

Original Article

Antimicrobial resistance and virulence associated genes in *Campylobacter jejuni* isolated from chicken in Côte d'Ivoire

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Abstract

Introduction: *Campylobacter jejuni* is one of the major causes of gastroenteritis worldwide of the last century. The aim of this study was to investigate the antibiotics profiles and the virulence gene in *C. jejuni* strains isolated from chicken in Côte d'Ivoire.

Methodology: A total of 336 chicken ceaca samples recovered from market of two municipality of Abidjan were examined by conventional microbiological methods and molecular test using PCR. The antibiotic susceptibility tests of the isolates were determined by disk diffusion method. The presence of virulence genes was examined using simple PCR method.

Results: Among of 336 samples, 168 (50%) were positives for *C. jejuni*. Among the *C. jejuni* isolates, 159 strains (94.64%) were resistant to one or more antimicrobial agents. The highest percentage of antimicrobial resistance was found for Nalidixic acid (85.33%), Tétracycline (71.76%) and Ciprofloxacin (55.65%). Moreover, MDR including 3, 4, 5 and 6 antibiotics families was detected in 16.66% of isolates. On the other hand, detection of virulence putative gene shows presence of *cadF* in 100% of tested strains. In addition, *cdtA*, *cdtB* and *cdtC* genes were detected in 100%; 89.51% and 90.32% respectively of *C. jejuni* isolates.

Conclusion: Because of the key role of broiler chicken in human campylobacteriosis infection, it will important in first time to monitoring using of antibiotics in chicken farms and in second time to verify presence of campylobacteriosis in country.

Key words: *Campylobacter jejuni*; antibiotics; virulence gene; Côte d'Ivoire.

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Introduction

Campylobacter is a food-borne zoonotic pathogen, and is considered to be one of the most common causes of bacterial gastroenteritis both in developed and developing countries [1,2]. Indeed, World Health Organization (WHO) [3] estimated that *Campylobacter* caused more than 37000 deaths per year worldwide. In developing countries, *Campylobacter* has been associated to 11.3 to 21% of diarrhea episodes in children under the age of two years [4]. Moreover, absence of epidemiological data could lead to the underestimation of the burden of *Campylobacter* infections in these regions [4]. Among all species of *Campylobacter*, *Campylobacter jejuni* is the mostly associated to humans campylobacteriosis with more 85% cases worldwide. In generally, *C. jejuni* pathogenicity is due to various factors such as cytotoxin production [5], intestinal cell invasion [6], extra-intestinal adherence and translocation [7]. Several genes have been linked to this pathogenicity but the most important are *Campylobacter* adhesin to

fibronectin F (*cadF*), *Campylobacter* invasion protein B (*ciaB*), which is vital for invasion and cytolethal distending toxin (*cdtA*, B and C), which disrupts mucosal barriers by causing host cell death [1,8]. Human campylobacteriosis due to *Campylobacter* in its acute phase is characterized by diarrhea, fever, abdominal cramps, and vomiting [9]. Moreover, *C. jejuni* infection may lead to extragastrointestinal manifestations, including bacteremia, lung infections, brain abscesses, meningitis, and reactive arthritis, in individual cases and small cohorts of patients [10]. In general, *Campylobacter* infections do not require antibiotic treatment, however the use of erythromycin, tetracycline, and quinolones is recommended in severe cases [3,11]. However, emergence of *Campylobacter* strains resistant to antibiotics since several years could make it difficult to treat human campylobacteriosis. Some studies reported that the misuse of antibiotics and the lack of control over their usage in poultry production systems promote the development of resistant and even multidrug-resistant bacteria [12].

Indeed, antibiotics are widely used to prevent, control, and treat bacterial infections as well as growth promoters in a large number of countries during poultry production [13]. These facts are of special relevance in developing countries where misuse of antibiotics and the lack of control over their usage is a problem to be addressed [12]. In Côte d'Ivoire, increased rates of antimicrobial resistant *Campylobacter* isolated from poultry have been reported [14,15].

Despite of the importance of *C. jejuni* as a foodborne pathogen [16] and the fact that chicken meat is frequently consumed and its demand increased over the years, little is known about *C. jejuni* strains contaminated poultry farms and slaughterhouses in the main market in Côte d'Ivoire. Yet, this information may help in first time to establish surveillance programs and intervention measures regarding to the presence and antimicrobial resistance of *Campylobacter* in Ivoirian poultry and in a second time to monitor human infections with *Campylobacter* in Côte d'Ivoire. The aim of this study was to investigate the prevalence, antimicrobial resistance and virulence genes profiles of *C. jejuni* in broilers slaughtered in two municipalities in Abidjan.

Methodology

Isolation and identification of *Campylobacter jejuni*

This study was conducted in two municipality of Abidjan including Adjamé and Abobo. Adjame is the principal market of Abidjan with more than 3 million visitors per day and Abobo is a commune with a large part of population compared to other in Côte d'Ivoire. During 2009 to 2010, samples were collected at the largest poultry slaughter sites in each municipality. A total of 366 samples of chicken caeca were collected over a period of one year and analyzed for *Campylobacter* isolation.

Isolation of *Campylobacter* was performed with passive filtration method preceded by enrichment as proposed by [14]. Briefly, 1 g of caeca contents was transferred to 9 mL of Preston enrichment broth base

(OXOID LTD., Basingstoke, Hampshire, UK) and 7% (v/v) defibrinated sheep blood. Incubation was performed in an anaerobic jar containing a packet generator microaerophilic atmosphere (5% oxygen, 10% carbon dioxide, 85% nitrogen) type CAMPYGen (Oxoid, Basingstoke, Hampshire, UK) during 24 hours at 37 °C. After enrichment, 300 µL of broth was filtered through acetate cellulose filter (0.45 µm) on Columbia agar (Sharlau; Barcelona, Spain) containing 5% (v/v) fresh sheep blood at aerobic conditions during 45 minutes. After this time, filter was removed and plates were incubated at 42°C during 2-5 days under microaerobic conditions. After incubation, five (5) presumptive *Campylobacter* colonies from each agar plate were identified as previously described [14]. After biochemical identification, each *Campylobacter* colony was cultured on Columbia agar supplemented with 5% of sheep blood and incubating at 42°C during 48 hours. The subculture from each positive sample was stored in 25% glycerol at -70°C until using for molecular identification and detection of virulence genes.

DNA extraction and molecular identification

DNA was extracted from freshly grown colonies on Columbia agar supplemented with sheep blood using Biomerieux DNA Bacteria Kits (Biomerieux, Marcy l'Etoile, France). In brief, colonies from on Columbia agar plates were suspended in 1mL of bidistilled sterile water and centrifuged at 14000 trs/minute for 10 minutes at 4 °C. After this, the pellet was suspended in 200 µL of bidistilled sterile water and incubated at -20°C for 1 hour. Then, the tube was immediately put at +95 °C during 10 minutes by using dry block incubator (Thermo Scientific, Dardilly, France). Samples were allowed to cool, centrifuged at 13000 × g for 5 minutes and then 100 µL of each sample was transferred in 1.5 ml microcentrifuge tubes and stored at -20°C for future use.

Molecular identification was performed as previously described [14] using PCR amplification of the *hipo* gene encoding *C. jejuni* hippurase. Sequences

Table 1. Primers sequences used in this study.

Target Gene	Primer	Sequence (5'→3')	Size (bp)	Reference
<i>cadF</i>	cadF F	TTGAAGGTAATTTAGATATG	400 bp	[19]
	cadF R	CTAATACCTAAAGTTGAAAC		
<i>cdtA</i>	GNW	GGAAATTGGATTTGGGGCTATACT	165 bp	
	IVH	ATCACAAGGATAATGGACAAT		
<i>cdtB</i>	VAT2	GTAAAAATCCCCTGCTATCAACCA	495 bp	[20]
	WMI-R	GTTGGCACTTGGAAATTTGCAAGGC		
<i>cdtC</i>	WMI-F	TGGATGATAGCAGGGGATTTTAAC	555 bp	
	LPF-X	TTGCACATAACCAAAAGGAAG		

of primers used for gene amplification are F 5' -GAA GAG GGT TTG GGT GGT G-3' and R 5' -AGC CGC ATA ATA ACT TAGCTTTG-3' [17]. PCR was performed in final volume of 50 µL mix containing 2.5 µL of each dNTP (10 mM), 2.5 µL of MgCl₂ (25 mM), 0.3 µL of Taq polymerase and 5 µL of each primer *hipo* (10 µM). PCR consisted of incubating for 5 minutes at 94°C followed by 30 cycles of 94°C for 1 minute, 49 °C for 1 minute and 72°C for 1minute.All PCR reactions were performed in thermal cycler (Gene Amp PCR system type 9700, Thermo scientific, Dardilly, France). The PCR products were stained with a 0.3% solution of SIBR Safe and were visualized under UV light after gel electrophoresis on 1.5 % agarose. Samples were confirmed as positive by comparing to the standard curve of the positive control *C. jejuni* strain (ATCC 33560).

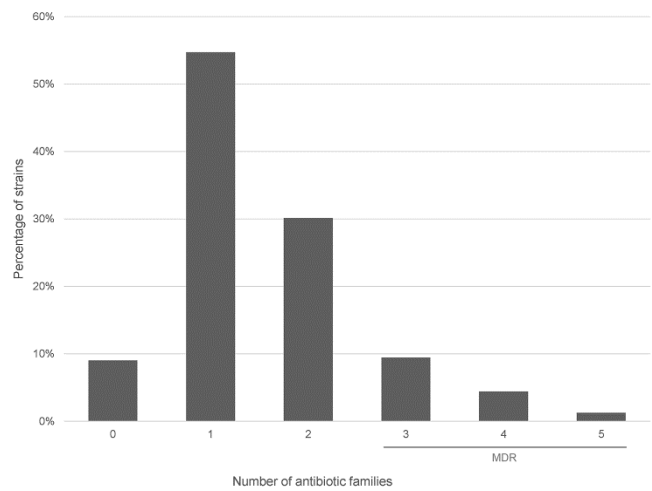
Antimicrobial Susceptibility

C. jejuni susceptibility was tested by using the disk diffusion method and the following antimicrobial disks (BioRad, Marnes-la-Coquette France) were included: Tetracycline (30 µg), Erythromycin (15 µg), Ciprofloxacin (5 µg), Azithromycin (15 µg), Gentamicin (10 µg) and Nalidixic acid (30 µg); Amoxicillin (20 µg). Susceptibility testing to all antimicrobial agents was carried out on Mueller-Hinton agar supplemented with sheep blood that were spread with a 0.5 McFarland standard suspension of *C. jejuni* in trypton saline buffered (Biorad, Marnes-la-Coquette, France) and incubated for 48 h at 37°C under microaerobic conditions. Zones of inhibition were measured and three isolates were classified as sensitive, intermediate, and resistant according to the clinical and laboratory standards institute (CLSI) [18] guidelines. *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were used as reference strains.

Potential virulence factors genes

A simple PCR method was used to detect the presence of four genes (*cadF*, *cdtA*, *cdtB* and *cdtC*), which are involved mainly in adhesion and cytotoxin production capacities. The primers used in this study

Figure 1. Percentage of resistant strains to 0 to 5 families of antibiotics.



were provided in Table 1. For *cadF* gene, amplification program consist of an initial denaturation 5 minutes followed by 30 cycles of denaturation at 94 °C for 1 minute, annealing at 49 °C for 1 minute and polymerization at 72 °C for 1 minute . A final extension was performed at 72 °C for 7 minutes. in case of *cdt* genes, PCR reactions were performed in same conditions except the annealing step which carried out at 42°C during 2 minutes.

Results

Prevalence

Among the 366 chicken's ceaca analyzed 296 (80.87%) were positives for *Campylobacter*. Moreover, biochemical and molecular tests show that 168 (56.75%) samples were positive for *C. jejuni*.

Antimicrobial susceptibility testing

Antimicrobial susceptibility was tested for all the isolates and antimicrobial resistance profiles were defined (Table 2 and Figure 1). Among 168 tested isolates, a total of 159 strains (94.64%) were resistant to one or more antimicrobial agents. The highest percentage of antimicrobial resistance was found for nalidixic acid (85.33%), Tetracyclin (71.76%) and

Table 2. Distribution of simple resistance in tested strains.

Antibiotics	Number of tested strains	Number (%) of resistant strains
Ciprofloxacin	168	93 (55.35%)
Nalidixic acid		143 (85.11%)
Tetracycline		120 (71.42%)
Erythromycin		31 (18.45%)
Azithromycin		27 (16.07%)
Amoxicillin		34 (20.32%)
Gentamicin		11 (6.54%)

Ciprofloxacin (55.65%). Comparatively, resistance levels observed to the other antibiotics were low with 20.12%; 18.5%; 16.51% and 6.71% for amoxicillin, Erythromycin, Azithromycin and Gentamicin respectively. On the other hand, 55.55% *C. jejuni* isolates were cross-resistant to ciprofloxacin and Nalidixic acid. Moreover, 32.25% of tested strains were also resistant to both Erythromycin and Azithromycin which belonging to macrolide antibiotics family. The multiple drugs resistance (MDR) was detected in 16.66% of the tested strains. Moreover, the MDR including three antibiotics families was detected 9.43% of these strains while 4.40% of them were resistant to four drugs families and 1.25% were resistant to five drugs families.

Prevalence of virulence gene

In this study, presence of genes coding for putative virulence factors in the 168 *C. jejuni* strains have been tested by PCR. As expected, *cadF* was detected in 100% of tested strains. Moreover, toxin virulence factors *cdtA*, *cdtB* and *cdtC* were present in 100%; 89.51% and 90.32% respectively of the *C. jejuni* isolates in this study.

Discussion

In this study, *Campylobacter* were isolated in 80.87% chickens ceaca analyzed and 56.75% of these were positive for *C. jejuni*. Studies from other countries showed also a high prevalence rates ranged from 77 to 100% [21,22]. Chicken's ceaca contamination by *Campylobacter* can occur during poultry production including breeding and transportation to slaughterhouse [23-26]. Furthermore, *C. jejuni* which contaminate chicken's Ceaca could also contaminate poultry carcass at the end of the slaughter process because of the noticeable lack of hygiene practice in these slaughterhouses. Indeed, some study reported that if some step of poultry slaughter process (scalding) would contribute to lowering *C. jejuni* contamination rate, others steps (plucking, evisceration and carcass washing) are important factors of the poultry meat campylobacter contamination at the end of the processing. In traditional slaughterhouses in general in Abidjan, poultry slaughterers are illiterate and have no knowledge of hygienic practices. For example, the plucking is carried out on the ground, the slaughtering equipment is not disinfected and many carcasses washing is carried out in the same water basin [21,27-29]. In these conditions, all poultry carcasses from these slaughterhouses will probably be contaminated with *C. jejuni*.

Moreover, these *C. jejuni* strains belonging high rate resistance to several antibiotics mainly with nalidixic acid, Tetracyclin and Ciprofloxacin. These high resistances obtained in our study are comparable to those observed in many countries [11,30-33]. On the other hand, low resistance has been also observed with amoxicillin, Erythromycin, Azithromycin and Gentamicin by Rivera *et al.* [34], in strains isolated in Chile and by Vinuesa-Burgos *et al.* [11] in Ecuador.

The multiple drugs resistance (MDR) corresponding to resistance to three or more families of antimicrobial agents [35] was detected in 16.66%, 9.43, 4.40% and in 1.25% of the tested strains. Those MDR strains were recovered from broilers worldwide [36]. The appearance of these combinations of resistance is a direct consequence of the uncontrolled use of antibiotics in the poultry sector in Côte d'Ivoire [37]. The antibiotics tested being the most used molecules in cases of *Campylobacter* infection in humans, so existence of these resistances could constitute a major problem of public health given the risks of therapeutic failure [11]. Therefore, the search for new approaches to limit the spread of these strains in poultry farming in first time and also on products derived from poultry likely to be contaminated is required. Indeed, data collected through the world indicate that poultry products are responsible for a significant proportion (20-40%) of *Campylobacter* human infections and many cases of others enteropathogens diseases such as salmonellosis [1].

In addition, virulence genes *cadF* and *cdtA* were detected in all tested strains while, *cdtB*, and *cdtC* were isolated in 89.51% and 90.32% respectively in these isolates. This high prevalence of these four (4) genes is in agreement with several studies that found it in nearly all of the *Campylobacter* isolates [1,38]. The *cadF* gene is coding for a putative OmpA-like protein that mediates bacterial adhesion by binding to host fibronectin. This ability of *Campylobacter* to bind to host epithelial cells is a key step in expression of pathogenicity especially since this ability contributes to colonization and invasion of host organism [39,40]. The high prevalence of this gene in our study could indicate this decisive role of the *cadF* gene in the virulence capacity of *C. jejuni*. However, Iglesias-Torrens *et al.* [38] studies suggest that *cadF* may not be essential for the ability of *Campylobacter* to cause infection in humans. Therefore, we hypothesize that the high prevalence of the virulence factors in our finding is more indispensable for these strain in the colonization of the digestive tract of broilers chicken as reported by Ziprin *et al.* [41] and Konkel *et al.* [42].

Regarding the genes *cdt* coding for synthesis and deliver of the cytolethal distending toxin, our findings are similar with those reported by Bang *et al.* [20], Datta *et al.* [43] and Al-Mahmeed *et al.* [44]. In our knowledge, activity of CDT toxin (consisting CDTA, CDTB and CDTC) lead to increase of intestinal epithelial cell volume by stopping cell division cycle at G2 step causing cell destruction [38,45-46]. Indeed, after adhesion to fibronectin, *C. jejuni* colonizes intestinal cells and then invasion occurs simultaneously with production of CDT toxin causing inflammatory-type diarrhea in the host organism [47]. Indeed, Purdy *et al.* [48] showed that in a mutant *cdt*; the invasion capacity of the mouse epithelial cells is considerably reduced compared to the non-mutant strain. These data show the key role of CDT toxin in the occurrence of *Campylobacter* infection. Thus, presence of these putative virulence factors in these *C. jejuni* strains may constitute a major health problem since customers of artisanal slaughterhouses generally consist of restaurant owners and the monitoring sanitary systems are non-efficient in Côte d'Ivoire.

Conclusion

This study revealed presence of *C. jejuni* with virulence gene including *cad F*, *cdtA*, *cdtB* and *cdtC*. Moreover, our results indicated a high level of resistance of tested strains to antibiotics generally used in treatment of diarrheal cases in Côte d'Ivoire. Because *C. jejuni* is the most common cause of bacterial gastroenteritis, it is crucial to investigate a thorough and reliable monitoring program to reduce the availability of contaminated chicken's products in our country.

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