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Review article
Ανασκόπηση

The genus *Campylobacter:* detection and isolation methods, species identification & typing techniques

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Το γένος *Campylobacter:* μέθοδοι ανίχνευσης και απομόνωσης, ταυτοποίηση είδους και τεχνικές τυποποίησης

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ABSTRACT. *Campylobacter* is well recognized as the leading cause of bacterial foodborne diarrheal disease worldwide; while, poultry has been identified as a significant cause of campylobacter infection in humans. The *C. jejuni* has been found to be the predominant species isolated from poultry samples and, yet, responsible for the majority of human campylobacteriosis. *Campylobacter* spp. are small, oxidase positive, microaerophilic, curved gram-negative rods exhibiting corkscrew motility and colonize the intestinal tract of most mammalian and avian species. From its very first description in late 19th century by Theodor Escherich until nowadays, a lot of research has been carried out providing a wealth of information regarding its microbiological properties. Since novel technologies constantly emerge, increasingly advanced methods for detection, identification and typing of *Campylobacter* spp. are becoming available. The aim of this article is to review the recent bibliography on *Campylobacter* focusing, especially, on its survival and growth characteristics, the laboratory methods used for its detection and isolation from clinical, animal, environmental, and food samples, the reported methods applied for its speciation, as well as the typing systems developed for subtyping of *Campylobacter.*

Keywords: *Campylobacter* spp., detection, isolation, species identification, typing.

ΠΕΡΙΛΗΨΗ. Το *Campylobacter* είναι παγκοσμίως αναγνωρισμένο ως ο συχνότερος αιτιολογικός παράγοντας της βακτηριακής αιτιολογίας, διαρροϊκής τροφοδηλητηρίασης, ενώ τα πουλερικά έχουν αναγνωριστεί ως η κύρια αιτία μόλυνσης του ανθρώπου. Το *C. jejuni* είναι το είδος που απομονώνεται συχνότερα από δείγματα προερχόμενα από πουλερικά και συνεπώς ευθύνεται για τα περισσότερα περιστατικά ανθρώπινης καμπυλοβακτηρίωσης. Τα *Campylobacter* spp. είναι μικροί, θετικοί στη δοκιμή οξειδάσης, μικροαερόφιλοι,gram-αρνητικοί, κυρτοί βάκιλοι που παρουσιάζουν χαρακτηριστική ελικοειδή κίνηση και αποικούν τον εντερικό σωλήνα των περισσότερων θηλαστικών και πτηνών. Από την πρώτη περιγραφή τους στα τέλη του 19ου αιώνα από τον Theodor Escherich έως σήμερα, έχει διεξαχθεί σημαντική έρευνα που πρόσφερε πλούτο πληροφοριών σχετικά με τα μικροβιολογικά τους χαρακτηριστικά. Χάρη στη συνεχή εμφάνιση καινοτόμων τεχνολογιών, όλο και πιο προηγμένες μέθοδοι ανίχνευσης, ταυτοποίησης και γενοτύπησης γίνονται διαθέσιμες. Σκοπός αυτού του άρθρου είναι η ανασκόπηση της πρόσφατη βιβλιογραφία σχετικά με το *Campylobacter* εστιάζοντας κυρίως στα καλλιεργητικά του χαρακτηριστικά, τις εργαστηριακές μεθόδους που χρησιμοποιούνται για την ανίχνευση και την απομόνωσή του από κλινικά, ζωικά, περιβαλλοντικά και δείγματα τροφίμων, τις καταγεγραμμένες μεθόδους που χρησιμοποιούνται για τη ταυτοποίηση του είδους, καθώς και τα συστήματα γενοτύπησης που έχουν αναπτυχθεί για την υποτυποποίηση του *Campylobacter*.

Λέξεις ευρετηρίασης: Campylobacter spp., τροφιμογενή παθογόνα, Ελλάδα, πτηνά, επιπολασμός, παράγοντες κινδύνου.

INTRODUCTION

Campylobacters are ubiquitous bacteria, able to colonize mucosal surfaces, usually the intestinal tract of most mammalian and avian species tested (OIE, 2008). *Campylobacter* is well recognized as the leading cause of bacterial foodborne diarrheal disease worldwide; while, the poultry has been identified as a significant source for *Campylobacter* infections in humans. The *C. jejuni* is the predominant species isolated from poultry samples, followed by *C. coli,* and other less-detected *Campylobacter* species such as *C. lari* (EFSA, 2010). The *C. jejuni* is considered responsible for the majority of human campylobacteriosis, followed by *C. coli*, and rarely by C. lari (Zhang and Sahin, 2013). The incidence of human campylobacteriosis has been steadily rising worldwide since 1990's (WHO, 2011). While in Greece there is a dearth of data (Natsos et al., 2016), in the European Union, campylobacteriosis has been the most commonly reported zoonosis since 2005 (EFSA, 2006; EFSA and ECDC, 2017), in the United States, the incidence of Campylobacter infections per 100,000 people was the highest along with Salmonella (CDC, 2018), in Australia *Campylobacter* has been found to be the most common cause of acute bacterial diarrhea among all

the notified enteric pathogens (Stafford, 2010), while human campylobacteriosis is hyperendemic in many developing areas of the world (Coker et al., 2002).

THE GENUS Campylobacter: A HISTORICAL OVERVIEW

The generic name *Campylobacter*, from the Greek kampylos (curved) and baktron (rod), was given by Sebald and Véron (1963) to the group of bacteria formerly known as the microaerophilic vibrios, due to their special *characteristics* (Moore et al., 2005). It is believed that Campylobacter species were first described by Escherich (1885) who observed non-culturable spiral-shaped bacteria in the large-intestinal mucus of infants who had died of cholera infantum (Vandamme, 2000), while McFadyean and Stockman (1913) were the first to isolate these organisms from the uterine exudate of aborting sheep. A few years later, the study of Butzler et al. (1973) raised the interest in *Campylobacter* as a cause of human disease by noting their high incidence in cases of diarrhea. The first successful isolation of Campylobacter from human faeces had been accomplished one year before by using a filtration technique (Dekeyser et al., 1972). Later, the isolation of Campylobacter became a routine in the field of clinical microbiology and Campylobacter spp. rapidly became recognized as a common cause of bacterial gastroenteritis (Fitzgerald et al., 2008a).

CLASSIFICATION

In the 1970s, there was much confusion over *Campylobacter* nomenclature (Skirrow, 1994); however, the classification of Véron and Chatelain (1973) forms the basis of currently approved nomenclature. The family Campylobacteraceae, proposed by Vandamme and De Ley (1991), consists of two genera, *Campylobacter* and Arcobacter (Vandamme, 2000); while, the genus of *Campylobacter* currently contains 34 species and 14 subspecies (Parte, 2014). The taxonomy of the Campylobacter genus, which has been revised many times (Debruyne et al., 2008), is reviewed by On (2001).

MORPHOLOGY

Members of the *Campylobacter* genus are slender, spirally-curved, and non-sporeforming gram-negative rods. The size of the 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). Some species, such as *C. hominis* and *C. gracilis,* form straight rods (Fitzgerald et al., 2008a). Most species are motile by means of a single polar unsheathed flagellum inserted at one or both poles of the cells (monotrichate or amphitrichate) (Vandenberg et al., 2005). The only exceptions are *C. showae*, which has up to five unipolar flagella, and C. gracilis, which has none and is immotile (Debruyne et al., 2008). Motility is rapid and darting, with the bacteria spinning around their long axes in a corkscrew fashion (Vandenberg et al., 2005). Because of their small size and motility, *Campylobacter* spp. are able to pass through membrane filters (0.45 to 0.65 μm) with relative ease, a property used for isolating Campylobacter spp. from clinical samples (Bolton, 2000; Steele and McDermott, 1984).

GROWTH AND SURVIVAL CHARACTERISTICS

Under ideal conditions, Campylobacters produce visible growth after 24 h at 37 °C, but colonies are not well formed until 48 h; however, it may take up to 72-96 hours of incubation to observe some slow-growing strains (Corry et al., 1995). Depending on the media used, the appearance of *Campylobacter* colonies may vary. If the agar is moist, the colonies may appear gray, flat, irregular, and thinly spreading; whereas, round, convex, or glistening colonies may be formed when plates are dry (Corry et al., 1995; Vandenberg et al., 2005). Since the pathogenic *Campylobacter* species grow at 37-42 °C, with an optimum growth temperature of 41.5 °C, they are used to be referred as thermophilic Campylobacters: although Levin (2007) suggested the term “thermotolerant” since they do not exhibit true thermophily (growth at 55°C or above). Campylobacters are incapable of growth below 30°C, as they lack cold shock protein genes which play a role in low-temperature adaptation (Silva et al., 2011).

These non-spore-forming and fastidious bacteria neither ferment nor oxidize carbohydrates; instead, they obtain energy from the degradation of amino acids, or tricarboxylic acid cycle intermediates (Kelly, 2001; Vandamme, 2000). They are essentially

microaerophilic, thus an atmosphere with low oxygen tension (5% O2, 10% CO2, and 85% N2) is regarded as the most suitable for *Campylobacter's* incubation (Garénaux et al., 2008). Oxidase activity is present in all species except for *C. gracilis* (Silva et al., 2011).

Except of their fastidious growth requirements, *Campylobacter* spp. are very fragile and more susceptible than most bacteria to many environmental conditions, such as temperature and pH changes, low humidity, presence of oxygen and UV irradiation, and to many chemical agents such as disinfectants (Isohanni, 2013). *Campylobacter* spp. are easily inactivated by heat treatments with their D-value being less than 1 min (Silva et al., 2011), while freezing and thawing causes a 1-2 log10 fall in viable numbers, yet bacteria remain alive for many months at -20 oC (Vandenberg et al., 2005). Most species have a pH growth range of 5.5-8.0, though optimal growth occurs at pH 6.5-7.5 and water activity (aw) equal to 0.997 (approximately 0.5% w/v NaCl), as mentioned by Silva et al. (2011).

In some species, notably *C. jejuni* and *C. lari,* cultures that are exposed to atmospheric oxygen (Vandenberg et al., 2005) or other unfavorable conditions, such as changes in temperature and pH, dehydration and low nutrient availability, may undergo coccal transformation (Jackson et al., 2009; Kassem et al., 2013; Oliver, 2010; Rollins and Colwell, 1986), which seems to be a degenarative process in response to these circumstances (Harvey and Leach, 1998; Reezal et al., 1998). Those viable but non-cultivable cells (VBNC) have been shown to be unable to grow in subculture; even though the possibility that they can revert to spiral forms after passing through the intestinal tract of chickens or humans remains unanswered (Oliver, 2010; Vandenberg et al., 2005) and even their existence is contentious (Silva et al., 2011).

LABORATORY ISOLATION AND DETECTION METHODS

In a clinical context, a laboratory is mainly asked to detect campylobacters in the faeces of patients with diarrhea. The same purpose also applies when it comes for samples derived from animal stool, environmental materials, or processed food. There are two main categories regarding the detection method used: the conventional culture-based isolation methods and the culture-independent methods.

Culture-based isolation methods

The conventional method for isolating the common enteric *Campylobacter* species from faecal samples is a primary plating on selective media followed by incubation at 42 oC in a microaerobic atmosphere (Vandenberg et al., 2006). Though faeces often contain large numbers of viable *Campylobacter* making their detection easily possible by direct plating on selective media (Fitzgerald et al., 2008b), food products and environmental samples tend to have fewer numbers of stressed *Campylobacter* cells, thus, an enrichment step in liquid medium before plating on solid agar plates is indicated (Corry et al., 1995). Several enrichment broths (e.g. Bolton broth, *Campylobacter* enrichment broth and Preston broth), that are available to be used before plating, have been compared for their efficacy (Baylis et al., 2000).

The first selective culture medium for culturing *C. jejuni* and *C. coli* was developed in 1977 by Skirrow. Since then more than 40 solid and liquid selective culture media for culturing *Campylobacter* from clinical and food samples have been reported and evaluated (Habib et al., 2008; Kiess et al., 2010; Potturi-Venkata et al., 2007). All the selective media contain a basal media, either blood or other agents such as charcoal, to quench oxygen toxicity (Fitzgerald et al., 2008a), and a variety of combinations of antibiotics to which thermophilic *Campylobacter* species are intrinsically resistant; such antibiotics (like polymyxin, vancomycin, trimethoprim, rifampicin, cefoperazone, cephalothin, colistin, cycloheximide and nystatin) suppress the growth of many background microbial flora present in samples allowing the isolation of slow-growing Campylobacter spp. (Vandenberg et al., 2005; Zhang and Sahin, 2013).

The most recent standard method (ISO, 2006a) for detection and isolation, as well as a direct plating method for enumeration of *Campylobacter* spp. (ISO, 2006b), use mCCDA as the selective agar, while Bolton broth is used for the enrichment step. Alternative enrichment and plating combinations for enumeration and detection of *Campylobacter* in chicken meat have

been evaluated (Habib et al., 2011) and seem to provide significantly better results.

Direct detection methods

Microscopic observation of direct smear or wet preparation, in the case of liquid feaces, may reveal the presence of curved rods characteristic of *campylobacters* (Vandenberg et al., 2005). Dark-field microscopy may also reveal – besides the characteristic morphology – the darting motility of Campylobacter species (Fitzgerald et al., 2008a). Moreover, the direct Gram-stain with carbol-fuchsin counterstain method, though underutilized, may provide a presumptive result within 30 minutes of receipt of a feacal sample in the laboratory with relatively high sensitivity and at low cost (Wang and Murdoch, 2004).

There are also nonculture-based methods for the direct detection of campylobacters in human or animal faeces and processed food samples, which allow the identification of this fastidious organism without the specialized media and equipment needed for Campylobacter culture. Several enzyme immunoassays (EIA), which are based on antigen-antibody interaction, have been developed for this purpose in human faeces and are commercially available in a form of kits (Bessède et al., 2018; Dediste et al., 2003; Granato et al., 2010; Tolcin et al., 2000). While the culture-independent diagnostic tests (CIDTs) are convenient to use, the sensitivity, specificity, and positive predictive value of *Campylobacter* stool antigen tests have found to be highly variable (Bessède et al., 2011; Giltner et al., 2013; Granato et al., 2010) and therefore their use as standalone tests for direct detection of *Campylobacter* in stool is questioned. In addition, the utility of these assays for detection of campylobacters in chicken faeces, which represent the main reservoir of pathogenic *Campylobacter* species, remains to be determined (Zhang and Sahin, 2013). Regarding the food samples, although commercial EIAs are available for culture-independent identification of *Campylobacter* spp., these assays have not been extensively validated (Oyarzabal and Battie, 2012) and are mainly applied to enriched cultures (Bailey et al., 2008; Bohaychuk et al., 2005). Commercial and/or published immunological methods used to identify *Campylobacter* spp. in food and stool samples have been reviewed by Oyarzabal and Battie (2012).

Many PCR-based assays have been described to directly detect campylobacters in human stools from clinical cases (Al Amri et al., 2007;Lin et al., 2008; Zhang et al., 2013), feacal samples from bovine (Inglis and Kalischuk, 2004) and pigs (Jensen et al., 2005; Leblanc-Maridor et al., 2011), ceacal and feacal samples from broilers (Al Amri et al., 2007; Lund et al., 2003; Rodgers et al., 2012), samples from poultry meat (Debretsion et al., 2007; Fontanot et al., 2014; Hong et al., 2007; Josefsen et al., 2010; Schnider et al., 2010) and environmental specimens (Rothrock et al., 2009; Waage et al., 1999); although, so far these have been used only for research applications. Advantages of using a PCR approach instead of culture include same-day detection and identification of *Campylobacter* to the species level, along with the identification of the less-common Campylobacter species that are often missed by conventional culture (Kulkarni et al., 2002). However, PCR methods are more expensive and labor-intensive than culture and do not provide an isolate for further characterization such as typing and sensitivity testing.

Finally, fluorescent in situ hybridization (FISH), with the application of highly specific oligonucleotide probes, may serve for the detection and identification of thermotolerant Campylobacter spp. in feacal and liver samples, and looks promising to become a future monitoring system in a logistic poultry slaughter concept (Schmid et al., 2005).

SPECIES IDENTIFICATION

Among the Campylobacter spp. growing at 42 °C, the most frequently encountered species from samples of animal origin are *C. jejuni* and *C. coli,* however, low frequencies of other species have also been reported. Speciation is difficult because of the complex and rapidly evolving taxonomy along with the biochemical inertness of *Campylobacter* spp., and these problems have resulted in a proliferation of phenotypic and genotypic methods for identifying members of this group (Fitzgerald et al., 2008a).

Campylobacters are biochemically inactive compared with many other bacteria, thus, few phenotypic tests

are available to identify them to the species level. Generally, *C. jejuni* can be differentiated from other species based on the hydrolysis of hippurate as this is the only *Campylobacter* species that expresses the N-benzoylglycine amidohydrolase (hippuricase) gene, giving hippurate-positive result. However, variability in the hippurate reaction has been observed in some strains of *C. jejuni* resulting in hippurate-negative results (Denis et al., 1999; Jensen et al., 2005; Rautelin et al., 1999). Nalidixic acid and cephalothin susceptibility testing have been used in species identification in the past (Barrett et al., 1988). Both *C. jejuni* and *C. coli* grow at 42 °C and are resistant to cephalothin and cefoperazone, which are valuable agents for inclusion in selective media (Vandenberg et al., 2006). Instead, *C. upsaliensis* is sensible to cephalothin (ISO, 2006a). Nowadays sensitivity to nalidixic acid may give difficulties in interpretation (OIE, 2008) since fluoroquinolone resistant and cross-resistant to nalidixic acid *Campylobacter* species have become increasingly common with rates reported to be as high as 80% (Engberg et al., 2001), therefore, antimicrobial susceptibility tests can no longer be relied upon for the phenotypic identification of *Campylobacter* isolates (Fitzgerald et al., 2008a). More biochemical tests may be applied for species identification, such as the detection of catalase which is absent in *C. upsaliensis,* and the detection of indoxyl acetate hydrolysis which is negative in *C. lari* (ISO, 2006a); whereas, more extensive speciation schemes have been described in the literature (On, 1996; Vandamme, 2000).

Because of the difficulties and the unreliability of the phenotypic identification, several molecular methods may be used as supplementary to biochemical tests or even to replace them. A variety of DNA probes and polymerase chain reaction (PCR)-based identification assays has been described for *Campylobacter* species (On, 1996; Vandamme, 2000). Detection of species-specific sequences via PCR can be helpful, especially, in cases where the differentiation between hippuricase-negative *C. jejuni* strains and *C. coli* – which are closely related species – is needed, and the application of biochemical tests alone is inadequate (Denis et al., 1999; Persson and Olsen, 2005).

TYPING AND SUBTYPING

Classification of bacterial strains at the species or subspecies level is generally known as bacterial typing or subtyping. The main purposes of bacterial subtyping are the evaluation of taxonomy, the definition of phylogenetic relationships, the examination of evolutionary mechanisms, and the conduct of epidemiological investigations (Van Belkum et al., 2001). Moreover, the use of typing methods provides the opportunity to apply more rapid, precise, and efficient foodborne pathogen surveillance and prevention practices (Wiedmann, 2002). The ability to discriminate or subtype campylobacters below the level of species has successfully been applied to aid the epidemiological investigation of outbreaks of campylobacteriosis (French et al., 2011; Sails et al., 2003a; Wassenaar and Newell, 2000), providing information to recognize outbreaks of infection, to match cases with potential vehicles of infection and to discriminate these from unrelated strains.

Typing of *Campylobacter* is a dynamic field with older methods continually being advanced and new methodologies constantly being developed (Ross, 2009). A multitude of typing systems have been developed over the last few years, however, no single technique has been declared as universally acceptable and applicable (Sails et al., 2003a), since each one has both advantages and disadvantages. A number of criteria are used to evaluate subtyping methods to define their efficacy and efficiency: two major properties that any typing system should possess in order to be adapted for further use (ECDC, 2009). The efficacy of any typing technique can be assessed in terms of typeability, reproducibility, consistency, and power of discrimination; while, the efficiency reflects the expertise required, time consumed or rapidity of the technique, flexibility, and suitability to carry out a certain investigation (Mohan, 2011).

Typing systems are based on the idea that clonally related isolates share common characteristics which can be tested to differentiate them from unrelated isolates (Eberle and Kiess, 2012). They are broadly classified into two major categories: phenotyping – applies phenotypic methods that detect the presence or absence of biological or metabolic activities

expressed by the bacteria, and genotyping – utilizes genotypic methods that involve analysis of genetic elements based on the bacteria's DNA and RNA (Arbeit, 1995).

Phenotypic methods

The most popularly used phenotypic methods to differentiate *Campylobacter* isolates include biotyping, serotyping, phage typing, and multilocus enzyme electrophoresis. Even though most of these methods lack discriminatory power, they are still applied and are quite efficient in characterizing foodborne bacterial pathogens (Wiedmann, 2002).

Biotyping schemes based on the identification of bacterial isolates through the expression of metabolic activities, such as colonial morphology, environmental tolerances, and biochemical reactions, can group *C. jejuni, C. coli* and *C. lari* in broad categories (Eberle and Kiess, 2012; Vandenberg et al., 2006). Biotyping is useful as a first step for epidemiological investigation as it is easy to perform, relatively inexpensive, and can quickly identify bacterial isolates for further testing, however, due to its poor reproducibility and stability, and low discriminatory power it is often combined with serotyping to make the scheme more useful (Sails et al., 2003b).

Serologic typing, or serotyping, is based on the knowledge that different strains of bacteria differ in the antigens they carry on their cellular surfaces. In serotyping, antibodies and antisera are used to detect these surface antigens, thereby, distinguishing strains by the differences in their surface structure (Arbeit, 1995; Wiedmann, 2002). There are two generally accepted and well-evaluated serotyping schemes that were developed in the 1980s for epidemiological characterization of *Campylobacter* isolates: the first one is based on the heat stable O antigens (LPS, LOS and CPS) using a passive hemagglutination technique and was described by (Penner and Hennessy, 1980), and the other one, developed by Lior et al. (1982), is based on heat labile antigens using a bacterial agglutination method. Since the two schemes are complementary, they can give good discrimination when used together even with restricted panels of antisera (Vandenberg et al., 2005).

Phagetyping was initially performed to characterize *C. jejuni* and by (Grajewski et al., 1985) and is often used as an adjunct to serotyping. Concisely, the technique utilizes a set of virulent phages on a bacterial host irrespective of any receptors for attachment. If the phages are able to attach and infect the bacterial hosts, they lyse the bacterial cells producing a characteristic lytic pattern on the cultured petri dishes, referred to as 'plaques' (Grajewski et al., 1985). Like serotyping, the usefulness of phagetyping is also limited by the occurrence of non-typeable isolates and problems with cross reactivity (Sails et al., 2003b).

In multilocus enzyme electrophoresis (MLEE), bacterial isolates are distinguished by variations in the electrophoretic mobility of different constitutive enzymes by electrophoresis under nondenaturing 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).

Genotyping methods

The limitations associated with phenotypic subtyping methods along with the rapid growth of molecular techniques have led to the development of a wide range of molecular subtyping methods (Fitzgerald et al., 2008a). While phenotypic traits form the basis of phenotyping, genes responsible for the production of those phenotypic characters form the foundation for genotyping (Mohan, 2011). Molecular methods have become widely applied to subtype *Campylobacter* jejuni since they provide more sensitive strain differentiation and higher levels of standardization, reproducibility, typeablility, and discriminatory power, when compared with phenotypic typing methods (Eberle and Kiess, 2012; Wassenaar and Newell, 2000; Wiedmann, 2002). 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 bacterial pathogens, including *Campylobacter* (Ahmed et al., 2012; Eberle and Kiess, 2012). The PFGE is considered the 'gold standard' for epidemiological investigations due to its enormous discriminatory power (Sails et al., 2003a). Although the interpretation of PFGE data is difficult, rendering this technique unsuitable as a tool for routine use during outbreak investigation (Sails et al., 2003a), it has been extensively used in genetic and epidemiological investigations of *C. jejuni* and *C. coli* (Ahmed et al., 2012; Mohan, 2011).

The polymerase chain reaction (PCR) has certainly revolutionized molecular epidemiological studies thanks to its versatility and 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 of the original PCR technique have been developed and are applied for detecting *Campylobacter* spp., including reverse-transcriptase PCR, multiplex PCR, and quantitative real-time (QRT)-PCR (Eberle and Kiess, 2012). Notably, multiplex PCR assays, which are used for simultaneous differentiation of *Campylobacter* spp., have replaced monoplex PCR assays which were widely used for detection and differential diagnosis of *Campylobacter* spp. in the past (Asakura et al., 2008; Yamazaki-Matsune et al., 2007). These techniques are easy to reproduce, highly discriminatory, available in most laboratories and though may be expensive, they are still one of the most commonly used genotypic methods for typing *Campylobacter* spp. (Eberle and Kiess, 2012).

Apart from PCR being used as a diagnostic tool itself, 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* genotyping which provide good discriminatory power, although, due to certain limitations, these are not used successfully as a routine genotyping tool (Mohan, 2011). Ribotyping is an rRNA approach for the identification of bacterial isolates, which though has a high level of typeablility for *Campylobacter* spp., its low number of ribosomal genes gives it poor discriminatory power (Eberle and Kiess, 2012). Flagellin typing, using restriction fragment length polymorphism (RFLP), is another technique used for typing of *Campylobacter* species. Although flagellin gene typing is quick and can have high discriminatory power, it is recommended that it should not be the sole technique used in epidemiological grouping of isolates, and, therefore, it is often used in combination with other typing techniques mostly MLST (Dingle et al., 2005; Eberle and Kiess, 2012; Mohan, 2011).

DNA sequencing of one or more selected bacterial genes represents another genetic subtyping method (Wiedmann, 2002), which is becoming increasingly automated and, consequently, is a reasonable alternative method for genotyping bacterial isolates (Wassenaar and Newell, 2000). Multilocus sequence typing (MLST) is a genotypic typing method that was first developed in 1991 based on the well-established principles of MLEE (Maiden et al., 1998). This technique differs from MLEE in that it assigns alleles directly by DNA sequencing of 7 to 11 housekeeping genes rather than indirectly through the electrophoretic mobility of their gene product (Eberle and Kiess, 2012). An important component of the MLST approach is the availability of databases (e.g. PubMLST) for use by public health and research communities, where the sequence data can be compared. In turn, researchers can submit the results of their findings to these databases (Maiden, 2006).

MLST is currently the leading molecular typing method for *Campylobacter* (Ross, 2009). An increasingly used in epidemiological studies MLST system specific for the characterization *C. jejuni* strains was developed by Dingle et al. (2001), while an extended MLST method able to characterize not only *C. jejuni* but also *C. coli, C. lari,* and *C. upsaliensis,* was designed by Miller et al. (2005). The advantages of using MLST include high discriminatory power, reproducibility, ease

of interpretation and transferability of information among laboratories (Dingle et al., 2001; Wassenaar and Newell, 2000), however, it is a complex and expensive technique to perform (Ahmed et al., 2012; Djordjevic et al., 2007; Lévesque et al., 2008). Moreover, recent work has shown that the seven loci used may be insufficient to provide an accurate picture of gene content in all areas of the *C. jejuni* genome (Taboada et al., 2008). MLST is also unable to distinguish closely related strains in short-term outbreak investigations, and additional methods like fla typing may be required in order to obtain sufficient resolution (Sails, et al., 2003b).

Comparative genomics, namely the analysis and comparison of two or more genomes, has also served to underscore some of the new challenges in bacterial genotyping and phylogenetic analysis (Ross, 2009). Comparative genomic fingerprinting (CGF) is a novel method of comparative genomics-based bacterial characterization which is based on the concept that differential carriage of accessory genes can be used to generate unique genomic fingerprints for genotyping purposes (Ross, 2009). Taboada et al. (2012) developed and validated a rapid and high-resolution 40-gene comparative genomic fingerprinting method for *C. jejuni* (CFG-40). The results obtained with this method suggest that it has a higher discriminatory power than MLST at both the level of clonal complex and sequence type; while, it is also rapid, low cost, and easily deployable for routine epidemiologic surveillance and outbreak investigations (Clark et al., 2012; Taboada et al., 2012). It was shown that CGF and MLST are highly concordant, and that isolates with identical MLST profiles are comprised of isolates with distinct but highly similar CGF profiles.

CONCLUSIONS

Campylobacteriosis has become the leading foodborne disease worldwide and therefore a lot of effort is being done to achieve early diagnosis of human cases using a wide variety of direct and indirect detection methods along with specific identification tests, while epidemiological investigations of campylobacteriosis outbreaks using the innovative and constantly developing typing and subtyping systems available are increasingly conducted, providing information to recognize outbreaks of infection and match cases with potential vehicles of infection. No sole technique is perfect, thus the development of a novel typing method that combines efficiency with efficacy, while overcomes the shortcomings of currently used methods, is considered crucial

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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