The Identification of Hyperinvasive *Campylobacter jejuni* Strains in Poultry and Human Clinical Isolates

Running Title: Hyperinvasive *Campylobacter jejuni* strains

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SUMMARY

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 one hundred and thirteen *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 INT407 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 7 genes previously reported to be associated with virulence. Most of these genes did not correlate with invasiveness, but gene Cj0486 was weakly overrepresented, and a negative correlation was observed for the gene *ciaB*. This trend was stronger when the two genes were analysed together, thus *ciaB*$^{-}$Cj0486^{+} was overrepresented 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 industrialised 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 colonisation 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 colonisation 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 (Fauchere *et al.*, 1986; Newell & Pearson, 1984), INT407 (Wassenaar *et al.*, 1991) and Caco2 (Everest *et al.*, 1992; Russell & Blake, 1994) cells. Several invasion-related genes have been proposed as a consequence of such studies. The *fla*A gene has been known for some time to be involved with invasion (Wassenaar *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 *cad*F, 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-Gruszczynska *et al.*, 2007); *cia*B, 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 *vir*B8, *vir*B9 and *vir*B11, which are present on the pVir plasmid, first identified in strain 81-176 (Bacon *et al.*, 2000; Bacon *et al.*, 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 (Abu Oun *et al.*, 2005; Bang *et al.*, 2001; Bang *et al.*, 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 were 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-Gruszczynska *et al*., 2007; Manning *et al.*, 2003a) suggest either that not all poultry *Campylobacter* strains possess the pathogenic potential to cause disease in man, or that 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 6 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. The remaining ten strains were isolated from the blood of hospitalised 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 ^{o}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, Basingstoke, UK) and Actidione (50 μgml^{-1}) (BASA) and grown under microaerobic conditions at 42 ^{o}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 ^{o}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 (Elsinghorst, 1994). INT407 cells, and later Caco2 cells, were obtained from the European Collection of Animal Cell Cultures (ECACC, CAMR, Porton Down, Salisbury). Note that it is now generally recognised that the INT407 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 Eagles Minimal Essential Medium (EMEM, Sigma) supplemented with 10 % (v/v) foetal bovine serum, 2 mM L-glutamine, 1 % (v/v) non-essential amino acids (NEAA) and 50 μg ml^{-1} gentamicin (complete media, all from Sigma) at 37 ^{o}C in a 5% CO_2 atmosphere. Confluent cultures were trypsinised, the cells counted and suspended in the above growth medium at a concentration of 2x10^{5} cells ml^{-1}. A 24 well tissue culture tray was seeded with 1ml per well and incubated at 37 ^{o}C for 48 h to establish confluent monolayers (approx. 5 x 10^{5} cells per well for INT407 cells; or 3 x 10^{5} cells per well for Caco2 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 log phase campylobacters were harvested by centrifugation at 2100 g at room temperature and re-suspended in 0.1 M phosphate buffered saline pH 7.2 (PBS). Further dilutions into prewarmed EMEM were carried out to give a bacterial 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 INT407 cells in 1 ml of maintenance medium. Tissue culture plates were centrifuged at 450 x 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 hours to allow the bacteria to invade the cells. After washing three times with HBSS, 2 ml of medium containing 250 µg ml^{-1} gentamicin was placed in each well and incubated for a further 2 hours to kill extracellular bacteria. Following incubation the monolayers were washed 3 times with HBSS and lysed with 1 % (v/v) Triton X-100 (Sigma) in PBS for 10 minutes 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 INT407 or Caco2 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 standardisation of procedures, inter-experimental variation remained considerable. Nevertheless, particular strains were consistently found low or highly invasive. One low invasive strain, *C. jejuni* NCTC 81116, was used as an internal control strain in all experiments and 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 hyper-invasive, highly invasive and low invasive strains were chosen as described in the Results section. 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 INT407 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 polarised 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 above but after the initial three hour incubation the cells were lysed with Triton X-100 and the total number of bacteria that were in association and internalised 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 600nm, and 1 μl of the suspension was stabbed into semi-solid media (0.4% Muller 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 hours at 42 °C and the diameter of the halo of growth measured for each strain. Each strain was tested in triplicate.

Scanning electron microscopy. Specimens were fixed for 16 hours in 3% (v/v) gluteraldehyde in 0.1M phosphate buffer, pH 7.4, washed in phosphate buffer and post fixed in 1% (w/v) osmium tetraoxide 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 Stereo-scan S250 MarkIII scanning EM 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 *cad*F *iam*A, *vir*B11 and *cia*B (Datta *et al.*, 2003), Primers for *vir*B8 and *vir*B9 were derived from the *C. jejuni* strain 81-176 sequence (accession number AF226280) (*vir*B8 FWD 5'-GCCATTACTTTTCTTGCCCC, *vir*B8 REV: 5'-CGCTCCTTTCGTTTGTTGTG; *vir*B9 FWD 5'-GTTCCTAACCCTAATGCAAAC, *vir*B9 REV 5'- CTACACATACATAACTATCTCC). 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' GATAGAGCATTAAATTGGGATG 3', and Cj0486 REV 5' CCTATAAAGCCATACCAAGCC 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, Crawley UK). PCR conditions were as follows: an initial denaturation step of 95 °C for 15 min, 25 cycles of: denaturation for 45 sec at 95 °C; annealing for 45 sec at the temperature used by the authors in the above references, or 50 °C, 50 °C and 58 °C for the *virB8*, *virB9* and Cj0486 genes respectively; extension for 90 sec at 72 °C; followed by extension for 10 min.

Multilocus sequence typing (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 INT407 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 Figure 1. From the distribution profile obtained, three classes of relative invasiveness were defined. Strains that were at least 25 times as invasive than NCTC81116 were classified hyper-invasive and strains more than 10 times as invasive were classified as highly invasive. Below this level, strains were classified as low invaders. The cut-off level of 10 times the reference strain was chosen to divide low from high invaders since the distribution of invasiveness seems to drop at this level (as seen in Figure 1); below this cut-off invasion steadily decreases. The vast majority of strains (88 %) were thus classified as low invaders (between 0.0006 % to 0.3 % of the bacterial inoculum internalised). Although these invasion efficiencies are low in comparison to those reported for invasive *Salmonella* (Finlay & Falkow, 1990; Huang *et al.*, 1998), *Shigella* (Honma *et al.*, 2000), *Yersinia* (Pepe & Miller, 1993) and *E. coli* (Boudeau *et al.*, 1999) they are similar to those previously reported for *C. jejuni* (Biswas *et al.*, 2000; Everest *et al*., 1992; Konkel & Joens, 1989; Tay *et al.*, 1996). The number of strains per isolation source in each invasion class is summarized in Table 1. Of the poultry-related isolates, 11 % (8 of 74 strains) were classed as highly invasive (between 0.3% and 1% of the inoculum internalised, whereas only EX114, a strain isolated from a puddle, possessed the hyper-invasive phenotype (1.2 % of the bacterial inoculum internalised). Interestingly, the other strains that were isolated at the same time from puddles surrounding the same poultry house were all found to be low invaders.

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 neutropoenia 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 those of animal and environmental isolates (Biswas e*t al*., 2000; Fernandez & Trabulsi, 1995; Konkel & Joens, 1989; Manninen *et al.*, 1982; Newell *et al.*, 1985; Tay e*t 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 animal isolates and our data are in accordance to these previous reports.

Confirmation of invasiveness using alternative cell lines. It is well recognised that such INT407-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 Caco2 cells grown as a monolayer (data not shown). Five out of the six strains were invasive in Caco2 cells, one of which was over 4 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 Caco2 cells. Three strains with a low invasive phenotype were also tested using Caco2 cells, all of which maintained this phenotype in the alternative cell line (data not shown). The different invasion phenotypes observed using Caco2 cells may be due to inherent differences in the cell lines used (Friis e*t al*., 2005).

Caco-2 cells under defined culture conditions can also be used to generate polarised and differentiated monolayers. Such organised 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 e*t al*., 1992; Konkel e*t 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 (Figure 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 four hours compared to the two low invasive strains, both of which had low levels of translocation. These data support the INT407 cell invasion data.

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 (Figure 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 the hyperinvasive strain.

The finding that selected hyperinvasive and low invasive strains retained their relative differences in invasiveness in these alternative tissue culture models provides supporting evidence that the INT407 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 visualise 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 e*t al*., 2003b).

The results of the PCRs are given in Table 2. The reference strain NCTC 11168 was found positive for all PCR reactions except *iamA* and the *virB* genes. Previous studies reported that NCTC 11168 does not contain the pVir plasmid (Bacon e*t 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, e*t 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 *iam*A gene as well as Cj0486 as was expected from their respective genome sequences (Hofreuter e*t al*., 2007). 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 e*t al*., 2003; Dorrell *et al.*, 2001; Müller e*t al*., 2006; Pearson *et al.*, 2003; Zheng e*t al*., 2006). In contrast the other genes tested varied in presence from 82 % (*cia*B) to 2 % (*virB9*) of strains. The observed frequency is summarised 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. 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 *cia*B gene showed a negative correlation to invasiveness as it was more common in low invasive strains compared to highly and hyperinvasive strains (Table 3). This finding contrasts with a prevalence approaching 100% for this gene, reported previously (Datta e*t al*., 2003; Dorrell e*t al*., 2001; Müller e*t al*., 2006; Pearson e*t al*., 2003; Zheng e*t 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 *cia*B 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 and 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 e*t 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 NCTC11168 (Parkhill e*t al*., 2000), with homology to *fuc*P, encoding L-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 L-fucose is a reported chemoattractant 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.

As the three genes *vir*B11, *vir*B8 and *vir*B9 have been reported to encode type IV secretion proteins (Bacon e*t al*., 2002) it seemed likely that these genes may play a role in cell contact or invasion. Indeed mutational analysis of *vir*B11 (Bacon e*t al*., 2002) and *vir*B9 (Bacon, e*t al*., 2000) have indicated a significant role in invasion for these two genes. Mutation of the *vir*B11 gene resulted in an 11-fold reduction in invasion, and reduced virulence in the ferret model. The *vir*B11 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 e*t 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 *virB*8 and *virB9* were even less prevalent (3 % and 2 % respectively) than *virB11*, indicating diversity in the pVir genetic content.

Surprisingly *iam*A 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 *iam*A gene in all strains (Müller e*t 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 orthologs. 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 redundant and others which may be interchangeable. More comprehensive studies in the future using DNA microarrays may more accurately identify correlations between genotype and phenotype.

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 strains selected above by MLST, to assess if 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 e*t al*., 2001; Manning e*t al*., 2003a). The remaining strains belonged to at least 16 ST complexes; with each complex represented by up to 5 isolates within the 62 strains tested. There were also 4 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 (3 were ST21 and one was ST916). Of the other two hyper-invasive 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

414 contains a number of isolates from wild bird sources (http://pubmlst.org/campylobacter/), suggesting that
415 Ex114, which was isolated from a puddle on a farm, may well have originated from a wild bird, rather than a
416 poultry source.
417
418 Overall these results show that hyperinvasiveness is not restricted to strains belonging to a particular ST
419 complex. All ST complexes represented in this study, excepting three (ST354, ST677, and ST682 complex)
420 contained isolates with a low invasion potential, suggesting that this phenotypic group, too, is genetically
421 diverse. Using Pearson's Chi-square test, no association was found between ST complex and invasion
422 potential. The majority of isolates belonging to the most common ST21 complex possessed *cad*F (100 %),
423 Cj486 (100%) and *ciaB* (90 %) but only a minority possessed of cases *iam*A (13 %) or *vir*B11 (13%). None of
424 the ST21 isolates were *ciaB*$^{+}$Cj486^{-} and only 14 % were *cia*B^{-}Cj486^{+}. These findings suggest that ST21
425 complex isolates are no more likely to be invasive than those of other clonal complexes and in fact the
426 combination *cia*B^{-}Cj486^{+}, overrepresented in low invasive strains, is also overrepresented in ST21 isolates
427 compared to the total number of isolates.
428
429 In conclusion, using a relatively large number of isolates (n=113), we have shown that *C. jejuni* strains
430 exhibit a range of invasion phenotypes, and that hyper-invasiveness is more frequently observed among
431 human clinical isolates. Nevertheless, 82 % of clinical isolates have a low invasion phenotype and a similar
432 proportion (88 %) was also found in poultry isolates. Attempts to correlate the hyper- or high-invasiveness
433 with the presence of putative invasion-associated genes indicated an association with the *ciaB*$^{-}$Cj0486^{+}
434 genotype but the molecular basis of this observation needs to be studied further. Overall these results
435 suggest that invasiveness in the host is a consequence of the interaction of multiple bacterial factors.
436 However, it must also be considered that the outcome of infection with *C. jejuni* is highly dependant on the
437 physiological and immunological status of the host.
438
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633 **Table 1:** The invasiveness of *C. jejuni* isolates from poultry, the poultry environment (surrounding a broiler
634 house) and human clinical isolates from both blood and faeces.
635

Source	Number of strains displaying invasiveness† (% per isolation source)			
	Low	**High**	**Hyper**	**Total**
Poultry cloaca	60 (88%)	8 (12%)	0	68
Poultry environment	5 (83%)	0	1 (17%)	6
Poultry-related total	**65 (88%)**	**8 (11%)**	**1 (1%)**	**74**
Human faecal	24 (83%)	2 (7%)	3 (10%)	29
Human blood	8 (80%)	0	2 (20%)	10
Human total	**32 (82%)**	**2 (5%)**	**5 (13%)**	**39**

636
637 † Strains were grouped into the three invasion phenotypic groups according to their invasiveness compared
638 to the control strain *C. jejuni* 81116 as rationalised in the text. Isolates were classified as hyper-invasive,
639 high and low invaders using the criteria explained in the text and as shown in Figure 1.
640
641
642

643 **Table 2:** MLST characterisation and prevalence of invasion-related genes amongst selected *C. jejuni*

644 isolates (n=62) of human, poultry and poultry-related sources.

Strain	Source	Clonal Complex	ST complex	Invasion Phenotype	*cadF*	*ciaB*	Cj0486	*iamA*	*vir*B11	*vir*B8	*vir*B9	*ciaB+ Cj0486*$^-$	*ciaB*$^-$ *Cj0486+*
01 10	Human	21	21	Hyper	+	+	+	-	-	-	-		✓
01 35	Human	21	21	Hyper	+	-	+	-	-	-	-		✓
01 51	Human	21	21	Hyper	+	-	+	-	-	-	-		✓
01 41	Human	21	916	Hyper	+	+	+	-	-	-	-		
01 04	Human	677	677	Hyper	+	+	-	-	-	-	-	✓	
01 37	Human	21	21	High	+	-	+	-	-	-	-		✓
01 38	Human	354	354	High	+	-	+	-	-	-	-		✓
01 50	Human	21	19	Low	+	+	+	-	-	-	-		
01 39	Human	21	53	Low	+	+	+	-	-	-	-		
01 40	Human	21	53	Low	+	+	+	-	-	-	-		
01 42	Human	21	104	Low	+	+	+	-	-	-	-		
01 33	Human	21	943	Low	+	+	+	-	-	-	-		
01 07	Human	22	22	Low	+	+	-	-	-	-	-	✓	
01 32	Human	22	22	Low	+	-	-	-	-	-	-		
01 11	Human	45	137	Low	+	+	-	-	-	-	-	✓	
01 30	Human	45	45	Low	+	+	-	-	-	-	-	✓	
01 48	Human	48	48	Low	+	+	+	-	-	-	-		
01 09	Human	52	52	Low	+	+	+	-	-	-	-		
01 52	Human	52	775	Low	+	+	+	-	-	-	-		
01 08	Human	61	61	Low	+	+	-	-	-	-	-	✓	
01 43	Human	257	257	Low	+	+	+	-	-	-	-		
01 36	Human	354	324	Low	+	+	+	-	-	-	-		
01 05	Human	508	508	Low	+	+	-	-	-	-	-	✓	
01 06	Human	677	677	Low	+	+	-	-	+	-	-	✓	
C322/12	Poultry	U/A	436	High	+	-	+	-	-	-	-		✓
C423/5	Poultry	U/A	942	High	+	-	-	+	-	-	-		
C423/7	Poultry	U/A	942	High	+	-	-	+	-	-	-		
C153/1	Poultry	21	21	High	+	+	+	-	-	-	-		
C223/10	Poultry	21	21	High	+	+	+	-	-	-	-		
C153/4	Poultry	52	63	High	+	+	+	-	-	-	-		
C181/12	Poultry	52	910	High	+	+	+	-	-	-	-		
C272/11	Poultry	U/A	586	Low	+	+	-	-	-	-	-	✓	
C2/2	Poultry	21	21	Low	+	+	+	-	-	-	-		
C201/8	Poultry	21	21	Low	+	+	+	-	-	-	-		
C2/3	Poultry	21	21	Low	+	+	+	+	-	-	-		
C13/4	Poultry	21	21	Low	+	+	+	-	+	-	-		
C39/10	Poultry	21	44	Low	+	+	+	+	-	-	-		
C1/1	Poultry	21	53	Low	+	+	+	-	-	-	-		
C3/4	Poultry	21	104	Low	+	+	+	-	+	-	-		
C27/7	Poultry	21	262	Low	+	+	+	-	-	-	-		
C1/2	Poultry	21	489	Low	+	+	+	+	-	-	-		
C216/11	Poultry	42	911	Low	+	-	+	+	-	-	-		✓
C187/5	Poultry	45	45	Low	+	+	-	+	-	-	-	✓	

Strain	Source	Clonal Complex	ST complex	Invasion Phenotype	*cadF*	*cia*B	Cj0486	*iamA*	*vir*B11	*vir*B8	*vir*B9	*ciaB+ Cj0486*$^-$	*ciaB*$^-$ *Cj0486+*
C4/1	Poultry	48	475	Low	+	+	+	-	-	-	-		
C4/6	Poultry	48	475	Low	+	+	+	-	-	-	-		
C1/8	Poultry	49	907	Low	+	+	+	+	-	-	-		
C70/6	Poultry	49	909	Low	+	+	+	-	-	-	-		
C196/14	Poultry	49	915	Low	+	+	-	+	-	-	-	✓	
C181/2	Poultry	52	52	Low	+	+	+	+	-	-	-		
C27/14	Poultry	257	257	Low	+	+	+	-	-	-	-		
C35/4	Poultry	283	267	Low	+	+	-	-	-	-	-	✓	
C39/12	Poultry	283	267	Low	+	+	-	-	-	-	-	✓	
C110/4	Poultry	433	433	Low	+	+	-	-	-	-	-	✓	
C148/2	Poultry	433	433	Low	+	+	-	+	-	-	-	✓	
C69/7	Poultry	443	393	Low	+	+	+	-	-	-	-		
C69/2	Poultry	443	393	Low	+	+	+	-	-	-	-		
C12/11	Poultry	658	908	Low	+	+	-	-	-	-	-	✓	
Ex114	Puddle	682	914	Hyper	+	-	-	-	+	+	-		
Ex403	Puddle	45	45	Low	+	-	-	-	-	-	-		
Reference strains													
11168	Human	21	21	High	+	+	+	-	-	-	-		
81176	Human	42	913	High	+	+	-	-	+	+	+	✓	
81116	Human	283	267	Low	+	+	-	-	-	-	-	✓	

645
646

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

	Number of isolates (%) positive for each gene by PCR								
Invasion Potential	***cadF***	***ciaB***	**Cj0486**	***iamA***	***virB11***	***virB8***	***virB9***	***ciaB*$^+$ Cj486$^-$**	***ciaB*$^-$ Cj486$^+$**
Hyper	6	3	4	0	1	1	0	1	2
(n=6)	(100%)	(50%)	(67%)	(0%)	(17%)	(17%)	(0%)	(17%)	(33%)
High	11	6	8	2	1	1	1	1	3
(n=11)	(100%)	(55%)	(73%)	(18%)	(9%)	(4%)	(4%)	(9%)	(27%)
Low	45	42	28	9	3	0	0	15	1
(n=45)	(100%)	(93%)	(62%)	(20%)	(7%)	(0%)	(0%)	(33%)	(2%)
Total	62	51	40	11	5	2	1	17	6
(n=62)	(100%)	(82%)	(65%)	(18%)	(8%)	(3%)	(2%)	(27%)	(10%)
	Number of isolates (%) positive for each gene by PCR								
Isolation Source	***cadF***	***ciaB***	**Cj0486**	***iamA***	***virB11***	***virB8***	***virB9***	***ciaB*$^+$ Cj486$^-$**	***ciaB*$^-$ Cj486$^+$**
Poultry†	35	29	23	11	3	1	0	8	2
(n=35)	(100%)	(83%)	(66%)	(31%)	(9%)	(3%)	(0%)	(24%)	(6%)
Human†	27	22	17	0	2	1	1	9	4
(n=27)	(100%)	(81%)	(63%)	(0%)	(7%)	(4%)	(4%)	(33%)	(17%)
Total	62	51	40	11	5	2	1	17	6
(n=62)	(100%)	(82%)	(65%)	(18%)	(8%)	(3%)	(2%)	(27%)	(10%)

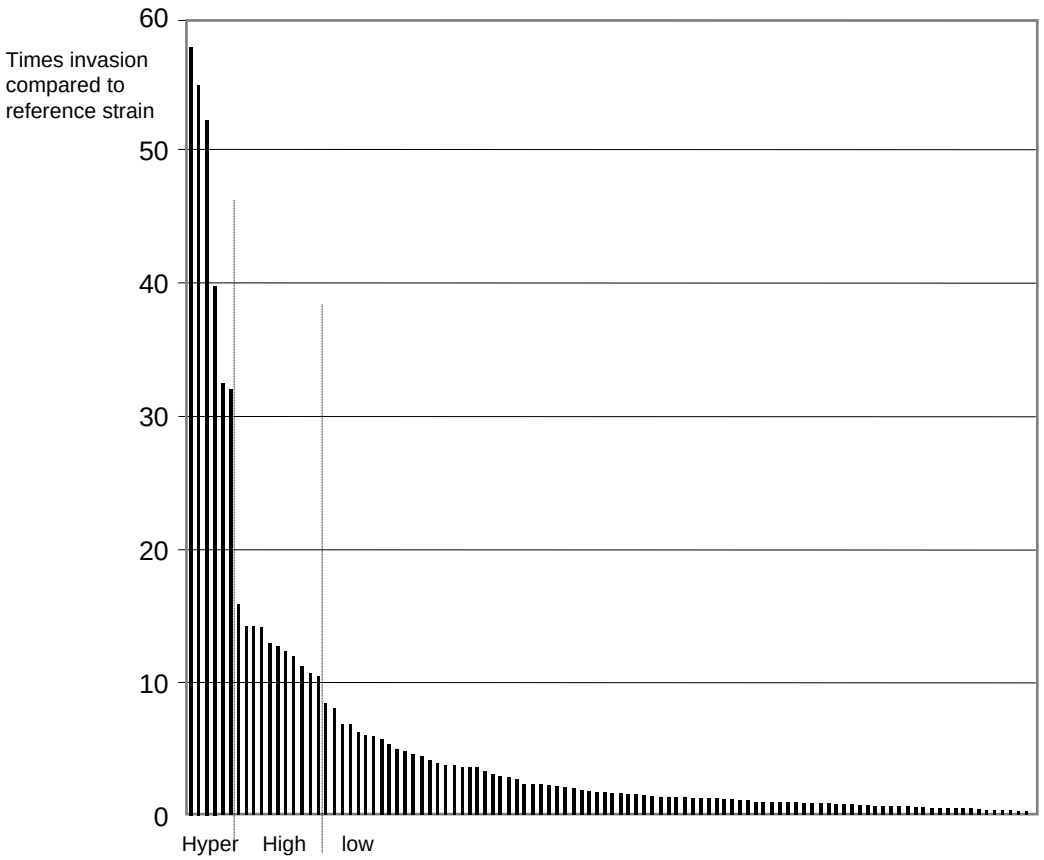
† Strains isolated from a puddle close to a poultry farm are included here as 'poultry' strains. Reference strains that were originally isolated from human clinical cases are included here as 'human'.

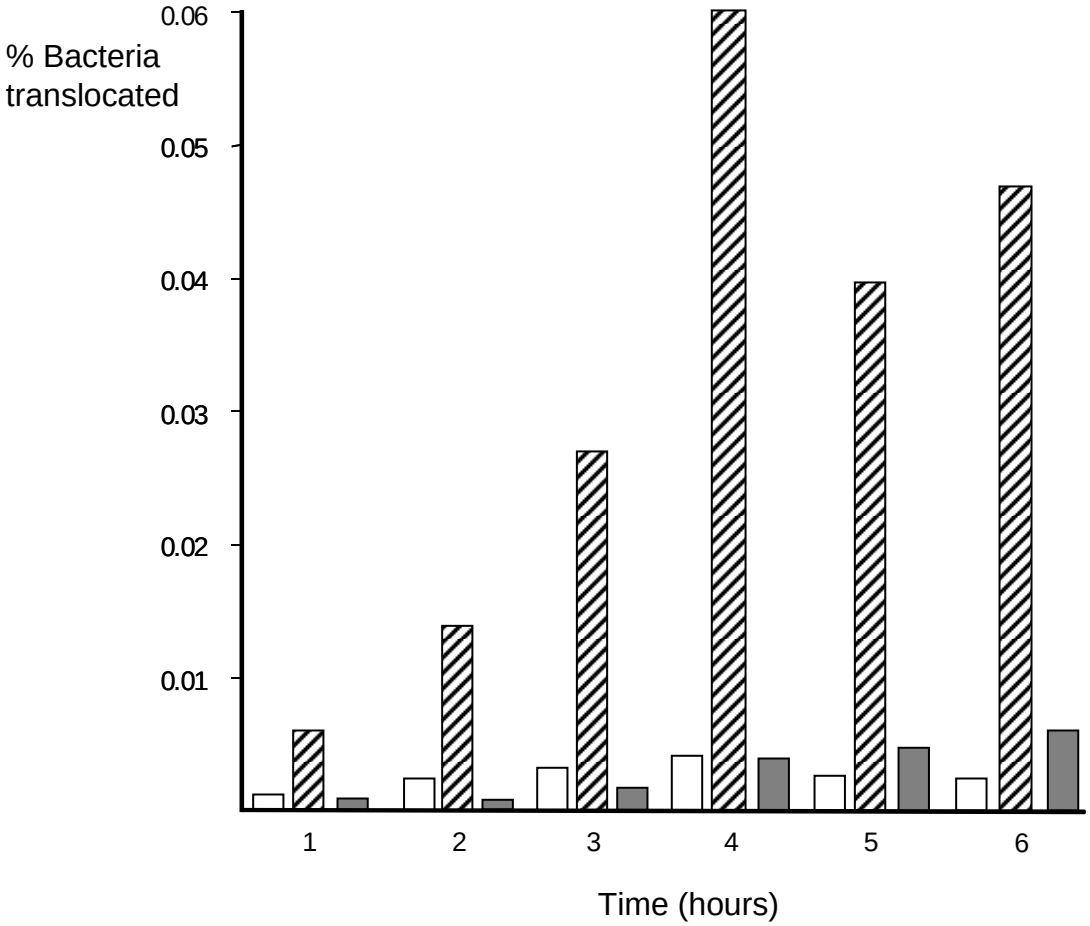
FIGURE LEGENDS

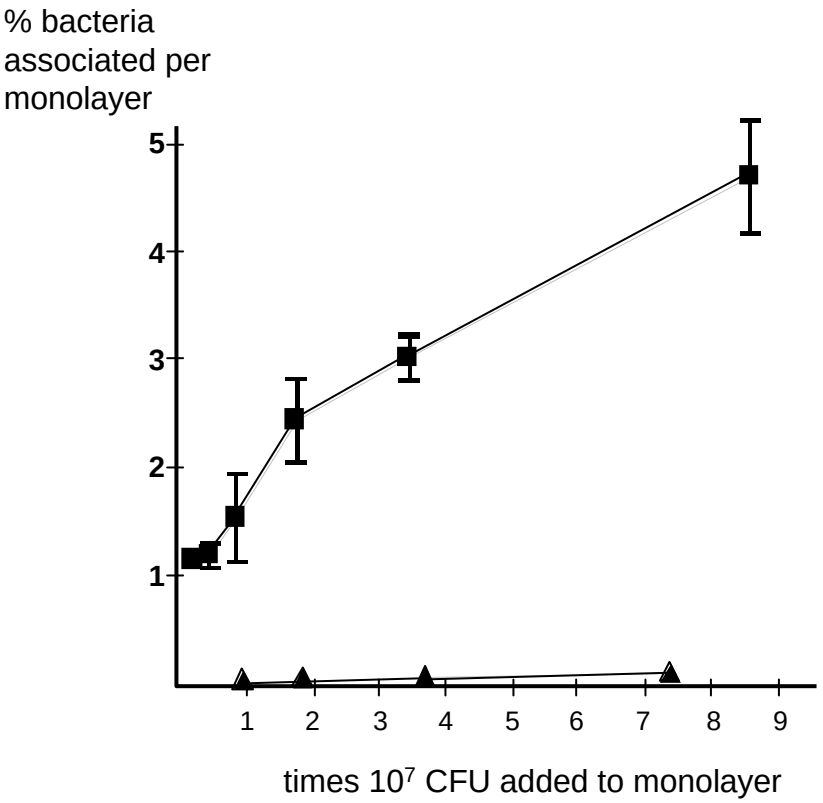
Figure 1. Distribution of the 113 isolates tested for invasion into INT407 cells. Invasion is expressed in relation to the reference strain NCTC 81116 which is a relatively low invader. Three classes of invasiveness were defined: hyper-invasive strains are at least 25 times more invasive than the reference 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).

Figure 2. Translocation of two *C. jejuni* strains through a monolayer of differentiated Caco-2 cells. The translocated fraction is shown for 6 time points. White bars: *C. jejuni* 81116; grey bars: *C. jejuni* strain Ex323; diagonal striped bars: *C. jejuni* Ex114.

Figure 3. 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.


Times invasion compared to reference strain
60
50
40
30
20
10
0
Hyper
High
low


% Bacteria translocated
0.06
0.05
0.04
0.03
0.02
0.01
1
2
3
4
5
6
Time (hours)


% bacteria
associated per
monolayer
5
4
3
2
1
1
2
3
4
5
6
7
8
9
times 10^7 CFU added to monolayer