyigor et al., 1999; Hänel et al., 2007; Johnson & Lior, 1986; Lindblom et al., 1990). Variation in invasion between strains of C. jejuni has also been demonstrated (Newell et al., 1985). Wide variation in adhesion and invasion was observed in isolates from retail meat (Zheng et al., 2006) and unsuccessful attempts were made to correlate the presence or absence of known virulence-related genes to the phenotypes observed. Similar results have been reported in other studies (Coote et al., 2007; Datta et al., 2003; Müller et al., 2006), suggesting that observable links between gene presence, genotype, isolation source or virulence potential are rarely observed, or extremely weak (Coote et al., 2007).

Risk attribution studies have identified poultry as a major source of human infection (Adak et al., 2005). Indeed, chickens are frequently colonized with Campylobacter and poultry meat is frequently contaminated (Jorgensen et al., 2002). Nevertheless, differences in the population structures of human and poultry strains (Dingle et al., 2001; Koenraad et al., 1995; Krause-Grusczynska et al., 2007; Manning et al., 2003a) suggest that either not all poultry Campylobacter strains possess the pathogenic potential to cause disease in man, or not all poultry isolates survive meat processing and storage, thus never reaching the human consumer. It seems likely that both explanations contribute to the observed differences in human and poultry C. jejuni populations.

In this study, we have investigated whether representative poultry isolates have the capacity to cause human disease using invasion as a surrogate marker of virulence. The invasion potential of 74 poultry and poultry-related isolates was compared with that of 39 human clinical isolates, some of which were from blood and were therefore assumed to be invasive to the human host. In contrast, the poultry isolates were, for the most part, epidemiologically unrelated and had been obtained from asymptomatic birds and their environments. The results confirm variation in invasiveness among C. jejuni strains. A hyperinvasive group of strains has been identified, a greater proportion of which were found among the human isolates. The genetic relatedness of these strains was determined by multilocus sequence typing (MLST). In addition, the presence of putative invasion-related genes was investigated by PCR.

**METHODS**

**Bacterial strains and growth conditions.** C. jejuni strains (n=66) were isolated from cloacal swabs of broilers, conventionally housed in farms within the South East of England, in 1996 and 1997. Two additional poultry cloacal isolates and six broiler house environmental strains (taken from puddles around the broiler house) were isolated from a farm in the South West of England and were thus temporally and geographically related.

Thirty-nine human C. jejuni clinical isolates were also investigated; 29 were isolated from the stools of patients with diarrhoea, who had presented to their general practitioner, and the remaining 10 strains were isolated from the blood of hospitalized patients with bacteraemia.

Three laboratory-adapted Campylobacter strains, originally of clinical origin, of which the genome sequences are now known were included as reference strains: C. jejuni strain NCTC 81116, originally isolated during a water outbreak in the UK in 1981 (Palmer et al., 1983; Pearson et al., 2007); C. jejuni 81-176, bearing the pVir plasmid (Bacon et al., 2000; Hofreuter et al., 2006), which has previously been reported to be invasive (Oelschlaeger et al., 1993; Russell & Blake, 1994); and C. jejuni strain NCTC 11168, for which the first complete Campylobacter genome sequence was obtained (Parkhill et al., 2000).

All strains used in this study were stored at –80°C in 1% (w/v) proteose peptone water containing 10% (v/v) glycerol until required. Strains had been minimally passaged in vitro before storage and subsequent testing. When required, bacteria were inoculated on blood agar containing selective Skirrow’s antibiotics (Oxoid) and actidione (50 μg ml⁻¹) (BASA) and grown under microaerobic conditions at 42°C. After 24 h growth, a loopful of bacteria was inoculated into pre-warmed brain heart infusion broth supplemented with 1% (w/v) yeast extract (BHI/YE) overlaying BHI/YE agar. This was cultured for 20 h at 42°C microaerobically for invasion assays. These conditions were determined in preliminary studies as optimum growth conditions for the invasion assay.

**Invasion assay.** The gentamicin protection assay used in this study was based on that of Elsinghorst (1994). INT 407 cells, and later Caco-2 cells, were obtained from the European Collection of Animal Cell Cultures (ECACC, CAMR, Porton Down, Salisbury, UK). Note that it is now generally recognized that the INT 407 cell line was contaminated with HeLa cells in the 1970s and therefore has cellular markers consistent with this contamination. Cells were maintained as a monolayer in Eagle’s Minimal Essential Medium (EMEM; Sigma) supplemented with 10% (v/v) fetal bovine serum, 2 mM l-glutamine, 1% (v/v) non-essential amino acids (NEAA) and 50 μg gentamicin
ml⁻¹ (complete media, all from Sigma) at 37°C in a 5% CO₂ atmosphere. Confluent cultures were trypsinized, and the cells were counted and suspended in the above growth medium at a concentration of 2 × 10⁵ cells ml⁻¹. A 24-well tissue culture tray was seeded with 1 ml per well and incubated at 37°C for 48 h to establish confluent monolayers (approx. 5 × 10⁵ cells per well for INT 407 cells or 3 × 10⁵ cells per well for Caco-2 cells). On the day of the assay, the monolayers were washed twice with Hanks’ Balanced Salt Solution (HBSS; Sigma) to remove any residual antibiotics and incubated with 1 ml of a maintenance medium of EMEM supplemented with 2 mM l-glutamine and 1% (v/v) NEAA before use. Mid-exponential-phase campylobacters were harvested by centrifugation at 2100 g at room temperature and resuspended in 0.1 M PBS (pH 7.2). Further dilutions into prewarmed EMEM were carried out to give a bacteria to cell ratio of 200:1. The viable count was determined retrospectively by culturing serial dilutions of the used suspension in PBS on BASA plates as before.

A volume of 0.1 ml of the bacterial suspension was inoculated into triplicate wells containing confluent monolayers of INT 407 cells in 1 ml maintenance medium. Tissue culture plates were centrifuged at 450 g at room temperature for 15 min to bring the bacteria in contact with the cells. Centrifugation was carried out to eliminate variations in motility between strains, which could influence the outcome of the assay. Inoculated monolayers were incubated for 3 h to allow the bacteria to invade the cells. After washing three times with HBSS, 2 ml medium containing 250 µg gentamicin ml⁻¹ was placed in each well and incubated for a further 2 h to kill extracellular bacteria. Following incubation, the monolayers were washed three times with HBSS and lysed with 1% (v/v) Triton X-100 (Sigma) in PBS for 10 min at room temperature to release the intracellular bacteria. Serial dilutions of the suspensions were made in PBS and inoculated onto BASA plates to determine the number of organisms that survived the gentamicin treatment and hence had invaded the INT 407 or Caco-2 cells.

The invasion efficiency of each isolate was expressed as a percentage of the number of bacteria added to the well at the start of the experiment with the standard error of the mean calculated from triplicate assays. Statistical analysis of the data was carried out using GraphPad Prism software version 2.01 (San Diego, CA, USA). Analysis of variance (ANOVA) with one factor was used to test for significant differences between the mean invasion efficiencies of the test isolates. Despite optimal standardization of procedures, inter-experimental variation remained considerable. Nevertheless, particular strains were consistently found to be low or highly invasive. One low-invasive strain, C. jejuni NCTC 81116, was used as an internal control strain in all experiments and the invasion potential of all other strains was related to this control strain using Dunnet’s post-test analysis. Invasiveness was then plotted for all investigated strains, and cut-off values for hyperinvasive, highly invasive and low-invasive strains were chosen as described in Results. The grouping of isolates into the invasion classes was reproducible irrespective of inter-experimental variation (data not shown).

Translocation and association assays using alternative cell lines. To confirm the invasive capacity observed with INT 407 cells, two more phenotypic characteristics were tested: the capacities to translocate across Caco-2 cell monolayers (Konkel et al., 1992) and to associate with HT29-Cl.16E mucus-secreting cells (Augeron et al., 1992). For translocation assays, Caco-2 cells were grown on porous membrane inserts (3 µm pores) that were immersed in complete media. The cells were allowed to differentiate into polarized monolayers for 14 days. Bacteria were placed in the upper compartment and allowed to associate with the apical surface of the Caco-2 cell surface. The ability of the bacteria to translocate was determined over time by enumerating the number of bacteria that had passed through the cell monolayer into the lower compartment below the porous membrane insert. HT29-Cl.16E is a homogenous colonic epithelial goblet cell line (Augeron et al., 1992). As gentamicin is unable to penetrate the mucus secreted by this cell line, total association of the bacteria with these cells was measured. The assay was carried out as described earlier but after the initial 3 h incubation the cells were lysed with Triton X-100 and the total number of bacteria that were in association and internalized was determined by viable count.

Motility assay. To test whether variation in invasion corresponded with variation in motility, the following motility studies were carried out. Bacterial strains were grown as described on BASA plates, adjusted to a similar concentration spectrophotometrically, at a wavelength of 600 nm, and 1 µl of the suspension was stabbed into semi-solid media (0.4% Mueller–Hinton agar). Both a test strain and a control strain were included on the same agar plate to avoid plate-to-plate variation. The plates were incubated as described for 48 h at 42°C and the diameter of the halo of growth was measured for each strain. Each strain was tested in triplicate.

Scanning electron microscopy. Specimens were fixed for 1 h in 3% (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), washed in phosphate buffer and post-fixed in 1% (w/v) osmium tetroxide in the same buffer. Specimens were rinsed in six changes of phosphate buffer, dehydrated in ethanol and placed in acetone. Specimens were subjected to critical point drying with liquid carbon dioxide. Dried specimens were fixed to aluminium stubs with silver conductive paint, sputter-coated with gold and examined using a Stereoh-scan S250 MarkIII scanning electron microscope at 10–20 kV.

PCR screening of isolates. PCR screening was conducted on a subset of strains (n=61), selected on the basis of their invasion phenotype to determine the presence of invasion-related genes. PCR primers, as previously published, were used for detection of cadF, iama, virB11 and cadB (Datta et al., 2003). Primers for virB8 and virB9 were derived from the C. jejuni strain 81-176 sequence (accession no. AF226280) (virB8 FWD 5’-GGCATATTCTTTCTTGCACC, virB8 REV 5’-GCCTCTCTTTGTTTGT; virB9 FWD 5’-GTTCTCCAACCTGATCCAAAC, virB9 REV 5’-CTACATACAATAACATCCTCC). In addition to the published invasion-related genes, the presence of another gene, cj0486, was determined since this gene was identified as having a potential role in the invasion of C. jejuni by transposon mutagenesis (Manning et al., 2003b). Primers for gene cj0486 were cj0486 FWD 5’-GATAGAGCATTAAATGTGATG-3’ and cj0486 REV 5’-CCTTAAAGGCCAATCCACCAGGC-3’. Primers were used at a concentration of 10 pmol µl⁻¹ and the pre-prepared PCR mastermix HotStar Taq Polymerase was used for the reactions (Qiagen). PCR conditions were as follows: an initial denaturation step of 95°C for 15 min; 25 cycles of denaturation for 45 s at 95°C, annealing for 45 s at the temperature used by the authors in the above references, or 50°C, 55°C and 58°C for the virB8, virB9 and cj0486 genes, respectively; extension for 90 s at 72°C; followed by extension for 10 min.

MLST. MLST was conducted on all the 61 isolates screened by PCR above using the primers and conditions previously described (Dingle et al., 2001; Manning et al., 2003a).

RESULTS AND DISCUSSION

Invasiveness of C. jejuni strains from poultry and poultry-related environments

Invasiveness of the 74 poultry-associated strains was tested using INT 407 cells and invasion was related to a low-invasive control strain, C. jejuni NCTC 81116, to correct for inter-experimental variation. Invasiveness varied
considerably between the investigated strains, as is shown in Fig. 1. From the distribution profile obtained, three classes of relative invasiveness were defined: hyperinvasive strains are at least 25 times more invasive than the reference strain; high-invasive strains are at least 10 times as invasive; and low invaders are lower than 10 times as invasive as the reference strain. These findings were reproducible between individual experiments (not shown).

**Table 1.** Invasiveness of *C. jejuni* isolates from poultry and the poultry environment (surrounding a broiler house) and human clinical isolates from both blood and faeces

<table>
<thead>
<tr>
<th>Source</th>
<th>No. of strains displaying invasiveness (% per isolation source)</th>
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<tr>
<td></td>
<td>Low</td>
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<tr>
<td>Poultry cloaca</td>
<td>60</td>
</tr>
<tr>
<td>Poultry environment</td>
<td>5</td>
</tr>
<tr>
<td><strong>Poultry-related total</strong></td>
<td><strong>65</strong></td>
</tr>
<tr>
<td>Human faecal</td>
<td>24</td>
</tr>
<tr>
<td>Human blood</td>
<td>8</td>
</tr>
<tr>
<td><strong>Human total</strong></td>
<td><strong>32</strong></td>
</tr>
</tbody>
</table>

Invasive potential of clinical isolates

Campylobacter strains (*n* = 39) isolated from patients with campylobacteriosis (enteritis and/or bacteraemia) also possessed a range of invasion phenotypes from low to hyperinvasive as previously defined (Table 1). However, a higher percentage of human isolates was found to be hyperinvasive (13%) compared to poultry isolates (1%). This difference was statistically significant (*P* = 0.0182). The proportion of low invaders varied very little between human and poultry isolates (82% and 88%, respectively), while of the clinical isolates only 5% were highly invasive (compared to 11% in poultry). The prevalence of hyperinvasive strains from patients with bacteraemia (20%; Table 1) was higher than among the stool isolates (10%) though this was not statistically significant (*P* = 0.3812). Medical records of the cases from which the blood isolates originated showed that 3 of the 10 bacteraemic patients had prior debilitating conditions, such as neutropenia or chronic renal failure, which may have rendered them more susceptible to bacteraemia.
However, at least in some cases, bacteraemia may have been the result of infection with a more invasive strain. Our data support a role for invasion in human disease as a greater proportion of human isolates were hyperinvasive compared with poultry isolates.

Several studies have compared the variation in invasiveness of clinical isolates with that of animal and environmental isolates (Biswas et al., 2000; Fernandez & Trabulsi, 1995; Konkel & Joens, 1989; Manninen et al., 1982; Newell et al., 1985; Tay et al., 1996). Despite a low number of isolates tested in each of these studies, all studies have shown that the prevalence of invasive isolates is higher among clinical isolates than among animal isolates and our data are in accordance with these previous reports.

**Confirmation of invasiveness using alternative cell lines**

It is well recognized that such INT 407-based invasion assays poorly reflect the in vivo situation as a result of their de-differentiated status. There are some cell lines which more closely mimic the differentiated intestinal tract. All six hyperinvasive strains identified within this study were subsequently tested for invasion of Caco-2 cells grown as a monolayer (data not shown). Five out of the six strains were invasive in Caco-2 cells; one was over four times more invasive than the control and the other four had invasion efficiencies greater than 10 times that of the reference strain NCTC 81116, including one human clinical isolate, 01/51, maintaining a hyperinvasive phenotype in this cell line (26 times more invasive than NCTC 81116). The sixth strain had a low-invasive phenotype in Caco-2 cells. Three strains with a low-invasive phenotype were also tested using Caco-2 cells, all of which maintained this phenotype in the alternative cell line (data not shown). The different invasion phenotypes observed using Caco-2 cells may be due to inherent differences in the cell lines used (Friis et al., 2005).

Caco-2 cells under defined culture conditions can also be used to generate polarized and differentiated monolayers. Such organized cell systems are considered models of the intestinal epithelium. The ability of *C. jejuni* to translocate may also be a virulence property (Lee et al., 1986), particularly to enable access to the underlying gut epithelial tissues (Bras & Ketley, 1999; Everest et al., 1992; Konkel et al., 1992). The hyperinvasive puddle isolate (Ex114) and two low-invasive strains (*C. jejuni* NCTC 81116 and a second puddle isolate, Ex323, from the same farm as Ex114) were tested in the translocation model. All three strains possessed the ability to translocate; however, large differences in their efficiencies were measured (Fig. 2). The hyperinvasive puddle isolate was the most efficient at translocating through the monolayer. Approximately 14-fold more bacteria had passed through the monolayer after 4 h compared to the two low-invasive strains, both of which had low levels of translocation. These data support the INT 407 cell invasion data.

![Fig. 2](image-url) Translocation of two *C. jejuni* strains through a monolayer of differentiated Caco-2 cells. The translocated fraction is shown for six time points. White bars, *C. jejuni* 81116; grey bars, *C. jejuni* strain Ex323; diagonal striped bars, *C. jejuni* Ex114.

The hyperinvasive strain Ex114 and the low-invasive reference strain *C. jejuni* NCTC 81116 were also tested for their ability to associate (adhere and invade) with HT29-Cl.16E mucus-secreting cells. NCTC 81116 demonstrated a low association with these cells while the hyperinvasive strain, Ex114, possessed a high association capacity (Fig. 3). Increasing the number of bacteria added to the monolayer did not significantly increase association of NCTC 81116, which was approximately 40-fold lower than that of the hyperinvasive strain.

The finding that selected hyperinvasive and low-invasive strains retained their relative differences in invasiveness in

![Fig. 3](image-url) Association of two *C. jejuni* strains to a monolayer of mucus-secreting cells. The fraction of associated bacteria, expressed as percentage of inoculum, is represented for triplicate experiments. Triangles, *C. jejuni* 81116; squares, *C. jejuni* strain Ex114.
these alternative tissue culture models provides supporting evidence that the INT 407 cell assay is a suitable surrogate and objective measure of the invasion potential of *C. jejuni*.

**The hyperinvasive phenotype is not due to enhanced adhesion or motility**

Scanning electron microscopy was used to visualize the number of bacteria of strains NCTC 81116 and Ex114 adhered to the mucous layer covering HT29-Cl.16E cells. No detectable differences in the abilities of these strains to adhere to the mucus were observed (data not shown). This suggests that the increased association demonstrated by the hyperinvasive strain to the HT29-Cl.16E cells is attributable to increased invasiveness, rather than to more efficient attachment.

The motility of the three *C. jejuni* strains representing the low and hyperinvasive phenotypes (Ex114, Ex323 and NCTC 81116) was determined used semi-solid motility agar. All tested strains were fully motile, with a diameter of growth varying between 5.0 and 5.8 cm. As there was little difference in motility between hyper- and low-invasive strains, it seems unlikely that motility influenced the invasion capacity.

**Prevalence of known virulence-related genes**

Attempts were then made to correlate the invasion phenotype to particular genetic characteristics. The presence of six previously reported invasion-associated genes was determined by PCR in 62 isolates (Table 2), selected to represent all sources and invasion phenotypes recognized. The three reference strains were also included. The genes studied included *cadF*, *ciaB*, *iamA*, *virB8*, *virB9* and *virB11*. In addition, gene *cj0486* was included as it had been identified as potentially related to invasion by transposon mutagenesis (Manning et al., 2003b).

The results of the PCRs are given in Table 2. The reference strain NCTC 11168 was found to be positive for all PCR reactions except for the *iamA* and the *virB* genes. Previous studies reported that NCTC 11168 does not contain the pVir plasmid (Bacon et al., 2000) and so absence of the *virB* genes was to be expected; however, NCTC 11168 was thought to contain the *iamA* gene. Comparison of the *iamA* gene sequence from NCTC 11168 with that previously identified in an invasive strain (Carvalho et al., 2001), from which strain the *iamA* PCR primers were derived, suggested that lack of conservation of the primer sequences could explain the absence of a PCR product from NCTC 11168. The other two reference strains NCTC 81116 and 81176 also lacked the *iamA* gene as well as *cj0486* as was expected from their respective genome sequences (Hofreuter et al., 2006). As expected, strain 81176 possessed the three pVir-derived genes. Although these three reference strains were originally isolated from clinical cases, their phenotypes may have changed over time as a consequence of multiple laboratory passages (Gaynor et al., 2004). However, because the genome sequences are known for all three strains, this provides evidence of the validity of the PCR tests.

Only *cadF* was present in all isolates tested regardless of invasive phenotype or isolation source. This confirmed previous studies of the prevalence of *cadF* (Datta et al., 2003; Dorrell et al., 2001; Müller et al., 2006; Pearson et al., 2003; Zheng et al., 2006). In contrast, the other genes tested varied in presence from 82 % (*ciaB*) to 2 % (*virB9*) of strains. The observed frequency is summarized for the three invasion potential classes and for the two main isolation sources (poultry and humans) in Table 3. None of the hyperinvasive strains possessed all of the genes investigated by PCR. The presence of the *cj0486* gene weakly correlated with invasive phenotype, in that 73 % of highly invasive strains were positive against 62 % of the low-invasive strains. In contrast, the *ciaB* gene showed a negative correlation with invasiveness as it was more common in low-invasive strains than in highly and hyperinvasive strains (Table 3). This finding contrasts with a prevalence approaching 100 % for this gene, reported previously (Datta et al., 2003; Dorrell et al., 2001; Müller et al., 2006; Pearson et al., 2003; Zheng et al., 2006). Considering these two genes together, a significant correlation was found (*P*=0.0064) for *ciaB* presence combined with *cj0486* absence: this pattern was found in 33 % of low invaders but only in 12 % of the combined highly or hyperinvasive strains. Conversely, *ciaB* absence combined with *cj0486* presence was found in 29 % of high or hyperinvasive strains, but only in 2 % of the low invaders (Table 3). The observed correlations between presence or absence of *cj0486* and *ciaB* may or may not be causative; the genes may either encode proteins that enhance or reduce invasiveness, or they may be genetic markers for such a phenotype without encoding a product functional in invasion. That protein CiaB is produced upon contact with host cells (Rivera-Amill & Konkel, 1999) suggests a functional relationship with cell contact for this gene. However, the observed correlation described in this study suggests that this protein may limit invasion rather than promote it. Interestingly, mutagenesis of *cj0486* in the hyperinvasive *C. jejuni* strain 01/51 resulted in a mutant with a reduced invasion potential of just 10 % of that of the wild-type (Manning et al., 2003b), indicating a functional, positive relationship between the *cj0486* gene product and invasion. The gene *cj0486* is annotated as a putative sugar transporter in NCTC 11168 (Parkhill et al., 2000), with homology to *fucP*, encoding β-fucose permease, in *C. jejuni* strain RM1221 (Fouts et al., 2005). It is likely that such a sugar transporter is located in the inner membrane and may be linked to chemotaxis as β-fucose is a reported chemotacticant of *C. jejuni* (Hugdahl et al., 1988). It should be noted that the correlation with invasiveness cannot be explained by differences in chemotaxis or motility, as the invasion assay included centrifugation to overcome such differences, and a correlation between invasiveness and motility was not found, as discussed above.
### Table 2. MLST characterization and prevalence of invasion-related genes amongst selected C. jejuni isolates (n=62) of human, poultry and poultry-related sources

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<tr>
<th>Strain</th>
<th>Source</th>
<th>Clonal complex*</th>
<th>ST complex</th>
<th>Invasion phenotype</th>
<th>cadF</th>
<th>ciaB</th>
<th>cj0486</th>
<th>iamA</th>
<th>virB11</th>
<th>virB8</th>
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</table>

... (remaining entries continue)
As the three genes virB11, virB8 and virB9 have been reported to encode type IV secretion proteins (Bacon et al., 2002), it seemed likely that these genes may play a role in cell contact or invasion. Indeed, mutational analysis of virB11 (Bacon et al., 2002) and virB9 (Bacon et al., 2000) has indicated a significant role in invasion for these two genes. Mutation of the virB11 gene resulted in an 11-fold reduction in invasion, and reduced virulence in the ferret model. The virB11 gene was present in only 5 of the 62 (8 %) isolates in our study, which is consistent with other reports (Bacon et al., 2000; Datta et al., 2003). These 5 strains included one of the hyperinvasive strains and one of the highly invasive strains; however, the numbers involved are too small to draw any conclusions about association with invasiveness. The three genes encoded by pVir were only rarely found (Table 2), presumably reflecting the low prevalence of pVir, and usually (but not always) detected together. Genes virB8 and virB9 were even less prevalent (3 % and 2 %, respectively) than virB11, indicating diversity in the pVir genetic content.

Surprisingly, iamA was not found in human isolates but was present in 31 % of poultry isolates (Table 3). Similar observations have been reported (Rozynek et al., 2005) in Poland, where 1.6 % of isolates from Polish children but 55 % of chicken isolates possessed this gene. In contrast, a study testing only 11 strains from various sources (human enteritis, milk and bovine sources) detected the iamA gene in all strains (Müller et al., 2006).

Clearly there are considerable discrepancies between studies attempting to correlate invasiveness with genomic content. One possible explanation is the inherent limitations of gene detection using PCR. False-negative results can be expected when a gene is polymorphic and the designed PCR primers do not detect the presence of particular orthologues. On the other hand, a gene may be present but mutated and non-functional or not expressed, leading to a lack of correlation with phenotype. However, it seems much more likely that invasiveness is the result of the interplay of numerous genes, some of which may be

---

**Table 2. cont.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
<th>Clonal complex*</th>
<th>ST complex</th>
<th>Invasion phenotype</th>
<th>cadF</th>
<th>ciaB</th>
<th>cj0486</th>
<th>iamA</th>
<th>virB11</th>
<th>virB8</th>
<th>virB9</th>
<th>ciaB +</th>
<th>cj0486 -</th>
</tr>
</thead>
<tbody>
<tr>
<td>C69/7</td>
<td>Poultry</td>
<td>443</td>
<td>393</td>
<td>Low</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C69/2</td>
<td>Poultry</td>
<td>443</td>
<td>393</td>
<td>Low</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C12/11</td>
<td>Poultry</td>
<td>658</td>
<td>908</td>
<td>Low</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ext114</td>
<td>Puddle</td>
<td>682</td>
<td>914</td>
<td>Hyper</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Ext403</td>
<td>Puddle</td>
<td>45</td>
<td>45</td>
<td>Low</td>
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<td>-</td>
<td>-</td>
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<td>-</td>
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<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**Reference strains**

<p>| | | | | | | | | | | | | | |</p>
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</thead>
<tbody>
<tr>
<td>11168</td>
<td>Human</td>
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<td>21</td>
<td>High</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>81176</td>
<td>Human</td>
<td>42</td>
<td>913</td>
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<tr>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
</tr>
</tbody>
</table>

*U/A, Unassigned to any clonal complex when database last searched.

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**Table 3. Presence of predicted invasion-related genes in C. jejuni isolates with varying invasion potentials (top) and isolation source (bottom)**

<table>
<thead>
<tr>
<th>Invasion potential</th>
<th>cadF</th>
<th>ciaB</th>
<th>cj0486</th>
<th>iamA</th>
<th>virB11</th>
<th>virB8</th>
<th>virB9</th>
<th>ciaB +</th>
<th>cj0486 -</th>
<th>ciaB -</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyper (n=6)</td>
<td>6 (100)</td>
<td>3 (50)</td>
<td>4 (67)</td>
<td>0 (0)</td>
<td>1 (17)</td>
<td>1 (17)</td>
<td>0 (0)</td>
<td>1 (17)</td>
<td>2 (33)</td>
<td></td>
</tr>
<tr>
<td>High (n=11)</td>
<td>11 (100)</td>
<td>6 (55)</td>
<td>8 (73)</td>
<td>2 (18)</td>
<td>1 (9)</td>
<td>1 (4)</td>
<td>1 (4)</td>
<td>1 (9)</td>
<td>3 (27)</td>
<td></td>
</tr>
<tr>
<td>Low (n=45)</td>
<td>45 (100)</td>
<td>42 (93)</td>
<td>28 (62)</td>
<td>9 (20)</td>
<td>3 (7)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>15 (33)</td>
<td>1 (2)</td>
<td></td>
</tr>
<tr>
<td>Total (n=62)</td>
<td>62 (100)</td>
<td>51 (82)</td>
<td>40 (65)</td>
<td>11 (18)</td>
<td>5 (8)</td>
<td>2 (3)</td>
<td>1 (2)</td>
<td>17 (27)</td>
<td>6 (10)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Isolation source</th>
<th>cadF</th>
<th>ciaB</th>
<th>cj0486</th>
<th>iamA</th>
<th>virB11</th>
<th>virB8</th>
<th>virB9</th>
<th>ciaB +</th>
<th>cj0486 -</th>
<th>ciaB -</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poultry* (n=35)</td>
<td>35 (100)</td>
<td>29 (83)</td>
<td>23 (66)</td>
<td>11 (31)</td>
<td>3 (9)</td>
<td>1 (3)</td>
<td>0 (0)</td>
<td>8 (24)</td>
<td>2 (6)</td>
<td></td>
</tr>
<tr>
<td>Human* (n=27)</td>
<td>27 (100)</td>
<td>22 (81)</td>
<td>17 (63)</td>
<td>0 (0)</td>
<td>2 (7)</td>
<td>1 (4)</td>
<td>1 (4)</td>
<td>9 (33)</td>
<td>4 (17)</td>
<td></td>
</tr>
<tr>
<td>Total (n=62)</td>
<td>62 (100)</td>
<td>51 (82)</td>
<td>40 (65)</td>
<td>11 (18)</td>
<td>5 (8)</td>
<td>2 (3)</td>
<td>1 (2)</td>
<td>17 (27)</td>
<td>6 (10)</td>
<td></td>
</tr>
</tbody>
</table>

*Strains isolated from a puddle close to a poultry farm are included here as ‘poultry’ strains. Reference strains that were originally isolated from human clinical cases are included here as ‘human’.

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http://jmm.sgmjournals.org
Distribution of strains with known invasion potential amongst the MLST clonal complexes

The strains selected for this study were, to the best of our knowledge, not epidemiologically related. Nevertheless, we determined the phylogenetic relationship of all 62 isolates selected above by MLST to assess whether the hyperinvasive strains were related. MLST analysis showed that the isolates were representative of 17 already-established sequence type (ST) complexes (http://pubmlst.org/campylobacter/) (Table 2). The ST21 complex was the most highly represented among the 62 isolates tested, with 22 strains belonging to this complex. This is in line with previous reports in which this complex is highly represented within the C. jejuni population (Dingle et al., 2001; Manning et al., 2003a). The remaining strains belonged to at least 16 ST complexes, with each complex represented by up to five isolates within the 62 strains tested. There were also four isolates with sequence types that are so far unassigned to any ST complex (database last searched August 2007). Four of the six hyperinvasive strains were part of the ST21 complex (three were ST21 and one was ST916). Of the other two hyperinvasive strains, one was ST914 (Ex114), which is part of the ST682 complex, and one was ST677 (0104), which is part of the ST677 complex. Interestingly, the ST682 complex contains a number of isolates from wild bird sources (http://pubmlst.org/campylobacter/), suggesting that Ex114, which was isolated from a puddle on a farm, may well have originated from a wild bird, rather than a poultry source.

Overall, these results show that hyperinvasiveness is not restricted to strains belonging to a particular ST complex. All ST complexes represented in this study, except three (ST354, ST677 and ST682 complex), contained isolates with a low-invasion potential, suggesting that this phenotypic group, too, is genetically diverse. Using Pearson’s chi-square test, no association was found between ST complex and invasion phenotype and a similar proportion (88%) was also found in poultry isolates. Attempts to correlate the hyper- or high-invasiveness with the presence of putative invasion-associated genes indicated an association with the ciaB− cj0486+ genotype but the molecular basis of this observation needs to be studied further. Overall, these results suggest that invasiveness in the host is a consequence of the interaction of multiple bacterial factors. However, it must also be considered that the outcome of infection with C. jejuni is highly dependent on the physiological and immunological status of the host.

ACKNOWLEDGEMENTS

This work was funded by the Department for Environment, Food and Rural Affairs (Defra), and the Food Standards Agency (FSA), UK. The authors would like to thank Jenny Frost (previously at HPA, Colindale) and Dorcas Hanson (Microbiology, Kingston Hospital) for kindly providing the human bacterial isolates.

REFERENCES


