Food Control 33 (2013) 227–231

Contents lists available at SciVerse ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Short communication

Campylobacter jejuni strains isolated from chicken meat harbour several virulence factors and represent a potential risk to humans

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ARTICLE INFO

Article history:
Received 27 December 2012
Received in revised form
14 February 2013
Accepted 19 February 2013

Keywords:
Campylobacteriosis
Transcription
Caco-2
RAPD-PCR

ABSTRACT

This study aimed to evaluate the virulence characteristics of 55 *Campylobacter jejuni* strains isolated from chicken carcasses. These characteristics included antibiotic resistance, the presence of virulence genes, and the transcription virulence genes, changes after the inoculation of Caco-2 cells and the phylogenetic relationship between strains. Resistance to amoxicillin and norfloxacin was observed in 34/55–61.8% and 26/55–47.3% respectively, and resistance to tetracycline was also observed (18/55–32.7%). The genes *flaA, pldA, cadF,* and *ciaB* and the CDT complex were detected in 41/55 (74.5%), 35/55 (63.6%), 37/55 (67.3%), 37/55 (67.3%) and 36/55 (65.5%) strains respectively, and transcripts for the *ciaB* and *dnaJ* genes evaluated in 46 strains were detected in 60.9%. In Caco-2 cells, loss of cell confluence was observed. Genetic heterogeneity among these strains was confirmed by RAPD-PCR. The data indicate the potential role of these *C. jejuni* strains in the pathogenesis of human diseases, emphasising the need for vigilance and strict control during production to protect the health of the consumer.

1. Introduction

Campylobacter jejuni is the most common cause of food-borne bacterial gastroenteritis in humans (EFSA – European Food Safety Authority 2009; Moore et al., 2005). Campylobacteriosis is a self-limiting disease in healthy adults, but in children, elderly individuals, and immunosuppressed individuals, it can become a severe illness requiring antibiotic therapy (Cox, 2002).

Poultry meat is the main disseminator of *C. jejuni*. The Panel on Biological Hazards presented by EFSA (2010) revealed a high prevalence of *Campylobacter* spp. in poultry meat, with alarming rates in Europe (of 10 carcasses, 8 were contaminated). In addition to testing for the presence of *C. jejuni* strains, virulence factor verification is a useful tool to assess the potential risk of chicken meat as a pathogen disseminator. This work evaluated five *C. jejuni* strains isolated from chicken carcasses to determine the levels of antimicrobial resistance, virulence, and damage to Caco-2 cells.

2. Methods

We used 55 *C. jejuni* strains derived from analyses of 420 chilled and frozen chicken carcasses samples for human consumption. The strains were isolated by the technique recommended by the ISO (ISO, 2006), and the species were identified by multiplex-PCR (Harmon, Ramsom, & Wesley, 1997). *C. jejuni* NCTC 11351 was used as a positive control. We evaluated *flaA, pldA, cadF,* and *ciaB* genes which are important genes for adherence, colonisation, and invasion (Zheng, Meng, Zhao, Singh, & Song, 2006), and the genes *cdtA, cdtB,* cdtC, which are related to the production of cytolethal distending toxin (CDT) (Martinez et al., 2006).

The antimicrobial susceptibility testing was performed by the disk diffusion method according to the protocol of the Clinical and Laboratory Standards Institute - CLSI (2010) for the following antimicrobials: amoxicillin (10 μg), erythromycin (15 μg), gentamicin (10 μg), neomycin (30 μg), norfloxacin (10 μg), sulphazotrim (25 μg), and tetracycline (30 μg) (Laborclin®).

Gene transcription was evaluated by rt-PCR (reverse transcriptase polymerase chain reaction). In this assay, the *ciaB* and *dnaJ* genes were used according to the method of Li, Ingmer, Madsen, and Bang (2008). The RNA was extracted and reverse transcribed. Then, we performed the PCR according to the method of Li et al. (2008) with some modifications.

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http://dx.doi.org/10.1016/j.foodcont.2013.02.032

To examine morphological changes, were selected five strains that had more virulence characteristics. Caco-2 cells were seeded onto coverslips in 24-well plates at a density of 3.5×10^5 cells/ml and then incubated for 14 days. After that period, each coverslip covered with Caco-2 cells was infected with 2×10^6 CFU *C. jejuni* strain per well; infection with each strain was performed in triplicate, and negative and positive controls were run in parallel. The culture method was performed as 67 described by Fonseca et al. (2012). After 72 h, each coverslip was evaluated by computer analysis of digitised images obtained from an Olympus BX 40 microscope with a 100x objective coupled to an Olympus camera (Oly 200) and connected to a PC via the PC card scanner Data Translation 3153.

The genetic diversity among the isolates was determined by RAPD-PCR (*Random Amplification of Polymorphic DNA*) according to the method of Akopyanz, Bukanov, Westblom, Kresovich, and Berg (1992). The results from the RAPD-PCR were evaluated using the GelCompar II programme (*Comparative Analysis of Electrophoresis Patterns*), version 1.50, *Applied Maths Korthrijk, Belgium*. The similarity matrix was obtained by comparing pairs of strains using the Dice similarity coefficient, with a 1% tolerance for each primer separately. We used the UPGMA (*unweighted pair group method with arithmetic mean*) method for the construction of the dendrogram (Madden, Moran, & Scates, 2007).

3. Results and discussion

The PCR showed that the genes *flaA, pldA, cadF, ciaB,* and *cdtABC* were present in 41/55 (74.5%), 35/55 (63.6%), 37/55 (67.3%), 37/55 (67.3%) and 36/55 (65.5%) of the studied strains of *C. jejuni*, respectively (Fig. 1). A total of 46 (83.6%) of these strains had at least one gene and 20 (43.6%) had all studied genes. Similar results to these were also found by Thakur et al. (2010), Biswas, Hannon, Townsend, Potter, and Allan (2011), Rizal, Kumar, and Vidyarthi (2010) and Hanning, Biswas, Herrera, Roesler, and Ricke (2010) that studied the same species.

Virulence potential observed in strains may explain the fact that *C. jejuni* be much more common as a cause of human infections (90%–95%) (Thakur et al., 2010). Moreover, differences in the presence of these genes in *C. jejuni* suggests that not all strains from chickens are capable to cause humans diseases.

FlaA gene, responsible for flagellar motility of the bacteria, is essential for cell adhesion and invasion (Malik-Kale et al., 2007), but its absence indicates severe reduction in motility and intestinal mucosa colonization of humans and chickens (Konkel et al., 2004). *PldA* is related to the phospholipase synthesis outer membrane and, consequently, to cell invasion (Ziprin et al., 2001). *CadF* presence indicates colonization through interaction with the host extracellular matrix (Monteville, Yoon, & Konkel, 2003). Protein encoded by CIAB promotes microtubules destruction that potentiates invasion (Rivera-Amill, Kim, Seshu, & Konkel, 2001). The activity of the toxin CDT occurs by blocking the cell cycle, inducing cell death (Martinez et al., 2006).

Table 1
Antimicrobial resistance 55 strains isolated from chicken carcasses.

Antimicrobial	*C. jejuni* N/55 (%)
Amoxiline	34 (61.8)
Eritromicin	14 (25.5)
Gentamicin	2 (3.6)
Neomicin	0
Norfloxacin	26 (47.3)
Sulphazotrim	12 (21.8)
Tetraciclin	18 (32.7)

N-number of resistant strains. % – Percentage in relation to total isolates.

Several strains were resistant to amoxicillin and norfloxacin (34/55–61.8% and 26/55–47.3%), as well as tetracycline (18/55–32.7%). A total of 14/55 (25.5%) strains exhibited resistance to erythromycin. For the antibiotic neomycin, resistance was not observed (Table 1).

Due to the self-limiting nature of campylobacteriosis, antimicrobials are generally not recommended for treatment except in severe cases, for which fluoroquinolones and macrolides are the treatment options of choice (Yates, 2005). Nevertheless, resistance to norfloxacin (fluoroquinolone) (47.3%) was confirmed in this study (Table 1). Studies conducted in the United States, Poland and other EU countries also confirm this trend (EFSA 2010; Gupta et al., 2004; NARMS 2007; Rozynek et al., 2008). In Brazil, amoxicillin and enrofloxacin are routinely used to treat poultry flocks, and this practice may favour the selection of resistant strains (Borges, 2009). The low levels of resistance to aminoglycosides (gentamicin and neomycin) can be explained by the fact that these antibiotics are not routinely used in chicken production, which reduces the selection pressure (Borges, 2009).

The rt-PCR assay (Fig. 2) and the inoculation of five strains into Caco-2 cells demonstrated that the important virulence-related genes are transcribed into mRNA and that these strains can damage intestinal epithelial cells, respectively (Fig. 3).

The presence of virulence transcripts were made in strains that had at least one of the genes studied (46 strains). There were virulence transcripts in a total of 28/46 (60.9%). Of these, 8/28 (28.6%) with *ciaB* transcripts, 2/28 (7.1%) *dnaJ* and 18/28 (64.3%) for both genes.

The transcription of *ciaB* is involved in the invasion of epithelial cells, and *C. jejuni* requires the *ciaB* protein for efficient

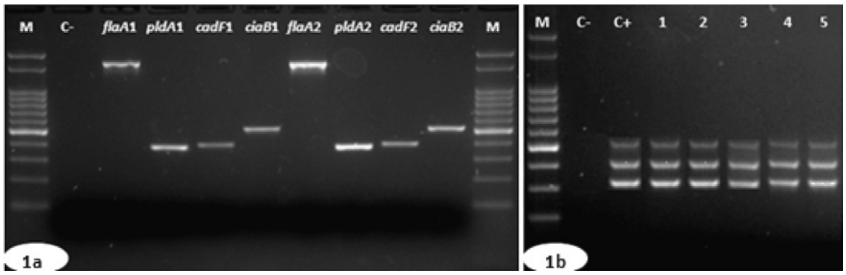

Fig. 1. PCR amplification of the *flaA, pldA, cadF,* and *ciaB* genes (1a) and the CDT complex (1b) from *Campylobacter jejuni* strains isolated from chicken carcasses. M (molecular weight marker, 100 bp), C- (negative control); *flaA*1, *pldA*1, *cadF*1, and *ciaB*1 (positive control, *C. jejuni* NCTC 11351) and *flaA2, pldA2, cadF*2, and *ciaB*2 (one *C. jejuni* strain isolated from a chicken carcass); C+ (positive control, *C. jejuni* NCTC 11351 positive for the CDT complex); 1–5 (*C. jejuni* strains isolated from chickens and positive for the CDT complex).

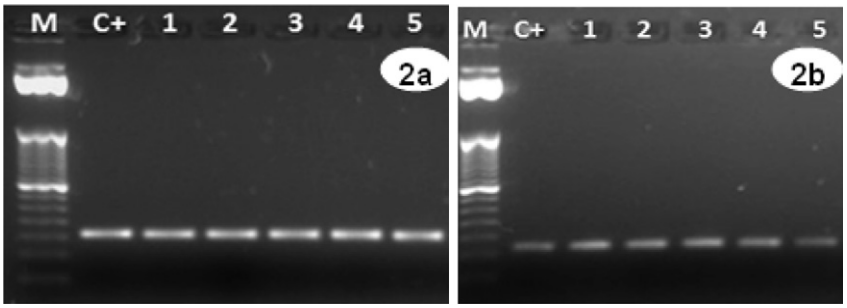

Fig. 2. rt-PCR for *ciaB* (2a) and *dnaJ* (2b) transcripts in *Campylobacter jejuni* strains isolated from chicken carcasses. M (molecular weight marker, 50 bp); C+ (positive control *C. jejuni* NCTC 11351); 1–5 (*C. jejuni* strains isolated from chicken carcasses and contain *ciaB* and *dnaJ* transcripts).

internalization into host cells (Poly & Guerry, 2008). The proteins derived from the *dnaJ* gene have an important role in overcoming sudden changes in temperature (Stintzi, 2003). The response to heat shock is associated with the ability to colonise the intestinal tract and with bacterial survival in high-temperature environments, such as in birds, wherein the temperature is approximately 41 °C (Konkel, Kim, Klena, Young, & Ziprin, 1998). The transcription of genes related to heat shock by these strains may have contributed to the survival of these bacteria during the sudden changes in temperature that chicken carcasses are subjected to and during the subsequent storage at low temperatures. Transcription absence in some strains may be associated with strain-dependant characteristics, which can take various properties to modulate their virulence may be more pathogenic than others depending on the situation they are submitted, so have different capacities to cause disease and deal with stress (Poli, Thorsen, Olesen, Wik, & Jespersen, 2012).

We selected five more virulent strains to verify its ability to alter the morphology of Caco-2 cells. We observe a loss of cell confluence for the five tested strains, similar to that observed for the positive control (*C. jejuni* NCTC 11351), which is a standard strain used in the evaluation of virulence (Fig. 3). The loss of intercellular junctions was also reported by Maccallum, Haddock, and Everest (2005), who detected the loss of occlusion junctions between cells after contact with *C. jejuni* for 24 h. The loss of cellular integrity, reflected by the loss of the ability to transport fluids and electrolytes between cells, contributes to the clinical symptoms of campylobacteriosis *in vivo*.

The junctional integrity of the cells is rarely mentioned in the context of campylobacteriosis *in vivo*. However, the histopathological analysis of acute intestinal biopsies showed intense infiltration of neutrophils into the infected mucosa and in the diarrhoeal faeces (Skirrow & Blaser, 2000). Presumably, these neutrophils had to cross the intestinal epithelium, and this action is facilitated by the enlargement of intercellular junctions by bacterial action (Maccallum et al., 2005).

The homology between the isolates studied was always less than 100%, but were identified 15 clusters with similarity greater than 80% considered as belonging to the same genotype (A–O) (Fig. 4). Besides these, there were 18 distinct genotypes, which show genetic heterogeneity. This genotypic variation may be due to exposure to over contamination sources during the chicken production process or genetic changes in bacterial population after settlement (Workman, Mathison, & Lavoie, 2008). Genetic diversity observed by RAPD-PCR was also reported by Workman et al. (2008) in isolates from chicken meat and medical patients in Bridgetown, Barbados. Aquino et al. (2010) found extreme heterogeneity in isolates of *C. jejuni* of humans and different animals in Rio de Janeiro, Brazil.

The presence of different clones of this virulent bacterium indicates that the sources of contamination were likely different. We can speculate that the lack of homology between these strains may lead to increased difficulty in controlling the presence of *C. jejuni* because these strains can acquire different profiles of antibiotic resistance and can express different virulence genes over time.

This work demonstrated that virulent *C. jejuni* strains from different backgrounds and with different antibiotic resistance profiles can survive on chicken carcasses that have been chilled and frozen, posing a risk for campylobacteriosis in humans.

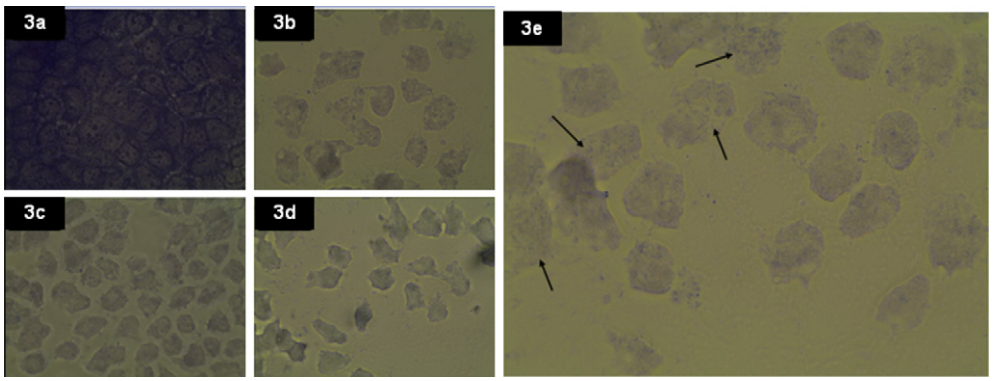

Fig. 3. Photomicrograph obtained by light microscopy with a 100x objective, illustrating the effect of *Campylobacter jejuni* on Caco-2 cells. 3a – Negative control (without the bacterium). 3b – Positive control, *C. jejuni* NCTC 11351. 3c and 3d – Caco-2 cells inoculated with *C. jejuni* strains isolated from chicken carcasses. 3e – Presence of *C. jejuni* in Caco-2 cells – these bacteria exhibited typical movements in phase contrast analysis, showing that they were still alive.

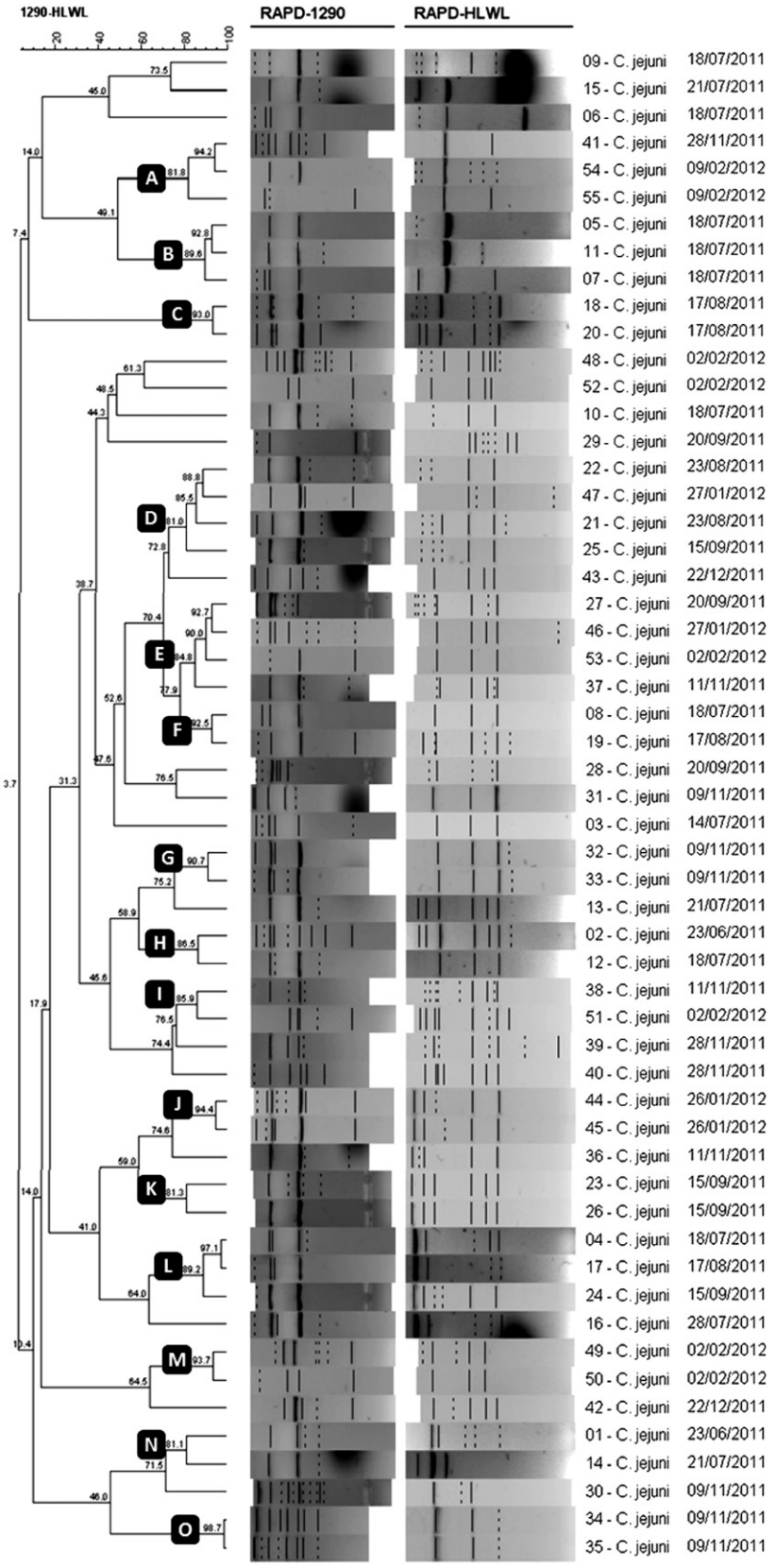

Fig. 4. Dendrogram of the 55 strains of *Campylobacter jejuni* used in this study. The RAPD-PCR *primers* 1290 and HLWL were used. A*verage from experiments,* tolerance 1.5% and UPGMA method and optimisation with 80%, using the programme GelCompar. Group A – *cluster* with 81.8% homology, composed of a subgroup with 94.2% similarity. Group B – *cluster* with 89.6% homology, composed of a subgroup with 92.8% de similarity. Group C – *cluster* with 93.0% homology. Group D – *cluster* with 81.0% homology, composed of two subgroups with 88.8% and 85.5% similarity. Group E – *cluster* with 84.8% homology, composed of two subgroups with 92.7% and 90.0% similarity. Group F – *cluster* with 92.5% homology. Group G – *cluster* with 90.7% homology. Group H – *cluster* with 86.5% homology. Group I – *cluster* with 85.9% homology. Group J – *cluster* with 94.4% homology. Group K – *cluster* with 81.3% homology. Group L – *cluster* with 89.2% homology, composed of a subgroup with 97.1% similarity. Group M – *cluster* with 93,7% homology. Group N – *cluster* with 81.1% homology. Group O – *cluster* with 98.7% homology.

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