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REVIEW ARTICLE

Jorge Barros-Velázquez[1]
Ana Jiménez[1, 2]
Tomás G. Villa[2]

[1]Food Technology Section, Higher Technological School, University of Santiago, Lugo, Spain
[2]Department of Microbiology, Faculty of Pharmacy, University of Santiago, Santiago de Compostela, Spain

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Correspondence to:
Tomás G. Villa. Department of Microbiology.
Faculty of Pharmacy.
5706 Santiago de Compostela. Spain.
Tel.: +34-981592490. Fax: +34-981594631.
E-mail: mpvilla@uscmail.usc.es

Isolation and typing methods for the epidemiologic investigation of thermotolerant campylobacters

Summary Thermotolerant campylobacters, *C. jejuni, C. coli, C. lari* and *C. upsaliensis*, are spiral bacteria involved in human enteric disease. The prevalence of these emerging pathogens, mainly *C. jejuni* and to a lesser extent *C. coli*, as etiologic agents of enteric disease in industrialized countries has increased over the last decade. The isolation and culture of these microorganisms is tedious and time-consuming mainly due to their complex nutritional and environmental requirements. This review discusses the techniques and methods developed for the selective isolation of thermotolerant campylobacters from food, environmental and clinical samples. Additionally, both traditional and newer molecular biology techniques applied to this group of thermophilic organisms for typing and taxonomic purposes are summarized.

Key words *Campylobacter jejuni* · *Campylobacter coli* · Typing · Enteritis · Food-borne disease

Introduction

Thermotolerant campylobacters, mainly *Campylobacter jejuni* and *Campylobacter coli*, are etiologic agents of human enteritis throughout the world. They are among the major microorganisms causing diarrhoea in Europe, the United States and other industrialized countries. Besides, campylobacter infections are hyperendemic among the infant populations of underdeveloped countries. In Galicia, like in other Spanish regions, the role of *C. jejuni* and *C. coli* in enteric disease is preponderant and is only surpassed by *Salmonella*, although *Campylobacter* infections are the major cause of enteric disease in children younger than five [22].

Species of the genus *Campylobacter* were initially considered as “spirilla” or “vibrio” but were later classified as *Vibrio fetus*. In 1947, Vinzent and Dumas reported the first case of human infection by this microorganism in a case of abortion. Later, in 1957 King linked the presence of this microorganism to certain types of human enteric disease. Determination of the guanine + cytosine (G + C) content of its DNA, indeed low (30–46%), together with its inability to ferment carbohydrates, prompted Sebald and Véron to introduce the new genus, *Campylobacter*, from the Greek *campylo* (curve-shaped) and *bacter* (rod). In 1991, Vandamme and De Ley proposed a new family, *Campylobacteraceae*, which would also include two species from the genus *Arcobacter*: *A. cryaerophilus* and *A. nitrofigilis* [60].

Structure and metabolism

The thermotolerant microorganisms belonging to the genus *Campylobacter* are spiral or curved Gram-negative non-sporeforming rods, 0.2–0.5 µm wide and 0.5–0.8 µm long. Their characteristic corkscrew-like motion is due to the presence of a single flagellum (occasionally multiple) at one or both cell poles. Nutrient limitation, aireation of the medium and the presence of free radicals all affect the transition from spiral to coccoid morphology (Fig. 1). This morphologic transition may be caused by certain changes in the structure of the peptidoglycan, mainly due to its enzymatic degradation [2].

Members of the genus *Campylobacter* are chemoorganotrophs, unable to ferment or oxidize carbohydrates. They obtain energy through the respiratory chain, from the metabolism of amino acids, and from the metabolism of intermediates of the tricarboxylic acid cycle. Menaquinone and its derivatives alkylated

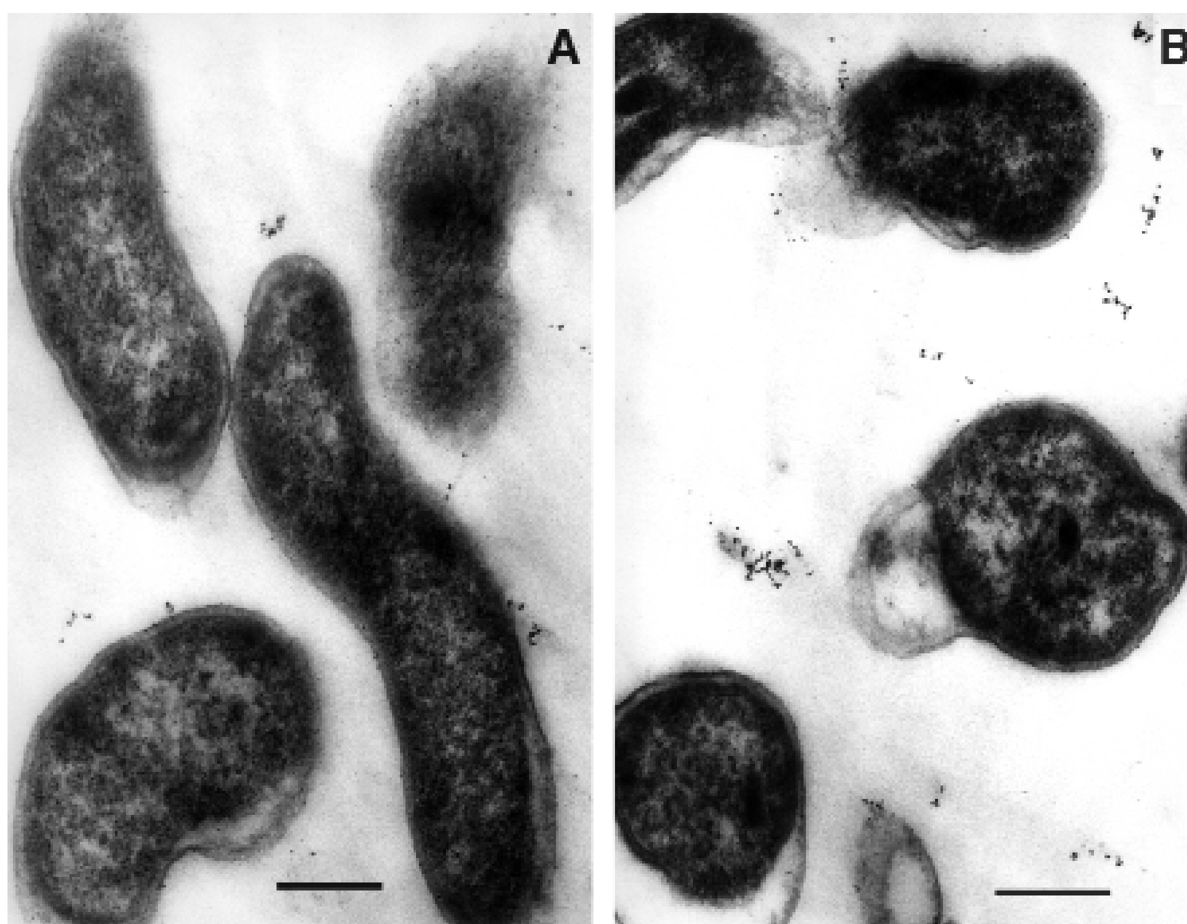

Fig. 1 Electron micrographs of axenic cultures of *Campylobacter jejuni* subsp. *jejuni* biotype 1 strain RSC-34. A: Characteristic spiral morphology corresponding to a fresh culture. B: Change to coccal cell morphology in an old culture exposed to oxidative stress. Scale bars represent 0.1 µm

at position 6 are the quinones involved in the respiration process. Additionally, cytochromes *b*, *c* and *d* have been found in *C. jejuni*. Apart from certain atypical strains of *C. lari*, the thermotolerant campylobacters hydrolize neither urea nor gelatine. They possess strong oxidase activity, but lack lipases. Several species —*C. jejuni, C. coli* and *C. lari*— produce catalase. The campylobacters are methyl red and Voges-Proskauer negative. They reduce nitrates but not nitrites. Hippurate hydrolisis has traditionally been the most reliable test to distinguish between *C. jejuni* (hippurate-positive) and *C. coli* (hippurate-negative), two of the species with high clinical incidence.

Sources of *Campylobacter* infection in humans

Campylobacter-due enteritis is caused by the consumption of food, mainly fresh and atmosphere-modified packed meat, raw milk and unchlorinated water. Other authors have demonstrated that people eating undercooked poultry are at a higher risk of infection. The personnel of poultry-processing factories are frequently infected by direct contact. Such operators have a higher incidence of antibodies against *C. jejuni* and *C. coli* than other people. The consumption of non-pasteurized contaminated milk is also a frequent source of campylobacteriosis, and outbreaks caused by contaminated milk have been reported. Other massive outbreaks (with up to 2,000 cases in a single outbreak) have been reported and associated to the consumption of contaminated water. Another major cause of infection is cross-contamination during the manipulation of food since campylobacters are usually found in meat and raw milk, as well as on the hands of people manipulating these foods. Nonetheless, person-to-person mechanisms of transmission seldom occur except in the cases of babies, who may acquire these microorganisms from their parents or minders.

Epidemiologic and clinical features

The clinical consequences of infection with a thermotolerant *Campylobacter* spp. strain include acute abdominal pain, fever, headache and profuse diarrhoea. Other clinical symptoms such as myalgia and fatiga have also been reported. Approximately half of the patients suffer from nausea and, to a lesser extent, vomiting. Acute diarrhoea generally lasts for two or three days, originating a considerable risk of dehydration in patients affected by *Campylobacter enteritis*. Abdominal pains and uneasiness may persist even after the diarrhoeic process has remitted.

Although *Campylobacter* spp. may cause severe infection that may last more than a week, they do not usually cause major complications. The most remarkable complication is the Guillain-Barré syndrome, which causes demyelinization of the peripheral nervous system, and has been associated with patients that have had *Campylobacter* infection [1]. Other complications are reactive arthritis, Reiter's syndrome, abortion in pregnant women, rheumatic manifestations and a type of polyneuritis called the Miller-Fischer syndrome. *Campylobacter enteritis* does not usually require further treatment apart from fluid and electrolyte restoration. The administration of antimicrobial agents to patients may eliminate the campylobacters from the faeces but does not reduce the duration or severity of the symptons. When the administration of antimicrobial agents is advisable, erythromycin is the drug of choice owing to the low level of resistance found and to its low toxicity.

The seasons with the highest isolation rates are late summer and early autumm, although *Campylobacter* strains can be isolated throughout the year. Enteritis associated with *Campylobacter* shows a different epidemiologic pattern in underdeveloped countries. Generally, these cases are restricted to children and no peak is usually observed among the young adult population, which is typical of industrialized countries, nor any clear seasonality. The higher incidence of *Campylobacter enteritis* in such countries is mainly due to a higher rate of asymptomatic carriers. This epidemiologic difference seems to be related to a higher exposure to *Campylobacter* in such countries, especially during the first years of life, which may lead to a different immunity profile.

The prevalence of infection with *C. jejuni* in patients affected with AIDS is about forty fold as compared to unaffected patients [52]. Such prevalence is especially high in patients in advanced phases of AIDS due to their low immunoglobulins levels, which make them especially sensitive to severe, persistent and recurrent *C. jejuni* infections.

Isolation of thermotolerant campylobacters

Culture techniques and media The chosen medium must comply with the criteria that allow the isolation of campylobacters from complex samples, such as faeces or raw meat, where the presence of a high microbial load of other bacteria might also occur, as well as the recovery of injured *Campylobacter* cells from processed food. To isolate *Campylobacter* spp. from samples in which the presence of competing microorganisms might occur, a filtration technique or selective agents should be used. The addition of certain agents able to inhibit the production of hydrogen peroxide and superoxide anions that might affect the growth of campylobacters has also been considered.

The first isolation of *Campylobacter* in faeces was carried out by Skirrow, who developed a selective medium based on blood-agar supplemented with trimethoprim, polymyxine B and vancomycin [49]. Blaser et al. developed the Campy-BAP medium, a selective medium now widely employed in clinical laboratories [4]. Another selective medium is Campylosel (BioMérieux), which comprises cefoperazone, vancomycin and amphotericine B as selective agents. Bolton and Robertson developed the Preston Medium, useful to isolate *Campylobacter* spp. from environmental samples in which these microorganisms may be outgrown by competing microorganisms [5]. The accuracy of this medium for the isolation of *Campylobacter* from faeces, water and from other ecosystems has been checked, but several authors have described possible failures in the isolation of certain strains of *C. coli* sensitive to polymyxine B, a component of the Preston Medium.

Since blood is an expensive component and its quality is not homogeneous, Bolton et al. replaced it with serum [7]. The medium (CCDA) described by these authors contained cefazoline and sodium deoxycholate, and proved to be even more selective if cefazoline was replaced by cefoperazone. Karmali et al. developed both CSM (this including vancomycin, cycloheximide and cefoperazone) and SKM media (this including trimethoprim, polymyxine B and vancomycin) for the selective isolation of *Campylobacter* [25]. Walmsley and Karmali succeeded in the direct isolation of *C. upsaliensis* in both CSM and SKM without preliminary enrichment or filtration steps [64]. Aspinall et al. reported another blood-free selective medium (CAT) for the isolation of *Campylobacter* at 37°C, this medium containing cefoperazone, amphotericine B and teichoplanin as selective agents [3]. This medium proved to be more accurate than CCDA for the isolation of *C. upsaliensis*. Another strategy that is currently employed is the addition of 5% hemolyzed horse blood to a blood-agar base supplemented with a commercial mixture of antioxidant agents such as SR84 supplement (Oxoid) and selective antimicrobial agents such as SR85 supplement (Oxoid) or SR98 (Oxoid).

It is sometimes advisable to consider an enrichment procedure for the isolation of *Campylobacter* spp. The enrichment protocol may increase the isolation rate of campylobacters, especially when the microbial load in the sample is low. Bolton et al. developed a "most probable

number" method, based on Preston enrichment broth and capable of detecting *Campylobacter* at concentrations as low as 10 CFU/100 ml [6]. Recently, Tran proposed a new blood-free enrichment broth (BFEB) to be used under aerobic conditions, which afforded good results in the isolation of *C. jejuni* strains [57].

Membrane-filtration techniques were initially designed for the isolation of *Vibrio fetus* (*C. fetus*) from cattle and later from human beings. Unlike many bacteria, campylobacters usually pass through 0.45 µm filters. Several membrane pore sizes, culture media and isolation techniques have been tested. Usually, 10% suspension of faeces is placed on the surface of a membrane-filter for some time and the filtered sample is seeded onto a plate containing selective medium. Ribeiro and Price reported that the introduction of the filters in Preston enrichment medium increased the isolation rate of *Campylobacter*. An alternative method consists of sample filtration followed by filtrate centrifugation and further seeding of the bacterial *pellet* in selective medium [45].

Incubation conditions. The thermotolerant campylobacters —*C. jejuni, C. coli, C. lari* and *C. upsaliensis*— grow well at 42–43°C. This temperature avoids the growth of a significantly broad spectrum of microorganisms. Although the optimum temperature is 42–43°C, the above four species also grow at 37°C but not below 30°C or above 47°C. Despite this, the isolation of *C. jejuni* or *C. coli* at 42°C is preferable since this temperature is more selective.

Species of the genus *Campylobacter* are strictly microaerophilic; i.e. they exhibit oxygen-dependent growth, oxygen being the final electron acceptor in the respiration process, but they do not tolerate the atmosferic oxygen concentration (21% v/v). *Campylobacter* spp. require oxygen concentrations ranging 5–10%. They also require 1–15% carbon dioxide concentration to grow. Nonetheless, several studies have reported the effects of the composition of the medium and even of the commercial brand of the components used in the formulation of the media on the oxygen-tolerance of campylobacters. A microaerophilic atmosphere selective for *Campylobacter* can be readily achieved by several methods and commercial systems such as the Gas Generating Kit (Oxoid) and the Campy-Pak system (BBL), which afford a selective microaerophilic atmosphere when introduced into an anaerobic jar system. Another useful method is the evacuation-replacement technique, in which air is removed from the incubating jar and replaced by a gas mixture containing (v/v) 15% carbon dioxide and 80% nitrogen, the oxygen concentration being reduced to 5%.

The survival of campylobacters depends on the temperature and the type of medium used. The optimum pH for survival is in the 6–8 range. Thermal treatment for more than 15 min at 60°C or for 30 min at 57.5°C destroys these microorganisms.

Rapid detection of thermotolerant *Campylobacter* spp.

Immunodetection An alternative technique for the detection of *Campylobacter* is immunoassay. This is sometimes used as a confirmatory test to detect the presence of *Campylobacter* spp. in food or clinical samples. The immunodetection protocol begins with the resuspension of a single colony in an extraction medium to obtain specific antigens, which are then screened by a detection reagent comprising specific polyclonal antibodies common to certain *Campylobacter* spp. and immobilized in latex [18, 20, 33]. The appearance of a precipitate indicates a positive antigen-antibody reaction, revealing the presence of *Campylobacter*. Several commercial systems for the immunodetection of *Campylobacter* spp. have been described. The Campyslide system (BBL), based on the detection of cell-wall antigens of *Campylobacter* by means of an anti-*Campylobacter* antibody immobilized in latex, allows confirmation of a microorganism as belonging to the genus *Campylobacter* in an easy, simple way. The Campyslide system to detect *Campylobacter* is reliable, although Hodinka and Gilligan found one false-positive (a strain of *Pseudomonas aeruginosa*) among 173 non-campylobacters assayed [20]. The Meritec-Campy system (Meridian Diagnostics) allows the immunodetection of *C. jejuni, C. coli* and *C. lari*. This method can be carried out on slides, where a colony is mixed with a drop of extraction reagent, followed by the addition of a detection reagent. This may lead to the observation of an antigen-antibody co-precipitate that indicates the presence of any of these three species. The Meritec-Campy system afforded 100% sensitivity and specificity values in the detection of *C. jejuni* and *C. coli* [33]. The Microscreen method (Mercia Diagnostics) proved to be more sensitive than the other two above commercial immunodetection systems, allowing the detection of up to ten-fold lower numbers of *Campylobacter* [18]. Recently, Buswell et al. used an immunofluorescent-antibody staining technique to detect the presence of *Campylobacter* spp. in water and aquatic biofilms [9].

Polymerase chain reaction (PCR)-based methods Lamoureux et al. introduced a rapid *Campylobacter* detection system based on the adsorption of RNA to wells in microtiter plates and further detection of the RNA:DNA hybrids labelled with digoxigenin by means of an enzyme-linked immunoassay using anti-digoxigenin antibodies [28].

Oyofo et al. directly detected the presence of *Campylobacter* in stools by PCR and their results agreed significantly with those obtained by traditional culture, although occasionally the confirmation of positive results required further investigation by hybridization studies [42]. Wegmüller et al. used PCR for the direct detection of *Campylobacter* in raw milk and dairy products [67]. The detection was based on the use of a primer for the amplification of the genes involved in flagellin biosynthesis in *Campylobacter*. Uyttendaele et al. examined 160 poultry products using the NASBA system for DNA

amplification after a 24 h broth enrichment step [58]. These authors failed to detect any false negative and achieved a sensitivity able to detect *Campylobacter* at numbers as low as 10 CFU/ml, even when a competing microbiota was present at concentrations of up to 10^5 CFU/ml [58].

Docherty et al. described a rapid and sensitive technique based on immuno-PCR for the detection of *C. jejuni* in poultry and milk. In this technique, the target cells of *Campylobacter* are captured from the food by means of magnetic particles coated with a specific anti-*Campylobacter* antibody; once recovered, the target cells are lysed and specific DNA sequences are amplified by the PCR [10]. Following a preliminary enrichment step, this technique allowed the detection of *Campylobacter* concentrations as low as 25 CFU/g in less than 30 h. In a collaborative study, Ng et al. reported new primers specific for *C. jejuni* derived from the plasmid pDT1720, which harbours the gene that codes for an outer membrane protein in *C. jejuni* [35]. This study allowed the selective detection of *C. jejuni* in milk and poultry, although it failed to distinguish between *C. jejuni* subsp. *jejuni* and *C. jejuni* subsp. *doylei*. Recently, Winters et al. have developed a new PCR-based system for the specific detection of *Campylobacter* in artificially contaminated foods [68].

Biotyping of *Campylobacter* spp.

The search for campylobacters necessarily involves examination of the colonies appearing in selective media after an incubation period of 48 h at 42°C under microaerophilic conditions. Non-hemolytic, grey or uncolored colonies, either plain-aqueous with irregular edges, or round-convex with regular round edges, should be investigated. Suspicious colonies are picked and subjected to at least three tests (i) direct microscopic examination of motility and cell morphology; (ii) Gram-staining, which should be negative, and (iii) oxidase production, which should be positive. Most thermotolerant *Campylobacter* spp. involved in food-borne bacterial enteritis, except for *C. upsaliensis*, also produce catalase. A simple scheme for the identification of thermotolerant campylobacters is presented in Table 1.

Skirrow and Benjamin introduced a scheme to distinguish *C. jejuni, C. coli* and *C. lari* based on the following tests: (i) growth at 25°C and 43°C; (ii) susceptibility to nalidixic acid; (iii) hippurate-hydrolysis, and (iv) hydrogen sulphide production in an iron-containing medium [50]. The scheme is accurate for routine work and is widely employed in microbiology laboratories. Another scheme, based on hippurate-hydrolysis, rapid production of hydrogen sulphide, and DNA hydrolysis was introduced by Lior [29].

The classification of campylobacters based only on biochemical tests is complex. However, the determination of biochemical features is the most widely employed identification strategy applied to *Campylobacter*, which justifies the optimization of phenotypic analysis for the differentiation of *Campylobacter* spp. The application of numerical analysis of phenotypic features to campylobacters was considered by Neill et al. [34]. More recently, On and Holmes designed a scheme comprising the investigation of 67 phenotypic characters in the genera *Campylobacter*, *Helicobacter* and other related taxa, obtaining a final scheme that proved to be a valuable tool for identification at species and subspecies level in most strains studied and whose results coincided with previous results obtained by RNA and DNA sequencing [38]. According to the study by On and Holmes, *C. coli, C. jejuni* subsp. *jejuni* and *C. lari* are closely related, a similar result having been obtained by Vandamme et al. from phylogenetic studies based on the analysis of 16S rRNA sequences [61].

Serotyping of *Campylobacter* spp.

The 1985 and 1987 Meetings of the International Committee on Serotyping *Campylobacter* recommended that only two systems should be considered. The first was based on the

Table 1 Differential features of the thermotolerant species of the genus *Campylobacter* involved in food-borne disease

	C. jejuni		*C. coli*	*C. lari*	*C. upsaliensis*
	subsp. *jejuni*	subsp. *doylei*			
Hippurate hydrolysis	+	+	–	–	–
Catalase production	+	+	+	+	–/W
Nitrate reduction	+	–	+	+	+
H_2S production (TSI)	–	–	–/W	–	–
Nalidixic acid resistance[1]	S	S	S	R	S
Cephalotin resistance	R	V	R	R	S

All species cited are able to grow at 42°C but not at 25°C.
[1]Increasing rates of nalidixic acid-resistant *gyrA* mutants of *C. jejuni* and *C. coli* have been reported [23, 63].
W: weak; V: variable; S: sensitive; R: resistant.

hemagglutination of heat-stable (HS or O) antigens, which were later confirmed to be the O somatic antigens. *C. jejuni* and *C. coli* have their own types of O antigen, although some cases (< 4.5%) of cross reaction have been reported. This system comprises 42 antisera against *C. jejuni* and 18 antisera against *C. coli*. Whereas 78% of the strains of *C. jejuni* corresponded to a single serotype, the remaining 22% were assigned to at least two different serotypes. Certain hippurate-negative strains, initially classified as *C. coli*, reacted against antisera raised against *C. jejuni*, suggesting that these strains were indeed hippurate-negative *C. jejuni* strains. The second system is based on the agglutination of heat-labile (HL) antigens, which considers whole cells as antigenic material. With this method, based on 22 antisera, it is not usual to observe multiple reactions. Each of the two systems described may be used alone, but as strains belonging to a single serotype according to one system may lead to different serotypes in the other system, it is advisable that both methods be considered with a view to obtaining complementary information. Patton et al. evaluated the serotyping schemes based on the HS and HL antigens and reported the advantages and disadvantages of each system [43]. Frost et al. modified the Penner serotyping scheme; they replaced passive hemagglutination by detection of O antigens by direct agglutination of *C. jejuni* and *C. coli* cells [14]. Recently, Jackson et al. reported a comparative analysis of O and HL serogrouping of human *C. jejuni* isolates which showed conserved associations between specific O and HL antigens [21].

Resistotyping and phage-typing

The study of susceptibility profiles with respect to several antimicrobial agents has also been considered for taxonomic purposes [46]. The methods of choice to evaluate susceptibilities to antimicrobial agents are: (i) determination of minimum inhibitory concentrations (MIC) in Mueller-Hinton broth, and (ii) standardized disk diffusion in agar on selected plate media. The increasing isolation rate of *Campylobacter* strains resistant to an increasing number of antimicrobial agents —especially the quinolones— together with the conjugational exchange of resistance against others —such as tetracycline or kanamycine— has reduced the taxonomic value of resistotyping studies. The interest for susceptibility studies, however, has increased from the therapeutic point of view. Acquired resistance of *Campylobacter* to fluoroquinolones, which depends mainly on a point mutation in the *gyrA* gene, has increased considerably over the present decade. The evaluation of susceptibility to nalidixic acid has ususally been considered as an important test to distinguish between traditionally sensitive species, such as *C. jejuni* and *C. coli*, and the resistant thermotolerant species *C. lari*. As a result, the differentiation of nalidixic acid-resistant strains of *C. coli* with respect to *C. lari*, another *Campylobacter* species causing enteritis, would need the introduction of additional tests, such as susceptibility testing against triphenyltetrazolium chloride.

Several authors reported the presence of specific phages in *C. jejuni* and *C. coli* [17, 48]. These authors proposed a phage typing system applicable for epidemiological purposes to the investigation of *Campylobacter* infection. The works of Salama et al. and Owen et al. allowed the discrimination of strains isolated during epidemic outbreaks [40, 48]. None of the phages isolated by Salama et al. turned out to be lysogenic, and treatment with mitomycin C did not allow the recovery of phages by induction of the lytic cycle [48].

Genotyping of *Campylobacter* spp.

DNA restriction profiles Owen et al. carried out chromosomal DNA restriction studies in *C. jejuni* and *C. coli* and observed that hydrogen sulphide-producing strains (Skirrow biotype 1-Lior biotype I) could be discriminated with respect to their non-producing counterparts (Skirrow biotype 2-Lior biotypes III and IV) [39]. Strains belonging to either Lior biotype III or Lior biotype IV could also be distinguished by genetic analysis. Owen et al. reported that strains belonging to different serotypes could be distinguished by analyzing their DNA restriction profiles, while strains belonging to the same serotype showed a high level of similarity [39]. Nonetheless, the simplicity of genetic analysis matched the tedious labour of accessing serological data, underlining the potential application of the analysis of DNA restriction profiles at least to the *Campylobacter* species and strains for which effective serotyping schemes were not available. Korolik et al. reported the usefulness of restriction enzymes *Cla*I, *Eco*RV and *Bgl*II for the typing of *C. jejuni* and *C. coli* [27]. Jiménez et al. achieved the differentiation of *Campylobacter* strains involved in mixed infections by a scheme based on the selective isolation of chromosomal DNA and further characterization of the RFLPs obtained by cleavage with *Eco*RV and *Bgl*II [24].

Hybridization studies Taylor and Hiratsuka designed a 1.5 kb DNA probe specific for *C. jejuni* and a 1.8 kb DNA probe for the detection of *C. jejuni* and *C. coli* [55]. Olive et al. developed a DNA probe capable of detecting even 5 ng of DNA from *C. jejuni* and *C. coli*, with a sensitivity of over 80% [36]. Wang and Taylor developed a colony-blot technique in which a 1.2 kb DNA fragment from the *gyrA* gene of *C. jejuni* was used as a probe [65]. Korolik et al. isolated a 1.8 kb DNA fragment, involved in the biosynthesis of a 31.5 kDa membrane antigenic protein in *C. jejuni* which, when used as a probe, allowed the effective classification of 169 strains of *C. jejuni* and *C. coli* [26]. This probe, besides being more specific than other previously-developed ones,

proved to be useful for the differentiation of *C. jejuni* strains isolated from humans or food.

Pulsed-field gel electrophoresis Unlike conventional electrophoresis, pulsed-field gel electrophoresis (PFGE) allows the separation of DNA fragments of high molecular weight with high resolution, this resulting in clearer restriction profiles. Additionally, PFGE genotyping studies may solve the problems deriving from the effect of environmental factors on the stability of certain phenotypic traits, this being the main problem associated with biotyping methods. PFGE has been applied to *C. jejuni* and *C. coli* [69]. The study of the PFGE restriction profiles for each strain was completed with hybridization studies based on 16S rRNA [69]. Lorenz et al. achieved the subtyping of *C. jejuni* Penner serotypes 9, 38 and 63 from human infections, animals and water by PFGE and flagellin gene analysis [30]. However, Wassenaar et al. and On reported spontaneous intramolecular genomic rearrangements which caused genetic changes on the PFGE profiles of *C. jejuni* and *C. coli*, respectively [37, 66]. Thus, data afforded by PFGE must be evaluated cautiously.

Ribotyping Yan et al. found that strains of *C. jejuni* and *C. coli* showing similar DNA restriction patterns also displayed similar rRNA hybridization patterns and that *Campylobacter* strains showing different DNA restriction profiles may display similar or different hybridization profiles [69]. This finding made the reliability of ribotyping for epidemiologic purposes questionable. Other authors, however, reported that ribotyping is highly discriminatory in *C. jejuni* [13]. Owen et al. carried out DNA restriction assays in *C. jejuni* and developed cDNA probes obtained by reverse transcription of 16S rRNA and 23S rRNA sequences [41]. Recently, Smith et al. concluded that ribosomal RNA gene-restriction endonuclease digest patterns and hybridization studies were more discriminatory than the Penner serotyping of *C. jejuni* and *C. coli* since strains of the same serotype were successfully distinguished [51].

PCR-based methods Giesendorf et al. sequenced part of the genes involved in the biosynthesis of the 16S rRNA in *C. jejuni* and *C. lari* and, after careful comparison of the variable regions, developed certain oligonucleotides that could be used both as primers of PCR and as DNA probes [15]. Van Camp et al. developed a genotyping system based on the amplification of genes coding for 16S rRNA in several *Campylobacter* spp., while Eyers et al. developed a system based on the utilization of a primer for the specific amplification of the genes that code for 23S rRNA in *Campylobacter* spp. [12, 59]

Endtz et al. carried out PCR studies based on specific DNA sequences reported for *C. jejuni* and compared the results with those obtained in biotyping and serotyping studies [11]. They found that, in strains displaying a variable serological reaction, the genetic profiles obtained by PCR amplification were not altered. This suggested that the variable serologic reaction might be due to altered gene expression rather than to genomic rearrangements [11]. Thomas et al. reported the amplification of the *flaA* and *flaB* genes of *C. jejuni* and the analysis of the RFLPs obtained after cleavage with *Alu*I, allowing the grouping of 300 strains in five groups [56].

Hernández et al. developed a decameric oligonucleotide (5'-CAATCGCCGT-3') for the amplification of specific sequences of *C. jejuni* and *C. coli*, allowing to classify the *Campylobacter* strains studied in five groups [19]. Note that the four strains of *C. jejuni* subsp. *doylei* typed were grouped together with 29 strains of *C. coli,* but were not in the same group as the strains of *C. jejuni* subsp. *jejuni* [19]. Stucki et al. isolated the *mapA* gene, which codes for a protein associated with the inner membrane of *C. jejuni* [54]. The MapA protein, encoded by *mapA*, allowed serologic differentiation between *C. jejuni* and other *Campylobacter* spp. Besides, the selective amplification of *mapA* sequences by PCR and its use as a probe in colony-blot assays proved to be of great value for the genotyping of *Campylobacter* strains [54]. Stonnet et al. carried out PCR studies using primers specific for *C. jejuni* as well as for *C. coli*, achieving accurate identification of all the strains studied, as well as the description of three hippurate-negative *C. jejuni* strains, previously misidentified as *C. coli* [53].

Bustamante et al. developed a genotyping system consisting of primers based on the probes CJ01 y CJ02, specific for *C. jejuni* and *C. coli*, respectively (these deriving from their respective *rpoB* genes, which encode their respective β subunits of the RNA polymerase) with relative success [8]. Madden et al. described the significant polymorphism of randomly amplified DNA (RAPD) in *Campylobacter* strains belonging to the same serotype, as well as the potential usefulness of this methodology to type strains that cannot be serotyped accurately [31]. Recently, Misawa et al. succeeded in the differentiation of *C. jejuni* serotype O19 strains, a group of strains which have been associated to Guillain-Barré syndrome, from non-O19 strains by PCR [32]. This method is based on the use of a 1.4 kb DNA fragment, specific of O19 strains, and highly similar to the *gyrB* gene of *Helicobacter pylori*. Other PCR-based methods, based on strain-specific sequences [47], *gyrA* and *pflA* genes [44], the putative GTPase genes [62], and genes encoding methyl-accepting chemotaxis-like proteins [16] have recently been developed with typing purposes.

Perspectives and future trends

New DNA-based systems for the detection of the thermotolerant campylobacters should be developed to achieve reliable and rapid tools for the diagnosis of infections

associated to this group of emerging pathogens in the clinical laboratory. Among these, the combination of DNA amplification with hybridization—using species-specific PCR products as probes—should improve the detection of these pathogens with respect to traditional culture-based techniques. From the clinical point of view, an increasing number of severe clinical syndromes —like Guillain-Barré syndrome— have been described to be associated to complications caused by *Campylobacter* spp. The investigation of the role of thermotolerant campylobacters on such clinical syndromes will surely take a great significance. The elucidation of toxin production by thermotolerant campylobacters and their role in human disease still constitutes another challenging project. From the taxonomic point of view, a more intense research effort should be conducted at both the genotypic and phenotypic levels to achieve a better knowledge of the phylogenetic relationships among *C. jejuni, C. coli*, *C. lari* and *C. upsaliensis*, and with respect to other campylobacters. In this sense, the taxonomic value of certain traditional phenotypic traits such as susceptibility to nalidixic acid will probably be reconsidered. From the epidemiologic point of view, the mechanisms of transmission of the thermotolerant campylobacters from food to humans would need an extensive research effort. In this sense, the dramatic increases in the resistance to antimicrobial agents by this group of pathogens —especially the unique ability of *C. jejuni* to develop resistance to quinolones by a point mutation in the *gyrA* gene— is a clear indicator of the extensive use of antimicrobial agents in animal feeding, a situation that the food administration should regulate and control.

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