



Genotyping and Antimicrobial Resistance of *Campylobacter Jejuni*: A Review

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Abstract | *Campylobacter* is one of the major cause of bacterial foodborne diarrheal disease all over the world. Different typing methods are used to differentiate between the bacteria at both species and subspecies levels and to identify the pathogenic microorganisms, as *Campylobacter jejuni* (*C. jejuni*) and *Campylobacter coli* (*C. coli*). During the past few decades, an increasing number of *Campylobacter* species (spp.) have developed resistance to fluoroquinolones and other antimicrobial agents like aminoglycosides, macrolides and beta-lactams. Herein, we discussed in details the genotypic techniques used for typing of *C. jejuni*. Moreover, the emergence of resistance of *C. jejuni* to the antimicrobial agents and their mechanisms of action were fully illustrated.

Keywords | Genotyping, *C. jejuni*, Campylobacteriosis, Antimicrobial resistance

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INTRODUCTION

Campylobacter species are ubiquitous bacteria that are able to colonize the mucosal surfaces, usually the intestinal tracts of most mammalian and avian species. The major sources of *Campylobacter* infection in humans are raw or uncooked meat (especially poultry meat), contaminated water, unpasteurized milk and contact with infected animals. *C. jejuni* is frequently isolated from poultry that is responsible for the majority of human campylobacteriosis, followed by *C. coli*, and less common *C. lari* (Blaser and Engberg, 2008; Hao, 2013). Human campylobacteriosis is characterized by mild to serious injuries to permanent neurological symptoms affecting all ages from children to the elderly (Silva *et al.*, 2011).

Most *Campylobacter* infections are self-limited and could be relieved in a short time without antibiotic treatment. However, severe or prolonged infections may occur specially in young, elderly and immunocompromized

individuals. In these circumstances, fluoroquinolones (FQ) and macrolides may be used for treatment of *Campylobacter* infections (Allos, 2001). However, beta-lactams are not recommended for the treatment of campylobacteriosis except the oral beta-lactam co-amoxiclav, which could be efficient against resistant *Campylobacter* isolates to FQ or macrolides (Wirz *et al.*, 2010).

Molecular methods have become widely applied to subtype *C. jejuni* since they provide more sensitive strain differentiation and higher levels of standardization, reproducibility, typeability, and discriminatory power when compared with phenotypic typing methods (Wassenaar and Newell, 2000; Wiedmann, 2002; Eberle and Kiess, 2012). These may be divided into two broad categories: macro-restriction mediated analyses based on separation of restriction enzyme digested nucleotide sequences, and polymerase chain reaction (PCR) based assays (Mohan, 2011). Pulse field gel electrophoresis (PGFE), also known as field alteration gel electrophoresis (FAGE) or macro-

restriction profiling PFGE, has emerged as one of the best molecular approaches to analyze the bacterial pathogens, including *Campylobacter* (Ahmed et al., 2012; Eberle and Kiess, 2012).

On the other hand, *Campylobacter* resistance to clinically important antibiotics is increasingly prevalent and considers one of the major public health concern. Although the vertical transmission of *Campylobacter* spp. is questionable, chicken breeder, which hosts antibiotic-resistant bacteria, can pose a public health threat because it can indirectly transfer antibiotic-resistant *Campylobacter* spp. to broiler chickens in the production chain, leading to human infection (Du et al., 2018). Therefore, development and transmission of antibiotic-resistant *Campylobacter* is complicated as *Campylobacter* is a zoonotic pathogen and is therefore exposed to antibiotics generally used in both animal production and human medicine.

This review will focus on different genotypic approaches for discrimination of *C. jejuni* at species and subspecies levels and the mechanisms of antimicrobial resistance associated with it.

CAMPYLOBACTER SPECIES CHARACTERISTICS

Members of the *Campylobacter* genus are slender, spirally curved, and non-spore forming Gram-negative rods. The size of bacterial cells is small and ranges from 0.2 to 0.9 μm in width and 0.5 to 5 μm in length (Silva et al., 2011). Certain species as *C. gracilis* and *C. hominis* form straight rods. Most species are motile by single polar unsheathed flagellum either at one or both poles of the cells (monotrichous or amphitrichous). On the other hand, *C. showae* has up to five unipolar flagella, while *C. gracilis* is non-motile (Fitzgerald and Nachamkin, 2015). Motility of the bacteria is rapid and darting with their pinning around their long axes in a corkscrew manner. Owing to their small size and motility, *Campylobacter* spp. can pass through membrane filters (0.45 to 0.65 μm) easily, allowing their isolation from clinical samples (Kramer et al., 2000).

Campylobacter spp. are non-fermentative, oxidase and catalase tests positive. They can survive in the environment with low oxygen concentration. However, *C. jejuni* can be changed to coccoid form when exposed to the atmospheric oxygen (Vandamme et al., 2006). *Campylobacter* spp. are best cultured at 42°C and can survive for a short time at a refrigerator temperature up to 15 times than at 20°C. However, their survival is poor at room temperature and die out slowly through freezing as well as the cells are heat sensitive and are destroyed at a temperature greater than 48°C (Crushell et al., 2004).

Campylobacter is considered as an etiological agent of gastroenteritis in humans that responsible for

approximately 166 million diarrheal cases and 37,600 deaths per year globally (Oh et al., 2018). *Campylobacter* species of clinical significance are *C. jejuni* and its closely connected *C. coli*, which represents more than 90% of human infections due to the consumption of contaminated meat and meat products (Mikulić et al., 2016). The natural reservoirs of *Campylobacter* species are the intestinal tracts of domesticated and wild birds as well as mammals. The consumption or the mishandling with raw or undercooked meat in particular poultry meat is considered to be the major risk factor for human campylobacteriosis (Szczepanska et al., 2017). Recently, the European Food Safety Authority (EFSA) described the factors influencing campylobacteriosis infections, namely the age (higher occurrence rates in children under 5 years old), the season (a higher number of campylobacteriosis cases is reported during the summer months), the strain variation (certain strains are less pathogenic than others), host immunity, travel and the demographic factors (i.e., the social economic status). Campylobacteriosis is estimated to affect over 2.4 million people causing diarrhea, abdominal pain, fever, headache, nausea, and vomiting and costing approximately \$1.2 billion annually (CDC, 2008; FDA, 2009). One out of every 1,000 cases may be affected by a serious demyelinating neuropathy known as Guillain-Barre syndrome (CDC, 2008). Infected individuals experience a rapid decline in muscle strength in the limbs and respiratory system (Nachamkin et al., 1998; Keener et al., 2004). In the United States, the mean cost per patient with Guillain-Barre syndrome is estimated around \$318,966, totaling to \$1.7 billion annually (Frenzen, 2008).

All *Campylobacter* spp. have two flagellin genes, *fla A* and *fla B*. Moreover, the cytolethal distending toxin (CDT) is the most common virulence factor which causes cellular distension that resulted in cell death (Lara-Tejero and Galán, 2001). Other virulence associated genes as *wla N*, *waa C*, *cgt B* lipo-oligosaccharides, phospholipase A (*pld A*), type IV secretory protein (*vir B11*) and invasion protein (*cia B*) genes were also reported (Konkel et al. 1999; Müller et al., 2006).

GENOTYPING METHODS

MULTILOCUS ENZYME ELECTROPHORESIS (MLEE)

In MLEE, the bacterial isolates could be discriminated by the difference in the electrophoretic mobility of diverse constitutive enzymes by electrophoresis under non-denaturing conditions (Wiedmann, 2002). This technique has been utilized to study the congruence between other typing schemes used for *C. jejuni* such as multilocus sequence typing (MLST) and pulse field gel electrophoresis (PFGE) (Sails et al., 2003b). Because of its limitations, MLEE has been rendered unsuitable for regular typing and has been superseded by a nucleotide-based technique MLST, which essentially mimics the MLEE's multi loci

principle (Mohan, 2011).

PULSE FIELD GEL ELECTROPHORESIS (PFGE)

The enormous discriminatory power of PFGE makes it the gold standard for investigations. However, the interpretation of PFGE data is difficult, this technique is appropriate as a tool for routine use during outbreak investigation (Sails et al., 2003a). It has been widely used in genetic and epidemiological examinations of *C. jejuni* and *C. coli* (Mohan, 2011; Ahmed et al., 2012).

POLYMERASE CHAIN REACTION (PCR)

PCR technology has the ability to detect the presence or absence of an organism in any sample by detecting a specific gene unique to the particular organism of interest (Mohan, 2011). Several variations in the original PCR technique are developed and are used for detecting *Campylobacter* spp. including multiplex PCR, reverse-transcriptase PCR and quantitative real-time (QRT)-PCR (Eberle and Kiess, 2012). Multiplex PCR assays which are used for simultaneous differentiation of *Campylobacter* spp. have replaced uniplex PCR, which were widely used for detection and differential diagnosis of *Campylobacter* spp. in the past (Yamazaki-Matsune et al., 2007; Asakura et al., 2008). These techniques are highly discriminatory and easily available in most laboratories. Although they may be expensive, they are still the most frequent genotyping methods for *Campylobacter* spp. (Eberle and Kiess, 2012).

Most of the genotyping techniques are PCR based since it is simple, rapid, and cost effective (Asakura et al., 2008). Random amplified polymorphic DNA analysis (RAPD) and amplified length polymorphism (AFLP) are two PCR-based methods used for *Campylobacter* spp. genotyping. They provide a good discriminatory power despite some limitations and they are not successfully used as routine genotyping tool (Mohan, 2011).

Ribotyping is a ribosomal RNA (rRNA) based technique used for identification of the bacterial isolates (Williams et al., 1998). Multiple copies of the rRNA gene loci coding for 5, 16, and 23S rRNA could be detected at different locations on the chromosome of *Campylobacter* spp. Strong conservative nature with the existence of the non-coding flanking regions are common features for the rRNA genes to be used in this typing approach (Wassenaar and Newell, 2000). The ribotyping method has a high degree of typability for *Campylobacter* spp. However, its small number of ribosomal genes giving a low discriminatory power. This method is also laborious, time-consuming, and expensive, thereby, it is unsuitable for routine genetic typing (Eberle and Kiess, 2012). Automated ribotyping (AR) systems have been developed to reduce labor and increase sensitivity in identification of foodborne pathogens. They combine molecular steps in one efficient device, making the test

method faster and more reliable (Pavlic and Griffiths, 2009). Flagellin typing using restriction fragment length polymorphism (RFLP) is another technique used for typing of *Campylobacter* spp. Although this technique is rapid and has a high discriminatory power, it is not recommended to be the only technique used in epidemiological investigation of the isolates. Subsequently, it is often used with other typing techniques mostly Multilocus sequence typing (MLST) (Eberle and Kiess, 2012; Mohan, 2011). The nucleotide sequence of a short variable region (SVR) of a gene provides important information on the *Campylobacter* spp. fingerprint. Recent studies stated that direct sequencing of SVR amplicons of both *fla A* and *fla B* genes is useful for *Campylobacter* spp. typing, especially in short-term and localized epidemiological investigations, which has similar or higher discriminatory power than MLST (Wassenaar et al., 2009; Wirz et al., 2010).

MULTILOCUS SEQUENCE TYPING (MLST)

It is a genotyping method that was first developed in 1991 based on the MLEE principles. This technique varies from MLEE in which it appoints alleles directly by DNA sequencing of 7-11 housekeeping genes and indirectly through the electrophoretic mobility of their amplicons (Eberle and Kiess, 2012).

Nowadays, MLST is the foremost molecular typing method for *Campylobacter* spp. (Ross, 2009). A specific MLST system has been developed and increasingly used in epidemiological studies to characterize *C. jejuni* isolates (Dingle et al., 2001), while the extended MLST method is able to characterize not only *C. jejuni* but also *C. coli*, *C. lari* and *C. upsaliensis* (Miller et al., 2005). However, MLST is also unable to identify closely related strains in short-term outbreak studies, additional methods such as *fla* typing may be needed in order to obtain sufficient accuracy (Sails, et al., 2003b).

COMPARATIVE GENOMIC FINGERPRINTING (CGF)

It is a novel method of comparative genomics based bacterial characterization that relies on the conception that differential existence of accessory genes could be used to generate unique genomic fingerprints for genotyping purposes (Ross, 2009). Taboada et al. (2012) developed and validated a fast and high-resolution, comparative genomic fingerprint method of 40 genes for *C. jejuni* (CFG-40). Results obtained using this method suggesting higher discrimination power than MLST at clonal complex and sequence type levels. Whilst, it is also quick, not expensive and easy to deploy for routine epidemiological surveillance and outbreak investigations (Clark et al., 2012; Taboada et al., 2012).

NEXT-GENERATION SEQUENCING OF *CAMPYLOBACTER* (NGS)

In this technology, whole genome sequencing (WGS) was actively used to illuminate the individual genomes of

foodborne bacterial pathogens in outbreaks in addition to the complex metagenomics of the microbiomes related to foodborne pathogens (Park et al., 2014; Cao et al., 2017; Ronholm, 2018).

As documented by Pendleton et al. (2013), WGS was the most discriminatory among PFGE and *fla* A typing providing supplementary data as the genome size and the genomic content (GC).

INFECTIONS MEDICATION AND EMERGENCE OF ANTIMICROBIAL RESISTANCE

Campylobacter spp. are generally susceptible to chloramphenicol, aminoglycosides, clindamycin, imipenem and nitrofurantoin. However, high rates of resistance make amoxicillin, tetracycline, ampicillin, cephalosporins and metronidazole bad choice to treat infections with *C. jejuni*. All *Campylobacter* spp. are resistant to rifampin, trimethoprim and vancomycin (Mandal et al., 1984).

Erythromycin has become the best medication for treatment of *Campylobacter* infection. Unlike fluoroquinolones, erythromycin and tetracycline can be safely given to children and pregnant women and is less likely than many factors to exert an inhibitory effect on other fecal normal flora (Ternhag et al., 2007). The newer macrolides, clarithromycin and azithromycin are effective against *C. jejuni* infection, but are more expensive than erythromycin and do not provide any clinical advantage. However, a recent study in Peru, eastern South America, reported that 77.4% of *C. jejuni* isolates and 79.8% of non-*C. jejuni* isolates were resistant to ciprofloxacin; 55.8% of *C. jejuni* isolates and 49.0% of non-*C. jejuni* isolates were resistant to tetracycline, whereas gentamicin resistance was detected in 15.8% of non-*C. jejuni* isolates. Moreover, 4.9% of *C. jejuni* isolates and 24.8% of non-*C. jejuni* isolates exhibited resistance to azithromycin (Schiaffino et al., 2019).

In Egypt, poultry meat is considered a serious vehicle of antimicrobial resistant *Campylobacter* transmission to humans due to the excessive use of the antimicrobial agents for treatment, prophylaxis as well as growth promotion. There was a remarkably high resistance rate exhibited by *C. jejuni* and *C. coli* to penicillin (95 and 90.1%, respectively), chloramphenicol (90 and 90.1%, respectively) and gentamicin (80 and 81.81%, respectively). However, lower resistance against nalidixic acid (30 and 36.36%) and ciprofloxacin (10 and 18.81%) were reported for *C. jejuni* and *C. coli*, respectively (Hafez et al., 2018).

ANTIMICROBIAL RESISTANCE MECHANISMS

FLUOROQUINOLONE RESISTANCE

In *Campylobacter*, point mutations in quinolone resistance-determining region (QRDR) of DNA gyrase A (*Gyr* A), and no mutations in DNA gyrase B (*Gyr* B) were reported

in FQ resistance (Piddock et al., 2003). Actually, *parC/parE* genes (encoding topoisomerase IV) are involved in FQ resistance in Gram-negative bacteria but their mutations are not responsible for *Campylobacter* resistance to FQ. Only one-point mutation in QRDR of *gyrA* is enough to decrease the sensitivity of *Campylobacter* spp. to fluoroquinolones. The predominant mutation in the FQ resistant *Campylobacter* isolates is the change of C257T in *gyrA* gene that results in replacement of T86I giving high resistance to fluoroquinolones (Thakur et al., 2010). Abuoun et al. (2005) and Zhong et al. (2016) reported that the resistance-associated mutations include D90N, A70T and T86K, which are less common and do not give high fluoroquinolone resistance such as those observed for T86I mutation.

MACROLIDE RESISTANCE

Macrolides prevent protein synthesis by binding to P site on 50S subunit of bacterial ribosomes. The main mechanisms for resistance to macrolides in *Campylobacter* spp. are target modification, flow and membrane permeability. They work synergistically to provide high-grade macrolide resistance. While enzymatic modification of macrolides, was not described in *Campylobacter* spp. (Zhao et al., 2016).

Campylobacter resistance to macrolide is associated with active efflux and target modification. Modification of the target ribosome leading to macrolide resistance, can occur either by point mutation in ribosomal proteins L4 and L22 and the 23S rRNA or enzyme mediated methylation (Florez-Cuadrado et al., 2015).

It was reported that mutations of A2074C, A2074G and A2075G yielded a high-level of resistance to macrolides (erythromycin MIC >128 µg/mL) in *C. coli* and *C. jejuni*. In *Campylobacter* spp., the mutation associated with macrolide resistance usually occurs in all 3 copies of 23S rRNA gene; whereas as A2074T mutation gives a low level of erythromycin resistance may not occur in all copies of 23S rRNA gene (Bolinger and Kathariou, 2017). In case of intermediate or low-level of macrolide resistance, failure to activate the *CmeABC* efflux pump will restore the isolation capability completely. In highly resistant *Campylobacter* strains with mutations of A2074G or A2075G, inactivation of the *CmeABC* also led to a significant reduction in the level of macrolide resistance, indicating that this flow system works synergistically with target mutation (Gibreel et al., 2007).

TETRACYCLINE RESISTANCE

Tetracycline resistance may be caused by efflux or the enzymatic change of antibiotics or ribosome protection. In *Campylobacter* spp., the resistance to tetracycline is induced by *tetO* gene. It is believed that there is no other *tet* resistance genes in *Campylobacter* spp.

(Wieczorek and Osek, 2013). Connell and his colleges (2003) proposed that the protein meet at an open A site on the bacterial ribosome and bind to it in such a way as to induce a conformational change that results in releasing of the associated tetracycline molecule. Moreover, this conformational change lasts for a long time to allow the continued elongation of the protein in an effective way. Based on codon use, G–C content, crossbreeding and sequential symmetry studies, it is clear that *Campylobacter tet O* might be acquired through horizontally transmitted genes from *Streptococcus*, *Streptomyces* or *Enterococcus* species (Liu et al., 2017). In most *Campylobacter* strains, *tet O* gene is encoded on plasmid, while, some isolates have a chromosomally encoded gene copy. In *C. jejuni*, tetracycline resistance is associated with a multidrug efflux pump known as *CmeABC* (Kempf et al., 2017).

AMINOGLYCOSIDE RESISTANCE

Campylobacter resistance to aminoglycosides provides drug modification proteins. Several enzymes are needed to modify the aminoglycosides, thorough 3', 9-aminoglycoside adenylyl transferase, 6-aminoglycoside adenylyltransferase and 3'-aminoglycoside phosphotransferase (types I, III, IV and VII) (Ramirez and Tolmasky, 2010).

β- LACTAM RESISTANCE

In general, β-lactams possessed limited effectiveness against *Campylobacter* spp. and resistance to these antibiotics is mediated by production of β-lactamase and intrinsic resistance. Three mechanisms for resistance to β-lactam in *Campylobacter* spp. are known: enzyme inhibition by chromosomal coding lactamases, reduced absorption due to changes in the permeability of outer membrane and the work of efflux pumps (Wieczorek and Osek, 2013).

RESISTANCE TO OTHER ANTIMICROBIAL AGENTS

Campylobacter spp. show substantial resistance to many antibiotics as novobiocin, bacitracin, streptogramin B, rifampin, vancomycin and trimethoprim. However, the mechanisms of this resistance are unknown and can be mediated by the low permeability of *Campylobacter* membrane and the active efflux provided by multidrug-efflux transporters (Denis et al., 2015).

CONCLUSIONS

Typing methods play an important role in the detection, monitoring and prevention of *Campylobacter* infections. Not a single technique is perfect, so developing a new typing method that combines efficiency with efficacy, while overcomes the shortcomings of currently methods used, is critical. Antimicrobials should be prudently used by the implementation strategies, and guidelines are required to control and limit the development and spread of resistant

bacteria and the genes encoded for this resistance especially in poultry farms and livestock.

AUTHORS CONTRIBUTION

Ahmed M. Ammar, Norhan K. Abd El-Aziz and Attia A. Elgdawy contributed to the design and implementation of the research. Mona S. Emara and Mona M. Hamdy collected literature and drafted the manuscript in consultation with Norhan K. Abd El-Aziz. All authors approved the final manuscript.

CONFLICT OF INTEREST

No conflict of interests is declared.

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