

## PCR Detection of Four Virulence-Associated Genes of *Campylobacter jejuni* Isolates from Thai Broilers and Their Abilities of Adhesion to and Invasion of INT-407 Cells

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**ABSTRACT.** *Campylobacter jejuni* is a major cause of food borne pathogens in humans and a major reservoir for this pathogen is poultry. The *C. jejuni* in broilers was investigated from in the caeca of broilers. Twenty broiler/flock samples from 7 flocks were assessed. The average prevalence of *C. jejuni* was 65% in the broiler flocks. The adhesion and invasion ability of 48 strains of *C. jejuni* on INT 407 were studied. The adhesion and invasion ability of 48 *Campylobacter* isolates from caecal contents were analyzed with Human embryonic intestine (INT-407) cells being used as a gentamicin resistance assay. The caecal isolates exhibited a wide range of adherence and invasion ability. There was a significant correlation ( $p < 0.01$ ) between the adherence and the invasion ability of the *Campylobacter* isolates. Each of the virulence-associated genes: *dnaJ*, *cadF*, *pldA* and *ciaB* was detected by polymerase chain reaction from 100, 76, 31 and 41% of the *Campylobacter* strains, respectively. All of four virulence-associated genes were detected in 11 isolates. However, there was unclear association between the invasion ability and the presence of virulence-associated genes in this experiment, suggesting that more genes may be involved in the invasion process.

**KEY WORDS:** adhesion, broilers, *Campylobacter jejuni*, invasion, virulence gene.

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*Campylobacter* is one of most leading causes of acute bacterial diarrhoea worldwide [29]. Infection with *C. jejuni* or *C. coli* is characterized by the sudden onset of fever, abdominal cramps and diarrhoea with blood and leukocytes [7, 8]. There are many possible sources of infection from *C. jejuni* and *C. coli*, as they are part of the normal intestinal flora in a wide range of birds and mammals and are transferred to other chickens during growth and processing [1, 36]. Large-scale outbreaks of human campylobacteriosis are rare and are usually linked to the consumption of polluted water or raw milk. Sporadic cases of campylobacteriosis are more common and are associated with the consumption of undercooked chicken. In the United States, case-control studies have attributed 48–70% of sporadic infections to the consumption of *Campylobacter*-contaminated chickens [11, 15]. The percentage of *Campylobacter*-contaminated chicken carcasses varies, often between 50 and 90%, depending on the time of year and the number of carcasses tested. One study has found that as many as 98% of chicken carcasses may be contaminated with *C. jejuni* by the time of sale [38]. *Campylobacter* organisms cause disease via at least three mechanisms: (i) intestinal colonization by ingested organisms and the production of bacterial cytotoxin [10, 14, 17], inducing diarrhoea, (ii) bacterial invasion of intestinal cells [35], resulting in damage to the mucosal surface cells of the jejunum, ileum and colon, and (iii) extra intestinal translocation [13, 19], in which the organisms cross the intestinal epithelium and migrate via the lymphatic system to various extra intestinal sites [42]. The adherence to and invasion of *C. jejuni* into host cells has been studied

in a variety of cell lines [11, 16, 19, 26]. Human embryonic intestine (INT-407) cells have been widely used to assess the ability of enteric bacteria to adhere to and invade the epithelium. The ability of *C. jejuni* to adhere to and invade the epithelial cells of the gastrointestinal tract is important to the development of *Campylobacter*-mediated enteritidis [27, 39]. Despite the recognition of *C. jejuni* invasion of the intestinal epithelium as a possible pathogenic mechanism, studies defining the precise mechanism of *C. jejuni* entry and the identification and characterization of entry-promoting proteins are in their infancy. The molecular basis of the pathogenicity of *Campylobacter* has not been clearly understood. Some genes are involved in *Campylobacter* adhesion and invasion. Four mutant strains derived from F38011, an excellent colonizer of chicken caeca, *dnaJ*, *cadF*, *pldA* and *ciaB* are incapable of colonizing the ceca [20, 23, 43, 44]. *cadF* (*Campylobacter* adhesin to fibronectin) is an outer membrane protein, which encodes a protein that interacts with a host extracellular matrix protein fibronectin [31], and is required for *Campylobacter* adherence to and colonization of the host cell surface [20]. Other genes such as *ciaB* (*Campylobacter* invasive antigen B) [21, 34] and *pldA* encoded outer membrane phospholipase A [34] are involved in host cell invasion and are important for caecal colonization [21, 43]. The *ciaB*, *pldA* and *dnaJ* (heat shock protein) genes are important to caecal colonization and mutations of these genes severely limit the ability of the mutant strains to colonize the chicken caeca [20, 43, 44]. The objective of this study was to study the prevalence of *C. jejuni* in Thai broiler caeca and their ability to adhere and invade and the presence of the 4 genes that are involved in the adherence and invasion of the *Campylobacter* in the INT-407 cell culture model.

*Bacterial strains and culture conditions:* The caecal con-

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tents of 7 broiler flocks were collected using a sterile swab from 20 samples from each flock that were located in Lopburi province, a dense area of broiler integrated farming in the central plains of Thailand. Samples were collected and sent to the laboratory within 3 hr and processed between June and October 2006. The chicken farms in this study were industrialized facilities and parts of an integrated production system. Strict biosecurity measures were applied in these flocks. Antimicrobials used on these farms were regulated by the companies purchasing these animals for slaughter. At least one week before sampling, broilers had not been treated with antimicrobial agents prior to sample collection. *Campylobacter* isolation and identification has previously been described by membrane filtration technique [24]. Briefly, the swabs were incubated in Preston broth (Nutrient broth no.2) (Oxoid, Hampshire, England) containing 5% lysed horse blood, *Campylobacter* growth supplement and modified Preston selective supplement (2500 IU of polymyxin B, and 5 mg each of rifampicin, trimethoprim and amphotericin B) (Oxoid, Hampshire, England) for 24 hr or overnight at 37°C. Eight drops of each broth culture were spotted on a cellulose acetate membrane with 0.45 µm pores of diameter 47 mm (Sartorius, Goettingen, Germany). The membrane was placed on the surface of a blood agar base no. 2 (Oxoid, Hampshire, England) containing 5% whole sheep blood and *Campylobacter* growth supplement (Oxoid, Hampshire, England). The membrane was left on the agar surface for 30 min to let all the fluid pass through. The pores allowed relatively slender and naturally spiralling 'cork screw' motiles to pass through whereas other bacteria harboured in the intestine were excluded by the 0.45 µm cellulose membrane. The culture plates were incubated for 48 hr at 37°C in an atmosphere of 5% O<sub>2</sub>, 10% CO<sub>2</sub> and 85% N<sub>2</sub> using an anaerobic jar with CampyGen (Oxoid, Hampshire, England). Colonies of *Campylobacter* were identified to the genus level by typical morphology on a Gram stain (slender, curved, 'seagull wing' shaped or spiral, Gram negative rods). Species differentiation was performed on the basis of nalidixic acid sensitivity and hippurate hydrolysis [28, 37] and PCR analysis [40]. A well-characterized human clinical strain, *C. jejuni* 81116 (kindly provided by Prof. Dr. J.P.M. van Putten, Utrecht University, The Netherlands) which has been described as a good colonizer of the chicken intestinal tract [2, 9] was used. Non-invasive *Escherichia coli* laboratory strain, DH5α, was used as positive and negative controls for all the analyses. The *C. jejuni* strains were suspended in Brain Heart Infusion containing 15% glycerol and stored at -80°C until use.

**Cell cultures:** The human embryonic intestine (INT-407) cells (ATCC CCL-6), kindly provided by Prof. Dr. J.P.M. van Putten, Utrecht University, The Netherlands, were maintained in DMEM (Gibco, Auckland, New Zealand) with a 5% FBS (foetal bovine serum), penicillin and streptomycin (Gibco, Auckland, New Zealand) in a 5% CO<sub>2</sub>-humidified incubator. Confluent stock cultures were trypsinized and new stock cultures were seeded with 10<sup>5</sup> cells/ml on to 24-well tissue culture plates (Corning,

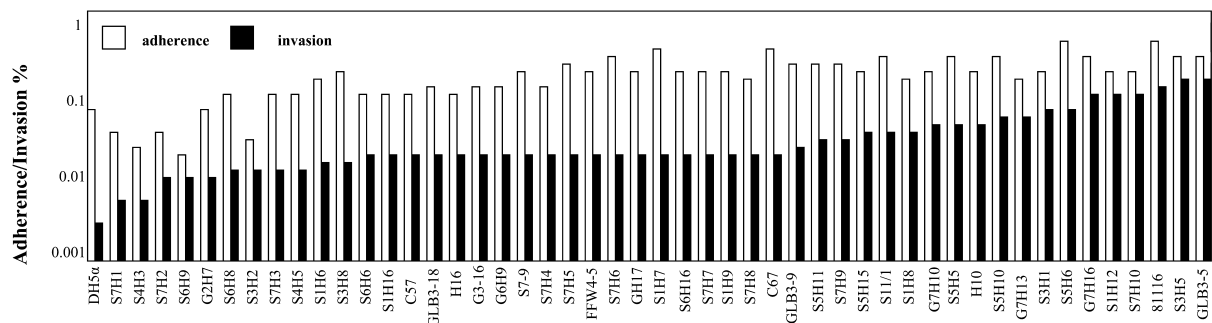
U.S.A.) for the adherence and invasion assay and incubated at 37°C in a humidified 5% CO<sub>2</sub> incubator for 48 hr, and a semi-confluent monolayer was obtained. Prior to the experiment, the monolayer was washed and incubated with DMEM without antibiotic and FBS.

**Adherence and invasion assay:** The adhesion and invasion assays were performed by the method of Konkel *et al.* [22] with some modifications. Briefly, *C. jejuni* strains were grown microaerobically on Blood agar No. 2 with supplement for 48 hr at 37°C. Bacteria were harvested from the plates with PBS (phosphate buffered saline) and adjusted spectrophotometrically to approximately 1 × 10<sup>7</sup> bacteria/ml. The containing CFU (colony forming unit) of approximately 100 times higher than the cell number, was inoculated into duplicated wells of a 24-well tissue culture plate containing semiconfluent monolayers of INT-407 cells. The infected monolayers were incubated for 3 hr at 37°C in a 5% CO<sub>2</sub> humidified atmosphere to allow for bacterial adherence and internalization. For determination of adherence, the cells were washed 3 times with PBS and the cell monolayer was lysed with 0.5% deoxycholate (W/V) (Sigma-Aldrich, Auckland, New Zealand) and the total bacteria associated with the cells (intracellular and extracellular bacteria) were enumerated by plating serial dilutions of the lysates on Blood agar No. 2 with 5% sheep blood and counting the resultant colonies. In order to measure bacterial invasion, the infected cells were washed 2 times with PBS and incubated in fresh PBS containing 1% foetal bovine serum (FBS) and 150 µg/ml gentamicin for 2 hr to kill the remaining viable extracellular bacteria. In preliminary experiments 150 µg/ml of gentamicin killed all bacterial strains 3 hr after exposure. Quantification of viable intracellular bacteria was performed by washing the infected eukaryotic cells twice with PBS and subsequently lysing with 0.5% deoxycholate (W/V). Following serial dilution in PBS, the released intracellular bacteria were enumerated as described for the adherence assay. The correlation between the adherence to and invasion of the INT-407 cells by *Campylobacter* isolates was analyzed by linear regression using SPSS (SPSS Inc., Chicago, Ill.).

**Detection of virulence-associated genes:** PCR (polymerase chain reaction) was used to detect the 4 *Campylobacter* genes that are associated with invasion in the genomic DNA of *Campylobacter* isolates: *cadF*, *ciaB*, *pldA* and *dnaJ*. PCR primers specific for these genes were designed based on the gene sequence information in the GenBank database and in previous published studies (Table 1). Conserved sequences of each gene were selected and a set of primers was used for each gene. Template DNAs for PCR were extracted by the boiling method described previously [30]. Fresh cultures of *Campylobacter* isolates were suspended in 1 ml of PBS and boiled at 95°C for 10 min. After centrifugation at 14,000 × g for 2 min, the supernatants were collected and stored at -20°C until use. PCRs were carried out using GoTaq Green MasterMix (Promega, U.S.A.) with 30 cycles of amplification in thermocycler (Biometra, Goettingen, Germany). The cycling program

Table 1. *Campylobacter* virulence genes and primer sequences used for PCR identification

Target gene	Primers	Sequence (5' to 3')	Annealing temperature (°C)	PCR Product (bp)	References
<i>cadF</i>	<i>cadF</i> -F2B <i>cadF</i> -R1B	TTGAAGGTAATTTAGATATG CTAATACCTAAAGTTGAAAC	42	400	21
<i>ciaB</i>	<i>ciaB</i> -652 <i>ciaB</i> -1159	TGCGAGATTTTTCGAGAATG TGCCCGCCTTAGAACTTACA	58	527	42
<i>C. jejuni</i> <i>pldA</i>	<i>pldA</i> -361 <i>pldA</i> -726	AAGAGTGAGGCGAAATTCCA GCAAGATGGCAGGATTATCA	58	385	42
<i>dnaJ</i>	<i>dnaJ</i> -F301 <i>dnaJ</i> -R477	ATTGATTTTGCTGCGGGTAG ATCCGCAAAAGCTTCAAAAA	42	177	This study

Fig. 1. The ability of *Campylobacter jejuni* isolates to adhere to and to invade INT 407 human intestinal epithelial cells.

was denaturation at 94°C for 1 min, annealing at a temperature specific to each primer pair for 45 s, and extension at 72°C for 45 s. In negative control reactions, the DNA template or the primer were replaced by sterile deionized water. PCR products were separated on 2% agarose gels and bands were stained with ethidium bromide and visualized using a gel document system (Vilber Lourmat, France).

**Prevalence of *C. jejuni* in broilers:** The *C. jejuni* in broilers was investigated from the caeca of broilers. Twenty broilers per flock from 7 flocks were assessed. The prevalence of *C. jejuni* in each flock was as follows 80%, 70%, 55%, 75%, 55%, 65% and 55%. The average prevalence of *C. jejuni* was 65% in the broiler flocks.

**Adhesion and invasion by *C. jejuni*:** To test the pathogenic properties of *Campylobacter* isolates, the adhesion and invasion abilities of 49 *Campylobacter* isolates were analyzed with Human embryonic intestine (INT-407) cells using a gentamicin resistance assay. After 3 hr incubation, the 49 *Campylobacter* isolates adhered to INT-407 cells between  $1.51 \times 10^4$  and  $3.65 \times 10^5$  from the isolates of S6H9 and 81116, respectively, and that could be expressed as 0.03 to 0.73% adherence of the starting viable inoculum. The invasion abilities of the 49 isolates to INT-407 were between  $3.5 \times 10^3$  and  $1.41 \times 10^5$  from the isolates of S7H1 and GLB3-5, respectively, and that can be expressed as 0.007 to 0.28% of the starting viable inoculum. The average percentages of 49 *C. jejuni* for adherence to and invasion were  $0.36 \pm 1.9$  and  $0.05 \pm 0.05$ , respectively (Fig. 1). There was a significant correlation ( $p < 0.01$ ) between the adherence ability and the invasion ability of the *Campylobacter* isolates.

**Presence of the putative virulence-associated genes:** Although the genes that determine the infectivity of *Campylobacter* have not been thoroughly studied, several genes are thought to be involved in *Campylobacter* adherence to and invasion of host cells. Forty-six percent of *Campylobacter* isolates examined in this study possessed two of the 4 virulence-associated genes. The percentages of *dnaJ*, *cadF*, *pldA* and *ciaB* genes of 49 isolates were 100, 76, 31 and 41%, respectively (Table 2). All 4 virulence genes were detected in the positive control strain and the other 11 isolates *dnaJ* gene was detected in all *Campylobacter jejuni* strains, in contradiction to *pldA* which was detected from 15 isolates.

*Campylobacter* is one of the major foodborne pathogens and is widespread in poultry meat and poultry products. Although the samples were collected from a strict biosecure farm, a high prevalence of *C. jejuni* was found. The average prevalence of *C. jejuni* isolated from the broiler caeca was 65% which is similar to a previous report that the prevalence of *Campylobacter* spp. in Thai broilers was 64% [32]. However, little is known about the pathogenicity of food-contaminating strains and the molecular basis of *Campylobacter* pathogenicity. In this study, we examined the ability of *Campylobacter jejuni* strains isolated from the caecal contents of broilers to adhere to and invade the cells of the INT-407 human intestinal cell line and determined the association with the presence of certain virulence genes which affect on poultry colonization [43, 44] and the adherence and invasion abilities of *Campylobacter jejuni* isolates. The adherence and invasion abilities of the caecal isolates varied considerably, from invasive levels similar to that of

Table 2. Detection of different putative virulence and in 48 *C. jejuni* isolates obtained from Thai broilers

strains	<i>cadF</i>	<i>pldA</i>	<i>ciaB</i>	<i>dnaJ</i>	strains	<i>cadF</i>	<i>pldA</i>	<i>ciaB</i>	<i>dnaJ</i>
DH5 $\alpha$	–	–	–	–	S1H7	–	–	–	+
S7H1	+	–	–	+	S6H16	+	–	–	+
S4H3	–	–	+	+	S7H7	+	+	+	+
S7H2	–	–	+	+	S1H9	–	–	–	+
S6H9	+	–	–	+	S7H8	+	–	+	+
G2H7	+	–	–	+	C67	+	+	–	+
S6H8	+	–	–	+	GLB3–9	+	–	–	+
S3H2	–	–	–	+	S5H11	–	–	–	+
S7H3	+	+	+	+	S7H9	+	+	+	+
S4H5	–	–	–	+	S5H15	+	–	–	+
S1H6	+	–	+	+	S11/1	+	+	+	+
S3H8	+	–	–	+	S1H8	+	+	+	+
S6H6	–	–	–	+	G7H10	+	–	–	+
S1H16	+	+	–	+	S5H5	+	–	+	+
C57	+	+	+	+	H10	–	–	–	+
GLB3–18	+	–	–	+	S5H10	+	–	–	+
H16	+	–	–	+	G7H13	+	–	–	+
G3–16	+	–	–	+	S3H1	+	+	+	+
G6H9	–	–	–	+	S5H6	+	+	–	+
S7–9	+	–	–	+	G7H16	+	–	+	+
S7H4	–	–	+	+	S1H12	+	+	+	+
S7H5	–	–	+	+	S7H10	+	+	+	+
FFW4–5	+	–	–	+	81116	+	+	+	+
S7H6	+	–	–	+	S3H5	+	+	+	+
GH17	+	–	–	+	GLB3–5	+	+	+	+

*C. jejuni* 81116 to 40-fold lower than that of *C. jejuni* 81116, indicating that not all *Campylobacter* strains that contaminate broiler caeca are able effectively to invade human intestinal epithelial cells. Moreover, 2 of 49 tested isolates had invasion abilities higher than that of *C. jejuni* 81116. Because animal models that completely mimic *Campylobacter* infection in humans are not available, the cell culture model using human intestinal epithelial cells is a useful tool for evaluating the abilities of *Campylobacter* food isolates to adhere to and invade the human intestinal epithelium. The INT-407 cell line has been widely used for studies of the pathogenicity of many human enteric pathogens, such as *Salmonella* Enteritidis, *Campylobacter jejuni*, and enteropathogenic *E. coli* [6, 25, 33]. Even though the process of *Campylobacter* adhering to and invading INT-407 human intestinal epithelial cells in culture does not exactly mimic the process *in vivo*, the cell culture model allowed us to determine the relative adherence and invasion abilities of the caeca isolates in comparison to well-studied human clinical strain *C. jejuni* 81116. From our study, a multiplicity of about 100 infections was used and the invasion and adherence levels of the human clinical strain *C. jejuni* 81116 reported here are slightly lower than a previous report by Biswas *et al.* [6] that 0.7416–2.1714% and 0.0012–0.4226% of the range of adherence and invasion, respectively. Colonization or adherence of microbial pathogens to mucosal surfaces is the primary step of infection and appears to be a prerequisite for invasion in most cases [3, 18, 41]. In this study, we analyzed the relationship between adherence and invasion efficiency in *Campylobacter* caecal

isolates using statistical tests. There was a significant correlation ( $p < 0.01$ ) between the adherence and the invasion ability of the *Campylobacter* isolates according to Zheng *et al.* [42]. Thus, the adherence of *Campylobacter* may facilitate invasion into host cells. Bacterial virulence is multifactorial and is affected by the expression of virulence genes. To determine the relationship between the presence of virulence genes in *C. jejuni* caeca isolates and the ability of these isolates to adhere to and invade human intestinal epithelial cells, the prevalence of the putative virulence genes *dnaJ*, *cadF*, *ciaB* and *pldA* among the 49 *Campylobacter* isolates was determined by PCR. Using PCR primers that targeted to conserved sequences for each gene, we detected all putative virulence genes in 12 of the 49 *Campylobacter* isolates including the positive control strain. The gene *dnaJ* was detected in all strains of *Campylobacter* caecal isolates. Only 76% of *cadF* genes were detected in all 49 chicken isolates which is contrary to the 100% detection of *cadF* genes from the isolates of Danish turkeys [4, 5]. This result may be caused by geographic and species differences. The previous studies revealed that *dnaJ*, *cadF*, *pldA* and *ciaB* genes are important to host cell invasion and caecal colonization [20, 31, 34, 43, 44]. However, we could not find a clear association between the invasion ability and the presence of virulence-associated genes in this experiment, suggesting that more genes may be involved in the invasion process. Furthermore, it can't be ruled out that the nucleotide sequences selected for PCR detection of these genes aren't conserved in the isolates studied herein. To confirm this point, therefore, southern blot analysis is necessary.

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