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A REVIEW OF CURRENT KNOWLEDGE AND GAPS ABOUT *CAMPYLOBACTER* METHODS: FROM CULTURE TO CHARACTERIZATION

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ABSTRACT

Campylobacteriosis is a zoonotic disease caused by *Campylobacter*, mostly associated with consumption of contaminated foodstuffs and water. *Campylobacter jejuni* and *Campylobacter coli* recognized as the leader of foodborne diarrheal illness in humans. The frequency of these microorganisms in poultry is fairly high than *Salmonella* and more challenging to measure, which represent an expensive burden charge on public health due to their difficulties to master them, especially with the fast increase rates of multidrug-resistant of thermophilic *Campylobacter* strains. It is well recognized that *Campylobacter spp.* is a fastidious cell, difficult to isolate in laboratories owing to their requirements and sensibility. That's why; these factors must be taken into consideration during recovery protocols. A variety of phenotyping tests have been reported and widely used for confirmation and identification of *Campylobacter* species. Nonetheless, Whole Genome Sequencing (WGS) and Culture-Independent Diagnostic Tests (CIDTs) are new eras of hopeful technologies, mainly involved in the detection and characterization of threaten public health pathogens. This review aimed to describe the culture methods, phenotypic and genotypic schemes used to isolate, identify, and characterize *Campylobacter* isolates, through discussing the current knowledge and gaps related to the application of these techniques over others performed for typing this microaerophilic genus.

Keywords: *Campylobacter*; CIDTs; public health; typing methods; WGS

INTRODUCTION

C. jejuni subsp. *jejuni* and *C. coli* have been known as the most frequent bacteria, causing food borne gastroenteritis in humans around world, found in intestinal tract of many domestic animals, especially in birds **(Keener *et al.*, 2004; Escher *et al.*, 2016; EFSA & ECDC, 2016)**. The common food vehicles of *Campylobacter* are poultry meat, unpasteurized milk, and untreated water **(Joensen *et al.*, 2020)**.
The analysis of historical events in *Campylobacter* taxonomy showed the difficulties to recognize these species **(Figure 1)**. Indeed, in 1886, *Campylobacter* was described by Escherich Theodor as spiral bacteria, but he failed to culture them by using standard methods **(Kist, 1986)**. After that, many successive observations for the same shape have been reported in bovine abortion and sterility **(Florent & De Keyser, 1964; Skirrow, 2006)**, which was formerly classified as *Vibrio fetus* **(Smith & Taylor, 1919; Theobald Smith, 1919)**. These micro-organisms have continued to cause more infections in humans such as abortion, bacteremia, and diarrhea **(Levy, 1946; Killam *et al.*, 1966)**.
Because of their growth under a reduced oxygen tension and non-saccharolytic metabolism, these bacteria were separated from *Vibrio* group **(Sebald and Veron, 1963)**. After their separation, many scientists have worked on the culture of *Campylobacter* **(Sandstedt *et al.*, 1983; Steele & McDermott, 1984)**. **Dekeyser *et al.* (1972)** have described the first successful membrane filtration method for the recovery of *Campylobacter* from diarrheal stools. Moreover, further investigations have contributed in developing selective culture media and methods to identify and characterize these organisms **(Khan *et al.*, 2009; On, 2013; Li *et al.*, 2018)**.
Campylobacter is biochemically inert and few phenotypic tests were established to distinguish these organisms from each other. As a result, it is extremely hard for practitioners to identify *Campylobacter* with such criteria **(On, 2013; Duarte *et al.*, 2016)**. Nowadays, the researchers in molecular biology field provide significant discoveries, aimed to facilitate diagnosis, control, and prevent outbreaks in public health system. The current molecular knowledge about foodborne bacteria offers fruitful outcomes.

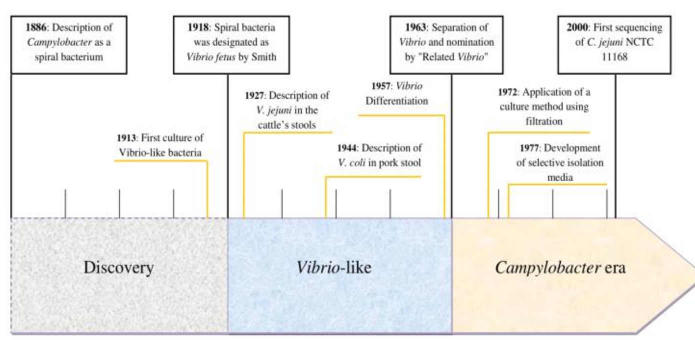


Figure 1 Historical events of *Campylobacter* taxonomy

For example, Whole Genome Sequencing (WGS) and Culture-Independent Diagnostic Tests (CIDTs) are new technologies intended to identify foodborne pathogens quickly and provide deep insights into their molecular mechanisms **(Shea *et al.*, 2017; Joensen *et al.*, 2020)**. Due to the scarcity of literature reviews about efficient tools to master *Campylobacter* spp. from culture until characterization and in order to provide epidemiological information, this review aimed to discuss the following questions; What are the appropriate methods performed to culture *Campylobacter*? What are efficient phenotypic tests to identify these bacteria? What are the current techniques to characterize these pathogens? What offered WGS compared to Pulse Field Gel Electrophoresis

(PFGE) and other molecular methods? How can the CIDTs help microbiologists to detect and control *Campylobacter*?

CULTURE OF *Campylobacter*

The bacterial culture aims to recover microorganisms and enhance their viability, through the use of specific culture media. The group of *Campylobacter* grow slowly (up to five days) even under optimal conditions **(Butzler, 2004; Fitzgerald, 2015)**. Although their widespread, they are very sensitive to many environmental obstacles, where usually cells cannot survive, for example, in ambient oxygen for more than 18 hours **(Garenaux *et al.,* 2007; Hilbert *et al.,* 2010)**.

CULTURE MEDIA

It has well recognized that the recovery of *Campylobacter* requires selective media, due to their inability to compete with other germs **(Josefsen *et al.,* 2003; Repérant *et al.,* 2016)**. Today, there is a wide range of plating media, whose selectivity differs from each other's, and usually grouped in two categories; this includes blood-based agar (Preston, Skirrow, Butzler, Campy-cefex agar) and others based on charcoal instead of blood, such as Karmali and modified charcoal cefoperazone deoxycholate agar (mCCDA). The best recommendation for isolation are mCCDA and Karmali agar since *Campylobacter* colonies were easily recognized, despite their poor sensitivity and productivity, especially in food specimens **(Chon *et al.,* 2011)**. Additionally, many formulations of enrichment broths were made, whose Bolton broth and Preston's formula were the most used in culture methods before isolation step, when bacteria are injured or/and when the number of cells expected in samples is small **(Bolton *et al.,* 1983; Repérant *et al.,* 2016)**. Overall, whether for enrichment or isolation of species, the *Campylobacter* culture media contains mainly the peptamin that provides amino acids (source of carbon), sulfide, and nitrogen required for making their energies, yeast extract provides B vitamins (co-enzymes), and sodium chloride to maintain osmotic equilibrium. *Campylobacter* do not ferment carbohydrates **(Epps et al., 2013)**; therefore etriphenyltetrazolium chloride was added to give contrasting color to colonies **(Brown *et al.,* 2013)**. Furthermore, defibrinated blood (horse or sheep), charcoals or other chemicals (e.g. ferrous sulfate, thioglycolate, and sodium metabisulfite or pyruvate) were inevitable in culture media formulas **(Corry *et al.,* 2003)**, owing to the fact that, during the respiratory chains, there is formation of toxic oxygen derivatives namely nitrite, nitric oxide, and peroxides sulfite (generating oxidative stress), harmful for *Campylobacter* cells, and therefore inhibit their growth **(Baaboua *et al.,* 2017)**. In contrast, some studies showed no significant differences in performance of enrichment broth supplemented or not with blood **(Odongo *et al.,* 2009; Gharst *et al.,* 2013)**.
For facilitating the isolation of all *Campylobacter* species, the selective media incorporates antimicrobial agents intended to suppressed background competing bacteria, present in normal faecal flora, food or in environments **(Chon *et al.,* 2013; Kim *et al.,* 2016)**. The *Campylobacter* culture media, whether used for isolation or enrichment, were typically supplemented with antibiotics mixture. For instance, cefoperazone, trimethoprim, and vancomycin inhibit Gram positive and/or negative aerobic and anaerobic bacteria, while amphotericin B and cycloheximide were incorporated to prevent yeasts and molds growth **(Kumar *et al.,* 2010)**. Nevertheless, other antimicrobial supplements like cephalothin, colistin and polymyxin B were also used, which may inhibit certain sensible strains of *C. jejuni* and *C. coli*, as well as other species that are less commonly encountered (i.e. *C. upsaliensis*, *C. hyointestinalis*, *C. fetus*) **(Corry *et al.,* 2003; Butzler, 2004)**. According to **Li *et al.,* (2018)** findings, the selection of the suspected colonies on the selective medium becomes more and more difficult due to the increased of multidrug-resistant strains, which reduced the efficiency of isolation media and enrichment broth as well. These might be the reasons of why there have been such variations in *Campylobacter* prevalence reported worldwide **(Biasi *et al.,* 2011; Szczepanska *et al.,* 2017; Es-soucratti *et al.,* 2020)** .
Chromogenic media were largely described for the recovery of *Campylobacter* in multiple samples (food, water, and in particular in clinical samples), based on the use of chromogenic substrate specific and selective for desired organism **(Perry and Freydière, 2007)**. CASA, Campy Food, and CHROM agar are some examples of *Campylobacter* chromogenic media, provided by BioMerieux, whose aimed to reduce time and cost of analysis by facilitating the recognition of presumptive colonies of *Campylobacter* visually and subsequently avoiding subculture and confirmatory tests **(Al-Wasify, 2013)**. Conversely, the bacterial detection was limited in one or two species, matter that limit their sensitivity compared to traditional media. Likewise, the chromogenic formulas incorporate antimicrobial molecules that can prevent the recovery of damaged cells, hence, the combination of an enrichment step remains required beforehand **(Malhotra-Kumar *et al.,* 2010)**.

INCUBATION CONDITIONS

These bacteria are microaerobic since they require an atmosphere depleted of oxygen. Many investigations have declaimed that 10% of carbon dioxide (CO_2), 5% dioxygen (O_2), and 85% of nitrogen (N_2) was the favorable gas mixture needed during incubation period of *Campylobacter* spp. **(Keener *et al.,* 2004; Macé *et al.,* 2015)**. For that, *and* after the development of jars containers, several microaerophilic atmosphere generation systems were described **(Haines *et al.,* 2011)** particularly, candle jar, chemical substances (Copper sulfate and sodium bicarbonate), gas generating envelopes, and tri-Gas CO_2 incubator **(Table 1)**. First, candle supplies a microaeophilic environment, nearly to 17–19% O_2 and 2–4% CO_2 appropriated for the isolation of *Campylobacter.* However, this system offer some advantages that are limited only in cost and simplicity **(Luechtefeld *et al.,* 1982; Hilbert *et al.,* 2010)**. Moreover, **Wang and others (1982)** were demonstrated that the increase in bacteria density in jars, only at 42 °C, decreases the oxygen tension, and consequently enhance the growth of *C. jejuni* strains. Secondly, the commercially available gas generating envelopes were used in bags and jars to provide the optimal microaerophilic atmosphere (5%O_2, 10%CO_2, 85% N_2) for a best growth of *C. jejuni* compared to candle jar **(Wang *et al.,* 1982)**. Other than cost, the limited numbers of incubated Petri dishes were the main described disadvantages of this system. Finally, and to overcome the inconvenience of gas generating envelopes, tri-Gas CO_2 incubator was invented to supply similar microaerophilic atmosphere conditions as the gas generating envelopes and also accommodate large number of Petri dishes, for a suitable routine use in laboratories **(Davis and DiRita, 2008)**.
Campylobacter requires favorable temperatures for growth. The incubation usually carried out at 37 °C or 42 °C, but it is very common, for routine diagnosis practice, to incubate at 42 °C to minimize the growth of contaminants and select the frequent thermo-tolerant species **(Silva *et al.,* 2011)**. Indeed, *C. jejuni* includes two subspecies *C. jejuni* subsp. *jejuni* and *C. jejuni* subsp. *doylei*, where subspecies *jejuni* are more frequent than subspecies *doylei* due to the fact that the temperature of incubation used during culture method is often 42 °C **(Miller *et al.,* 2007; Parker *et al.,* 2007)**. In addition, other species of *Campylobacter* (e.g. *C. helveticus, C. fetus,* and *C. hyoinestinalis*) grow better at 37 °C **(Corry *et al.,* 1995; Macé *et al.,* 2015)**.

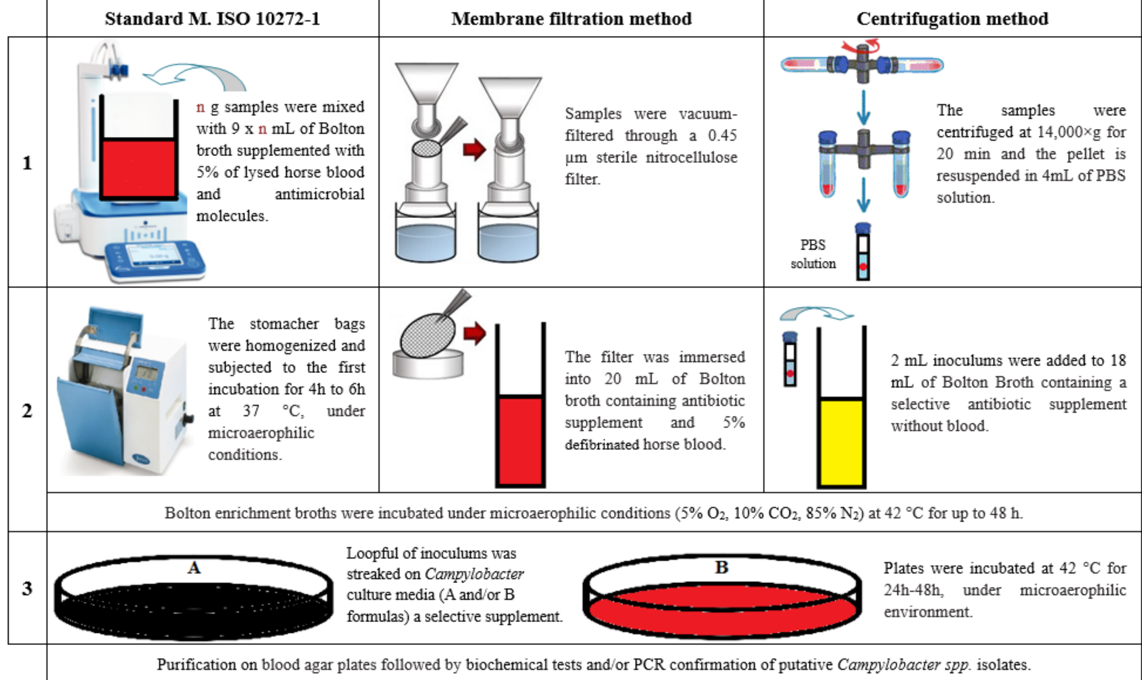
Table 1 Comparison of three microaerophilic atmosphere generation systems

System	Advantages	Disadvantages	References
Candle jar (17-19% O_2, 2-4% CO_2)	Simple, inexpensive	Cannot produce optimum microaerophilic condition, Cannot detect *C. jejuni* at 37 °C	**(Wang *et al.,* 1983; Hilbert *et al.,* 2010)**
Gas generating envelopes (5%O_2, 10%CO_2, 85% N_2)	Simple, large size of colonies	Expensive, Number of plates incubated were limited	**(Wang *et al.,* 1982)**
Tri-Gas CO_2 incubator (5%O_2, 10%CO_2, 85% N_2)	Reliable, productive	Expensive	**(Davis and DiRita, 2008)**

CULTURE METHODS

As other pathogenic micro-organisms, many culture methods were reported to isolate *Campylobacter* strains from three distinct sources (Food, stools, and water specimens). The protocol of standard method ISO 10272-1, membrane filtration **(Khan *et al.,* 2009)**, and centrifugation method **(FDA, 2020)** were based on samples preparation, enrichment, and isolation stage **(Table 2)**. Overall, through the observation of these protocols, it seems that the three methods have similar incubation conditions (Period and temperature), formulations of enrichment medium (Bolton broth), as well as culture media used for isolation of *Campylobacter* genus. However, the preparation stage was the only difference, in which *Campylobacter* was concentrated using centrifugation or membrane filtration, but not in the case of standard method ISO 10272-1. **Li *et al.* (2018)** have indicated that the enrichment with filtration method was the most effective culture method for *Campylobacter* genus from the diarrheal stool samples. Likewise, in their comparative study between centrifugation (CF) and membrane filtration (MF) method for isolation and detection of thermophilic *Campylobacter* in agricultural watersheds, **Khan *et al.* (2009)** have found that both methods detected similar frequency occurrence of *C. jejuni*. Nonetheless, the CF method detected significantly higher frequencies of *C. coli* (17%) and other *Campylobacter* species (13%) compared to the MF method (11% and 3%, respectively). Based on their results, it has been showed that the recovery effectiveness of *Campylobacter* species depend on culture method used, caution that must be taken into consideration when comparing studies that report on the occurrence of *Campylobacter* at the genus level **(Khan *et al.,* 2009).**

Table 2 Description of three *Campylobacter* spp. culture methods, according to standard method ISO 10272-1, NARMS-Methodology (Medicine, 2020) and Khan *et al.,* (2009). 1: Sample's preparation, 2: Enrichment, and 3: Isolation. A: Charcoal agars (Karmali and mCCDA) and B: Blood-based agars (Preston, Skirrow, Butzler, Campy-cefex agar).



	Standard M. ISO 10272-1	Membrane filtration method	Centrifugation method
1	n g samples were mixed with 9 x n mL of Bolton broth supplemented with 5% of lysed horse blood and antimicrobial molecules.	Samples were vacuum-filtered through a 0.45 µm sterile nitrocellulose filter.	The samples were centrifuged at 14,000×g for 20 min and the pellet is resuspended in 4mL of PBS solution. PBS solution
2	The stomacher bags were homogenized and subjected to the first incubation for 4h to 6h at 37 °C, under microaerophilic conditions.	The filter was immersed into 20 mL of Bolton broth containing antibiotic supplement and 5% defibrinated horse blood.	2 mL inoculums were added to 18 mL of Bolton Broth containing a selective antibiotic supplement without blood.
	Bolton enrichment broths were incubated under microaerophilic conditions (5% O_2, 10% CO_2, 85% N_2) at 42 °C for up to 48 h.		
3	A. Loopful of inoculums was streaked on *Campylobacter* culture media (A and/or B formulas) a selective supplement.	B. Plates were incubated at 42 °C for 24h-48h, under microaerophilic environment.	
	Purification on blood agar plates followed by biochemical tests and/or PCR confirmation of putative *Campylobacter spp.* isolates.		

Recently, the qualitative detection method of *Campylobacter spp.* ISO 10272–1: 2017 was amended and adapted according to resistance rates against β-lactams antibiotics of third generation, used in selective culture media, and because of non-efficiency of one culture method for the recovery of these species **(Acke *et al.,* 2009; Griggs *et al.,* 2009; Casagrande Proietti *et al.,* 2020).** In 2019, seventeen laboratories from 13 different countries in Europe have participated in inter-laboratory study of validation of the three procedures described in the revised standard method ISO 10272–1:2017. They concluded that the revised procedures of standard method were satisfactory for the detection of *Campylobacter* in different matrices. However, the values of LOD_{50} derived from this inter-laboratory study may not be applicable to food type or strains other than the ones carried out in the study (**Biesta-Peters *et al.,* 2019**). So that, each monitoring laboratory must validate appropriate culture method according to the samples analyzed for a future surveillance program.

IDENTIFICATION AND CHARACTERIZATION METHODS

In order to control the risks associated with prevalence, transmission mode, and resistance, the identification and characterization of these species provide very useful information. To our knowledge, there is no accepted standard scheme to process the identification and characterization of *Campylobacter* **(Hiett, 2017) (Figure 2)**.

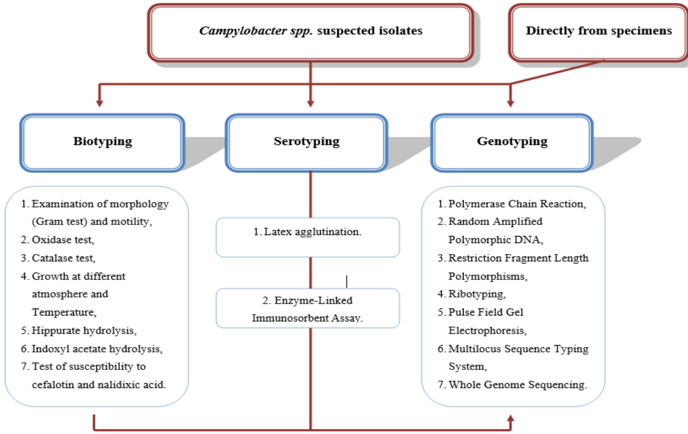


Figure 2 Identification and characterization methods for *Campylobacter* spp.

BIOTYPING

According to the traditional school, the first confirmation stage consists to distinguish the characteristic colonies of *Campylobacter* present on the isolation media, which usually appears after 24 to 72 hours **(Rahimi *et al.,* 2010; Szczepanska *et al.,* 2017)**. On charcoal agar plates, the characteristic colonies were greyish to white with a metallic sheen, round, high, convex, smooth, shiny, and moistened with a regular edge **(Figure 3)**. On blood agar plates, *Campylobacter* colonies are slightly pink, round, convex, smooth, and shiny with a regular edge. After the recognition and purification of suspected colonies of *Campylobacter* on blood base agar (oftenly Columbia base supplemented with 5% of defibrinated horse blood), a microscopic examination for morphology (Gram staining), motility, oxidase, catalase test, and growth under different conditions of suspicious colonies of *Campylobacter* were the most acceptable techniques reported in biotyping scheme in order to confirm the genus **(Duarte *et al.,* 2016)**. Moreover, the susceptibility against cefalotin and nalidixic acid, indoxyl acetate, and hippurate hydrolysis were recommended in standard method ISO 10272–1 to identify *Campylobacter* species **(Colles *et al.,* 2010; Singh, *et al.,* 2011)**. Other studies have suggested complimentary biochemical methods such as H_2S production in triple sugar iron medium, growth in NaCl 3.5%, growth in glycine 1%, growth on MacConkey agar, growth on nutrient agar, and nitrate reduction **(Kiehlbauch *et al.,* 1991; Silva & Van Dender, 2013; Silva *et al.,* 2020)**. The hippurate hydrolysis was reported as the only biochemical criterion that can differentiate *C. jejuni* from other species **(Adzitey and Corry, 2011)**, while other scientists found that hippurate hydrolysis test gives false positive and negative results **(Nakari *et al.,* 2008)**. Today, the test of sensitivity to nalidixic acid recommended by standard method ISO 10272–1 should be revised due to the increase of resistant isolate to nalidixic acid in *C. jejuni* and *C. coli* **(Biasi *et al.,* 2011; Vinueza-Burgos *et al.,* 2017)**.

For a long time, *Campylobacter* has been confused with group of bacteria named related organisms **(On, 2001; Lastovica *et al.,* 2014)**. In this regard, several studies have cited critical biochemical tests to differentiate *Campylobacter* genus from *Helicobacter* and *Arcobacter* (**Table 3**). In the light of previously demonstrated finding on growth temperature**, Vandamme and collaborators (1991)** showed that *Campylobacter* cannot grow at 25 ºC compared to *Helicobacter* that cannot grow at 42 ºC and *Arcobacter* that grown at 15 to 25 ºC. Except *C. lari,* all other species of *Campylobacter* are unable to metabolize urea, contrary to *Arcobacter* and *Helicobacter.*

The shortcomings associated with biochemical identification scheme were usually noted in discriminatory power that is only limited to the known species of *Campylobacter* and also time consuming (**On, 1996; Ugarte-Ruiz *et al.,* 2013**). The API Campy strip (BioMérieux, Marcy l'Etoile, France) is miniaturized gallery which contains eleven conventional tests and nine inhibition tests. It was developed to save space and time of materials preparation, so the results were obtained more rapidly, and without forgetting to mention the eliminated risk linked to improper sterilization and preparation of media during the use of classic biochemical

methods (**Huysmans *et al.*, 1995; Martiny *et al.*, 2011**). Despite the clear advantages of API Campy, this approach is not ideal. Firstly, the inappropriate concentration of bacteria may give false results. Secondly, the misidentifications of species and lack of certain strains in identification key of API Campy have been proved (**Reina *et al.*, 1995; On, 1996**). This means that further investigations are required to continue to use this kit.

Figure 3 Characteristic colonies of *C. jejuni* on *Campylobacter* Blood Agar Medium (red medium) and on *Campylobacter* Blood Free Medium (black medium). These pictures were taken in regional laboratory of analysis and research of Tangier, Morocco.

Table 3 Biochemical differences between *Campylobacter* related organisms

Test	*Campylobacter*	*Helicobacter*	*Arcobacter*	References
Growth temperature	Not at 25 ºC	Not at 42 ºC	Growth at 15 ºC and 25 ºC	(**Vandamme *et al.*, 1991**)
Urea degradation	+ Only *C. lari* biovar VPTC	+	+	(**Marshall *et al.*, 1990; Miller *et al.*, 2007**)

+ : positive

SEROTYPING

Serological tests have been performed for long time in clinical diagnostic and epidemiological studies to define the prevalence of serotype related to disease. All serologic techniques have in common, antibody or antigen detection. The extra-intestinal invasion of *C. jejuni* pathogenesis mechanism can cause bacteremia. In that case, and in order to protect its entity, the body produces specific antibodies against *C. jejuni* (**O'Hara *et al.*, 2017; Pacanowski *et al.*, 2008**). Thus, numerous rapid immunological methods have been developed, namely latex agglutination, Enzyme-Linked Immunosorbent Assay and others (**Tuuminen *et al.*, 2013**).
Latex agglutination tests are one of the easiest serological tests, which help in determination of the presence or absence of specific surface antigens. The reaction is based on the detection of target strain clumping particles with polyclonal or specific antibodies of the bacterial membrane or of the flagellum. For this purpose, several kits have been evolved following two schemes (**Frost *et al.*, 1998; Woodward & Rodgers, 2002**). The Penner and Hennessy scheme, established in 1980, uses thermostable antigens highly immunogenic, essentially lipopolysaccharide (LPS) of the outer membrane, flagellum (AGH), or capsule (AGK). The Lior scheme, founded in 1982, corresponds to thermolabile antigens of lipopolysaccharide nature (LPS/AgO), but also comprises certain components of the cytoplasm and proteins of the outer membrane. The agglutination latex test is carried out after culturing bacteria, which encumber up time. Likewise, the kit is not available for all *Campylobacter* species (**Nachamkin and Barbagallo, 1990**).
Other than auto-agglutination of *C. jejuni* isolates, it has been also recognized that *C. jejuni* and *C. coli* failed to agglutinate because of flagellin glycosylation phenomenon, the facts that limit latex agglutination uses (**Guerry *et al.*, 2006**).
Enzyme-Linked Immunosorbent Assay (ELISA) is an immunological method aimed to visualize the antigen-antibody reaction of target microorganisms. The presence of antigen was indicated by color change of chemical substrate. ELISA test can cost-effectively shorten the time for analysis by screening out presumptive positives specimens in less than 24 hours (**Quetz *et al.*, 2010; Turonova *et al.*, 2015**). In fact, many authors have been reported the high sensitivity and specificity of antigen selection, which have been successfully used in serodiagnosis of *Campylobacter* infections (**Hum *et al.*, 1991; Quetz *et al.*, 2010**). Another advantage that ELISA offers is the capacity of differentiating between non-exposure, chronic exposure, and recent exposure to *C. jejuni* antigens. Both ELISA and latex agglutination tests, are particularly important for the presumptive identification in case of suspicious post-infection or in case of complications cause by *C. fetus* and *C. jejuni* (Guillain-Barré syndrome, reactive arthritis, and bacteraemic), especially in *immunocompromised* individuals (**Ang *et al.*, 2007**).

GENOTYPING

Molecular biology have been widely improved worldwide to overcome the inconveniences of biotyping and serological tests (e.g. time, reliability, limited selectivity, and cost-effectiveness) (**Alves *et al.*, 2016; Gosselin-Théberge *et al.*, 2016**). These methods are based on nucleic acid detection and genes characterization that confer mobility, adhesion, *invasion,* toxin production, resistance and others. The most relevant genotyping methods reported for characterization of *Campylobacter* are Polymerase Chain Reaction (PCR), *Restriction Fragment Length Polymorphism* (RFLP), PFGE, MLST, and WGS (**Table 4**) (**Leblanc-Maridor *et al.*, 2011**).

Table 4 Main methods used for characterization of *Campylobacter* isolates

Methods	Advantages	Limitations	References
PCR[1]	Quick, easy and high sensitivity.	Cost and inhibitors, Cannot distinguish between VBNC and viable cells.	(**Law *et al.*, 2015**)
RFLP[2]	Discriminatory, Identification of new species.	Laborious and time-consuming process.	(**Botstein *et al.*, 1980**)
PFGE[3]	Provide meaningful evolutionary analyzes, Discriminate between outbreak associated and sporadic strains	Cost and reproducibility, Need bioinformatics analysis for interpretation.	(**Goering, 2010**)
MLST[4]	Discrimination and reproducibility, Easy for comparison of nucleotide sequence-based.	Cost and labour.	(**Dingle *et al.*, 2008**)
WGS[5]	High discriminatory power, Evolutionary understanding.	Cost and speed.	(**Lindsey *et al.*, 2016; Llarena *et al.*, 2017**)

[1]Polymerase chain reaction; [2]Restriction fragment length polymorphism; [3]Pulsed field gel electrophoresis; [4]Multilocus sequence typing ; [5]Whole Genome Sequencing.

The PCR innovative types (Conventional, qPCR, multiplex, and others) have been largely known for their high sensitivity, ease of operation, and fast results (**Garibyan and Avashia, 2013**). In fact, the specific 16S rRNA gene was described

to confirm *Campylobacter* genus, while *hip*O, CadF, CeuE, mapA genes and other specific-genes were reported to identify *Campylobacter* species **(Ghorbanalizadgan *et al.,* 2014; Begum *et al.,* 2015; Ricke *et al.,* 2019)**. PCR methods can be used directly (Enriched samples and suspected isolates), or in a combination with biochemical tests for further reliability and speed **(Taboada *et al.,* 2013; Efimochkina *et al.,* 2019)**. Despite the aforementioned advantages, PCR has many disadvantages such as capacity to detect the DNA of dead bacteria, viable but non-cultivable strains, and the limited number of microorganisms detected in reaction **(Liang *et al.,* 2018)**. Further, the presence of lysed blood in *Campylobacter* enrichment broths may inhibit the amplification **(Law *et al.,* 2014)**.
Ribotyping refers to the first process describing the specific recognition between labeled 16S, 23S, or 5S ribosomal RNA (rRNA) gene probes and digested genomic DNA by single endonuclease **(Wassenaar and Newell, 2000)**. The generated ribotypic sequences reflect the diversity of rRNA operons present in a bacterial species **(Bouchet *et al.,* 2008)**. Indeed, the name of this technique was amended according to the polymorphism observed in rRNA analyzed of species and was, lately, recognized by RFLP. Briefly, this molecular marker method is based on fragmentation of DNA with one or more of restriction enzymes and then the fragments are separated by electrophoresis, blotted onto a membrane, and hybridized, or exposed to a labeled probe of rRNA for visualization. The first application of RFLPs analysis on polymorphic sites of evolution of rRNA genes, have revealed that polymorphisms were within chromosomal genes **(Bouchet *et al.,* 2008)**. Moreover, the characterization of *Campylobacter* flaA gene by RFLP was appeared to be suitable as a preliminary typing method based on ease of manipulation, equipment availability, and cost compared to MLST, PFGE **(O'reilly *et al.,* 2006; Vinueza-Burgos *et al.,* 2017)**. Nowadays, this technology has been combined with PCR for studying deeper the diversity of specific genes. By applying PCR-RFLP, **Yadav et al., (2018)** compared three different restriction endonucleases (DdeI, HinfI and DpnII) to analyze the flagellin gene polymorphism of *C. jejuni* isolates and have determined that DdeI yielded high polygenic pattern (15 clusters) comparable to DpnII (7 clusters) and HinfI (6 clusters).
Pulsed-field gel electrophoresis is a variety of RFLP that consists to separate large fragments of genomic DNA, generated from enzymatic digestion of DNA (up to 10Mb) **(Sabat *et al.,* 2013)**. PulseNet surveillance systems is a global network laboratories of public health, aim to monitor foodborne illnesses detected in human outbreak and control new hazardous cases **(Fontanot *et al.,* 2014)**. Indeed, PulseNet uses PFGE to subtype foodborne pathogens isolates with the high discriminatory power to differentiate clustered cases of campylobacteriosis sporadic disease and outbreak occurring in the community and to characterize isolates from their environment **(Bakhshi et al., 2016; Di Giannatale et al., 2019)**. Annually, this system prevents an estimated 270,000 illnesses and saves more than $500 million in medical costs and lost productivity **(Scharff *et al.,* 2016)**.
After the development of first generation of sequencing technology by Sanger and Coulson in 1975, a variety of sequencing techniques have been described **(Sanger and Coulson, 1975)**. For instance, MLST is based on DNA sequencing of seven conserved housekeeping genes **(Dadar *et al.,* 2018; Aksomaitiene *et al.,* 2019)**. This technology represents a valuable understanding of the variability and relationship of *Campylobacter* isolated from outbreaks cases or food. Therefore, the sequence types can be shared between laboratories worldwide **(Lytsy *et al.,* 2017)**. **Dingle *et al.,* (2008)** have emphasized the easy comparison of nucleotide sequence-based for MLST method, a good discrimination, and reproducibility power.
Whole genome sequencing (WGS) is new sequencing generation for analyzing the entire genome of pathogenic bacteria and thus improves our knowledge in evolutionary biology as well as makes clinical progress easy. This genome analysis method gives very precise and more detailed information about bacteria compared to old methods (i.e., MLST and PFGE), and generating stable data, easy to share and compare at the international level **(Lytsy *et al.,* 2017)**. Many researchers suggested that the integration of genome sequencing in routine public health surveillance of *C. jejuni,* would facilitate the identification of possible case clusters, which should allow the implementation of more effective intervention strategies in prevention and control of campylobacteriosis cases **(Ghatak *et al.,* 2017; Llarena *et al.,* 2017)**. For these reasons, Centers for Disease Control and Prevention (CDC) are proceeding to develop analysis, to validate, and to establish a standardized procedure to make it easier for network members to exchange and compare data, within laboratory networks that support foodborne disease surveillance and outbreak. Indeed, the standardization of such data across laboratories, for routine surveillance, will provide deep understanding about outbreak identification, source attribution, antimicrobial resistance, serotype, and virulence factors that can be extracted at the same time **(Ellington *et al.,* 2017; Ghatak *et al.,* 2017)**. Regardless of these existing highlighted advantages, aforementioned, the adoption of whole genome sequencing in clinical laboratories remain challenging because of culture dependent, limited speed, and very high-cost. Another significant challenge is the lack of systematic surveillance of *Campylobacter*, as well as the significant resources required to set up for routine analysis, which may limit the success of this approach **(Joensen *et al.,* 2020)**.

CULTURE-INDEPENDENT DIAGNOSTIC TESTS

Culture-independent diagnostic tests (CIDTs) are an alternative technology based on molecular applications such as ELISA and PCR, intended to offer rapid results as compared to standard microbiological culture **(Imdad *et al.,* 2018)**. These tests can screen or/and identify disease-causing foodborne illnesses quickly, without need to culture bacteria in laboratory. Hospitals use this new technology because they are less labor-intensive and provide faster results than culture methods **(Singh *et al.*, 2011)**. Moreover, some CIDTs are able to detect more than single pathogen simultaneously, within hours, which results in faster public health action and prevention, compared to the days using traditional culture techniques **(Janda and Abbott, 2014; Shea *et al.,* 2017)**. These tests can improve the speed of diagnosis but may limit the ability to survey outbreaks **(Langley *et al.,* 2015)**. In reality, laboratories need isolated strains in order to provide further characterization *via* PFGE, WGS, and susceptibility tests against antimicrobial molecules. By skipping the step of producing a bacterial isolate, many consequences will be faced. For example, DNA fingerprints produced by PFGE won't be able to continue detecting clusters and such valuable opportunities for improving the food safety and emerging pathogens will be lost. Moreover, the information gathered about antibiotic resistance will not be available and no antibiotics resistance state will be known. As results, it will be difficult to treat disease and monitor resistance trends over time **(Couturier, 2016; Marder *et al.,* 2017; McAdam, 2017)**. Whenever the clinical laboratories embrace these new culture-independent diagnostic tests, the challenge increase to more study and harmonize this available technology for public health.
Owing to the increased use of CIDTs by clinicians and public health practitioners, the authorities takes advantages of this and try to accommodate these new technological tests not only to speed up the microbes screening but also prompting thorough outbreak detection in emerging infections program surveillance **(Couturier, 2016)**. In this context, CDC is encouraging clinics and hospitals to use them as primarily test and then send positive clinical specimens to the state health laboratory for pathogen isolation by culture **(Marder *et al.,* 2017; McAdam, 2017)**. Nonetheless, the approach of sending clinical specimens to another laboratory after they get a diagnosis using CIDTs has added additional challenges, including receiving CIDTs reports from hospitals, the potential loss of pathogen viability during transport, setting case during outbreaks, communicating CIDTs-positive results with patients, and interpreting results that are positive for multiple pathogens **(Atkinson *et al.,* 2013)**. As a result, these concerns should be balanced carefully against the said advantages.

CONCLUDING REMARKS AND PERSPECTIVES

A standard method for future surveillance systems is crucial to any monitoring laboratory. For that fact, the present review aimed to discuss the current knowledge and challenges of culture, identification, and characterization methods of *Campylobacter* species. Apparently, the selection of the appropriate methods is not always easy; owing to the different levels of discriminatory power, purpose, as well as the data needed to interpret. The classical biotyping have been described useful for preliminary surveillance and simple to perform, despite the fact that, they are heavy and labor. Also, there is possibility of providing false negative results due to transformation of viable but non-culturable state under unfavorable conditions. Furthermore, the identification of *Campylobacter* species has always been difficult using phenotypic methods, owing to their low metabolic activity and time-consuming. Nevertheless, molecular methods come to exceed these limitations and provide more beneficial techniques. Such advanced tests are extremely useful to clinicians, especially, PFGE and MLST that have been contributed to the current knowledge on *C. jejuni* outbreaks in human. The recent evidence suggests that whole genome sequencing is the most likely method to be used routinely in *C. jejuni* isolates for the detection and investigation of outbreaks. This new technology reveals crucial insights on various aspects of biology and epidemiology of these important pathogens, including, antimicrobial resistance, serotype and virulence factors traits.
For now, these innovations are in the early stages of development and could need more implementation work for sustainable use, thus we invite researchers to carry out studies regarding culture methods as fundamental processor to get deep insights into their metabolic mechanisms and therefore to speed up the growth phenomena of these strains as well as better understanding of their biochemical profiles. Also, the aspect of molecular identification techniques of *Campylobacter* species needs further studies to learn more about neglected species and to avoid false results that may be occurs during this operation.

COMPETING INTERESTS

All authors have declared no competing interests exist.

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