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Overview of methodologies for the culturing, recovery and detection of *Campylobacter*

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ABSTRACT

Campylobacter species are responsible for human gastroenteritis with diverse clinical spectra, ranging from acute watery or bloody diarrhea to life-threatening autoimmune disorders. Given the importance of *Campylobacter* in causing human illness, this article has reviewed the transmission and attribution sources as well as methodologies for the detection and virulence characterization of campylobacteria. The recovery and detection of *Campylobacter* from clinical, food and environmental samples has been achieved by the combinatorial use of selective enrichment and culturing methods. Biochemical, immunological, and nucleic acid-based methodologies have enabled the detection and differentiation of closely related *Campylobacter* isolates in foodborne outbreak investigations and have assessed the diversity and phylogenetic relationships of these bacterial pathogens. Analyses of motility, adherence, and invasiveness in host cells have assessed the pathogenic potential of campylobacteria. Further examination of determinants conferring antimicrobial resistance in *Campylobacter* have supported the growing need to closely monitor antimicrobials use in clinical and agricultural sectors.

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Campylobacter; food safety; zoonosis; foodborne pathogen; epidemiology; genotyping

Introduction

Campylobacter species are commonly reported as a significant cause of bacterial gastroenteritis in developing and industrialized countries (EFSA 2021). Campylobacteriosis has diverse clinical spectra, ranging from acute watery or bloody diarrhea, fever, and cramps. In some cases, *Campylobacter* infections can subsequently result in the life-threatening autoimmune disorders, Guillain-Barré and Miller Fisher syndromes (Chiba et al. 1992; van Belkum et al. 2009), or other gastrointestinal conditions including inflammatory bowel disease, esophageal diseases, periodontitis, celiac disease, cholecystitis, and colon cancer (Verdu et al. 2007; Kaakoush et al. 2015). The *Campylobacter* genus belongs to the family *Campylobacteraceae*, the order *Campylobacterales*, and the class *Epsilonproteobacteria*, which comprises other closely related genera, including *Arcobacter, Dehalospirillum* and *Sulfurospirillum* (Vandamme et al. 2015). To date, the *Campylobacter* genus is currently comprised of 32 officially described and 9 subspecies and 4 biovars (ITIS 2020). Campylobacters are

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microaerophilic Gram-negative bacteria with a corkscrew-shape, ranging in size from 0.5 to 5 µm in length and 0.2 to 0.9 microns in width. The temperature for optimal growth ranges from 37–42°C for thermotolerant *Campylobacter* species (*C. jejuni, C. coli, C. lari, C. upsaliensis, C. helveticus*, and *C. insulaenigrae*) (Wassenaar and Newell 2006; Vandamme et al. 2015). Other *Campylobacter* species, not listed above, are considered non-thermotolerant and have an optimal growth temperature of 37°C. Most species in the *Campylobacter* genus are fastidious organisms, and growth generally requires microaerophilic conditions.

Studies on the prevalence in various animal and environmental reservoirs have been employed to categorize the species within the *Campylobacter* genus. These zoonotic species are commensal organisms and reside in the intestinal tract of mammals, birds, and reptiles (Whiley et al. 2013; Vandamme et al. 2015). In addition, other environmental sources such as potable and surface water can also harbor campylobacters. Among the zoonotic species, *C. jejuni* and *C. coli* are considered to be responsible for the majority of the reported gastrointestinal-related *Campylobacter* infections (Kaakoush et al. 2015). Other species *C. concisus*, *C. lari, C. upsaliensis* and *C. ureolyticus* are defined as emerging *Campylobacter* species and have been underestimated as causative agents of human gastroenteritis due to a bias in the current testing methods to favorably identify *C. jejuni* and *C. coli* than the emerging species (Lastovica 2006; Man 2011; Costa and Iraola 2019). Given the importance of *Campylobacter* as a leading cause of human gastrointestinal disease (EFSA 2021), techniques for the typing of *Campylobacter* isolates were examined to further assess the diversity, pathogenesis, and phylogenetic relationships of these bacterial species. The information discussed in the present article is also aimed at providing an assessment of risks associated with *Campylobacter* infections for the development of efficient and targeted intervention strategies.

Epidemiology and disease manifestations

Human infections caused by *Campylobacter* are endemic worldwide, and in the recent years, the incidence of campylobacteriosis prevailed throughout North America, Europe and Australia and remained at stable levels (EFSA 2021; Hoffmann et al. 2021). Foodborne illness has been estimated to account for US$90 billion annually (Scharff 2020). Based on a report summary by the European Union, campylobacteriosis was the most reported cause of gastrointestinal infections in humans for the past 15 years and was the third most frequently reported pathogen for foodborne outbreaks (EFSA 2021). In a more recent study, the combination of results from food attribution, human illness and economic models revealed that *Campylobacter* was found to be significantly responsible for $6.9 billion in economic costs associated withcontaminated poultry products regulated by the United States Department of Agriculture (Scharff 2020). As a food commodity, contaminated meat and poultry products are estimated to account for 30.9% of all foodborne illnesses.

Campylobacter infections can occur with a dose as low as 800 colony forming units (CFU); however, it is possible that a lower dose of 360 CFU of *C. jejuni* can result in the development of campylobacteriosis (Hara-Kudo and Takatori 2011). The incubation period leading to the onset of diarrhea, ranges between 24 and 72 hours with most symptoms (Blaser 1997), and typical initial symptoms within 48 hours include acute watery or bloody diarrhea, fever, weight loss, and cramps. Gastroenteritis due to infections with *C. jejuni* and *C. coli* are more common in the summer months affecting children younger than 4 year of age and young adults than in older patients (Bessède et al. 2014). Among campylobacters, *C. jejuni* has been previously implicated in the development of other chronic inflammatory conditions of the gastrointestinal tract such as Crohn's disease and ulcerative colitis. The use of genomics enabled the identification of an association between the development of inflammatory bowel disease and *C. jejuni* genetic determinants implicated in stress response, adhesion, and core biosynthetic pathways (Peters et al. 2021). Additional evidence has demonstrated that emerging *Campylobacter* species, including *C. concisus, C. showae, C. hominis, C. gracilis, C. rectus*, and *C. ureolyticus*, can significantly contribute to the development of these

inflammatory bowel diseases (Zhang et al. 2009). Microbiome studies demonstrated these emerging *Campylobacter* species to be also been linked as important pathogens contributing to periodontal diseases (Kaakoush et al. 2015; Al-Kamel et al. 2019). One of these emerging species, *C. concisus*, commonly present in the human oral cavity, has been further implicated in the chronic disorders leading to the damage of the esophagus due to the high intracellular fitness in esophageal epithelial cells (Deshpande et al. 2021), and the expression in *C. concisus* of zonula occludens (*zot*) toxin, proposed to contribute to increased intestinal permeability, may lead to the development of inflammatory bowel disease (Liu et al. 2016). Although a few reports have suggested an association of *C. jejuni* infection in contributing to celiac disease inflammation of the gall bladder and colorectal cancer (Verdu et al. 2007; Vaughan-Shaw et al. 2010; He et al. 2019), more epidemiological data is thus warranted to further determine an association between campylobacteriosis and these other gastrointestinal disorders.

Besides causing gastrointestinal infections, reports have attributed *C. jejuni* as the causative agent for the postinfectious autoimmune disorders known as Guillain-Barré syndrome (GBS) and Miller Fisher syndrome (Chiba et al. 1992; van Belkum et al. 2009). In particular, outbreaks of GBS result as a subsequent consequence of *C. jejuni* infections due to human antibodies cross-reacting between *C. jejuni* sialylated lipooligosaccharides and human gangliosides. This *C. jejuni* molecular mimicry of the human ganglioside motif leads to the neurodegenerative autoimmune conditions characterized by weakness of the limbs and in some cases paralysis due to nerve damage. The proportion of *C. jejuni* infections leading to the onset of GBS is considered on average relatively low at 0.07%, and the development of severe symptoms in GBS outbreaks can vary widely worldwide (0.4–4%). In developing countries, GBS outbreaks have been associated with death in approximately 4–15% of the patients within the first year after onset (Keithlin et al. 2014). Moreover, *C. jejuni* infections in children in developing countries are prevalent, and the high rate of infections are thought to be the primary cause of paralysis in children due to GBS (Nachamkin et al. 2007; Kaakoush et al. 2015). As a clinical variant to GBS, Miller Fisher syndrome is due to molecular mimicry displayed by *C. jejuni* lipooligosaccharides (Chiba et al. 1992). The clinical manifestations of Miller Fisher syndrome include weakness of various body muscles, leading to loss of bodily movements (Chiba et al. 1992), and different classes of lipooligosaccharides in *C. jejuni* are associated with the development of Miller Fisher syndrome when compared to GBS (Parker et al. 2005; Quiñones et al. 2008). Ongoing research is investigating whether *C. coli* and other *Campylobacter* species can conclusively be responsible for promoting the onset of GBS by expressing ganglioside mimics in the lipooligosaccharide.

Routes and sources of transmission

In analyses of risk factor attributions for human campylobacteriosis, a large proportion of *Campylobacter* infections mainly occur due to the consumption of contaminated raw or undercooked poultry products (Figure 1). In particular, chicken meat is readily consumed in many industrialized and developing countries due to the high-quality protein and vitamins as well as low cost, taste and quick preparation time when compared to beef (Silva et al. 2011). As the main *Campylobacter* reservoir, chickens harbor in their cecum and intestinal tract a large number of *Campylobacter* populations, estimated to be 10^6-10^8 CFU per gram (Beery et al. 1988), and these large population sizes of *Campylobacter* can contaminate chicken meat and skin when the intestinal tract is ruptured during processing (Berrang et al. 2001). Other avian species, such as ducks and commercial turkeys, and domesticated animals, such as cattle, sheep, goats and pigs, can also serve as key animal reservoirs of *C. jejuni and C. coli* and emerging *Campylobacter* species. When compared to chicken, lower amounts of *Campylobacter* species have been detected in wildlife, including wild birds, insects, rodents as well as other land and marine mammals (Wacheck et al. 2009; Sippy et al. 2012; Whiley et al. 2013; Baily et al. 2015).

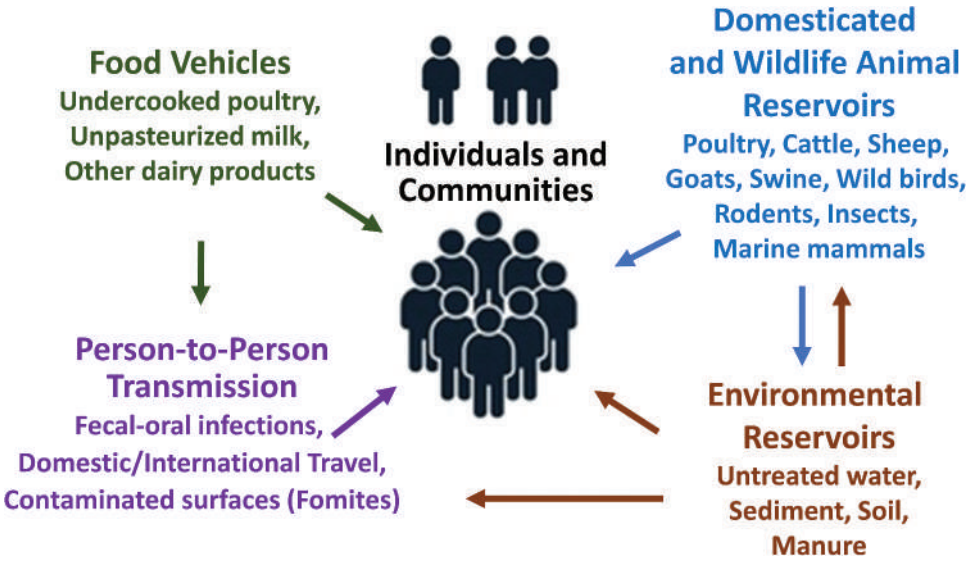

Figure 1. Potential routes of transmission for *Campylobacter* infections in humans. Several risk factors are attributed as sources of human campylobacteriosis. Avian species, in particular chickens, contribute to most human infections in addition to dairy products. Domesticated and wild animals can also serve as key reservoirs for zoonotic *Campylobacter*. Other attribution risks include environmental sources, and to a lower amount, person to person transmission.

Other environmental sources such as untreated water or unpasteurized milk as well as direct contact with feces from farm animals are considered relevant vehicles for campylobacteriosis (Figure 1). Moreover, untreated water has been overlooked as a leading source of *Campylobacter*. Ground/well water has been more likely to be a risk factor in the dissemination of human infections when compared to municipal surface waters (Hyllestad et al. 2020). In addition, the use of untreated water has been shown to lead to the transmission of *Campylobacter* in domesticated farm animals during the outdoor grazing period, contributing to the increase of positive samples in the tested animal hosts (Kaakoush et al. 2015). Moreover, *C. jejuni* outbreaks due to the consumption of unpasteurized milk have increased steadily during the recent years (Mungai et al. 2015), and some of these outbreaks have led to GBS in some patients in the United States (FIOD 2008). Contamination with animal feces or udder infection has been attributed as the source of *C. jejuni* in milk outbreaks. Infections due to non-food exposure, transmitted by the fecal-oral route, can occur because of contacting animal or human feces and is believed to result in uncommon/sporadic campylobacteriosis (Figure 1). Campylobacteriosis also can occur from travel-related infections associated with the consumption of contaminated food and water in some travel destinations with higher risk in the Americas, Asia, and some European countries (Kaakoush et al. 2015). Travel per se does not contribute to transmission but infected individuals can help disseminate strains with novel antimicrobial or virulent profiles to new and unexposed geographical locations.

Methods for isolation and culturing

Culturing and isolation methods are considered the traditional procedure for recovery of most foodborne bacterial pathogens (Silva et al. 2011; Fung et al. 2018). As the initial step in these traditional recovery methods, an enrichment is conducted, followed by selective and differential plating for isolation and then by immunological or molecular tests for confirmation (Figure 2). The enrichment broths resuscitate the bacteria exposed to stress or growth inhibitors in the tested matrix and enables the recovery of isolates when present at low concentration in the tested sample (FDA 2017; ISO 2017, 2019; USDA 2020). Common enrichment media for campylobacteria include

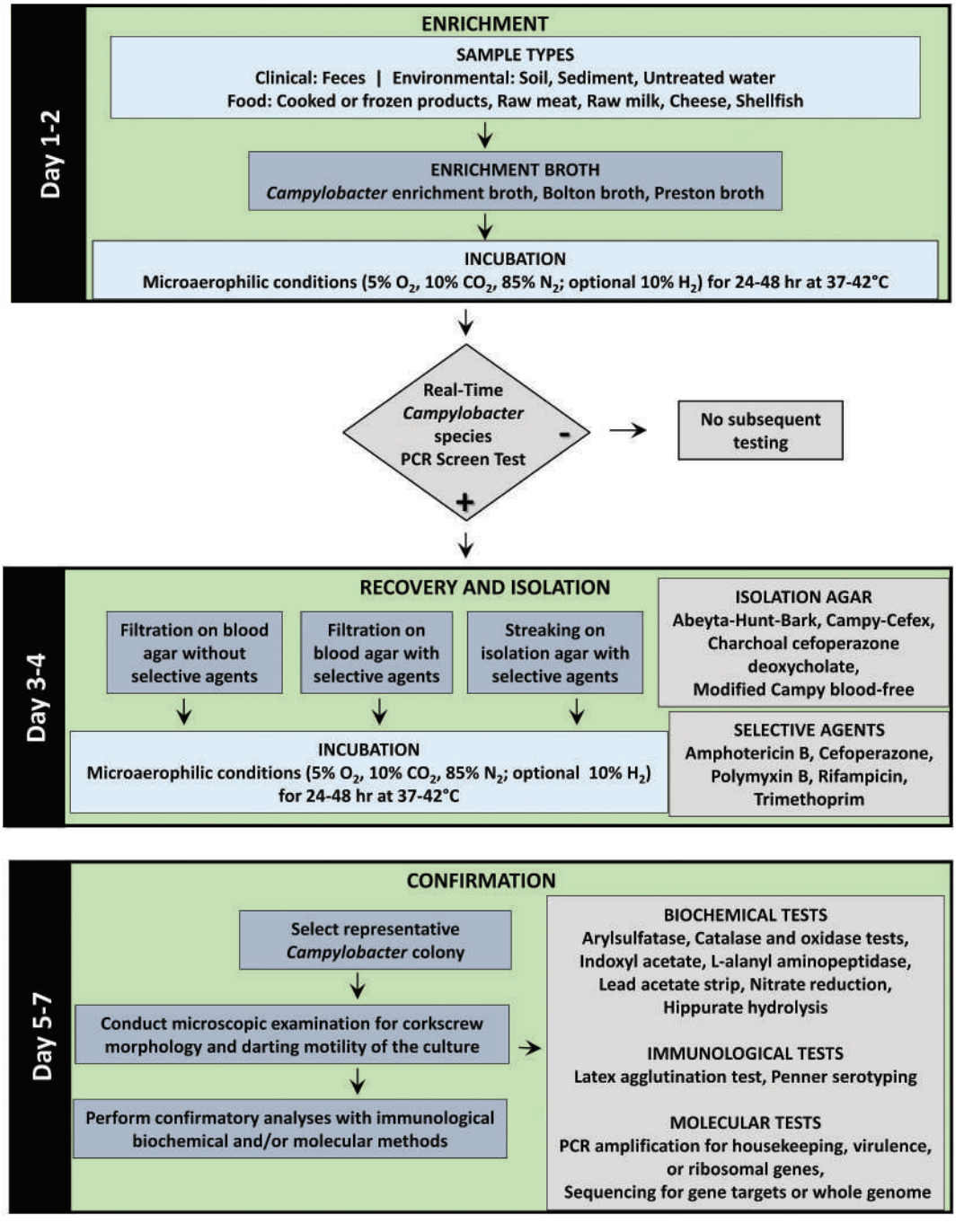

Figure 2. Culture and isolation methods for the efficient recovery of *Campylobacter* from clinical, food and environmental samples. The established methods, employed by food safety programs in the public and private sectors (FDA 2017; ISO 2017, 2019; USDA 2020), perform culturing and isolation methods for the effective recovery of campylobacteria from different types of samples. As the initial step, an enrichment is conducted to promote the resuscitation of bacterial cells in low concentrations when exposed to stress and/or growth inhibitors in the tested sample matrix. Selective and differential plating is performed for isolation, followed by immunological and/or molecular tests for confirmation.

Bolton broth, *Campylobacter* enrichment broth, Preston broth, charchoal cefoperazone deoxycholate, Abeyta-Hunt-Bark and Butzler agar (Figure 2). Given that the enrichment step also promotes the growth of microflora in the tested samples, there is a need for optimization of growth conditions in conjunction with the use of selective agents for the effective recovery of campylobacteria since there is no one single standard for the isolation of all *Campylobacter* species, particularly non-thermotolerant species. To aid the effective recovery of campylobacteria, chromogenic agar media have been incorporated in the isolation protocols for the identification of *Campylobacter* species with a distinctive colony color. Chromogenic media are both selective and differential due to the addition of synthetic chromogenic enzyme substrates, which enable the identification of the targeted isolate based on the organism's enzymatic activity (Perry 2017). Examples of chromogenic media successfully employed for the recovery of *Campylobacter* from clinical and food samples are *Campylobacter* selective chromogenic medium, RAPID *Campylobacter* agar, and CHROMagar *Campylobacter* (Le Bars et al. 2011; Seliwiorstow et al. 2014; Ma et al. 2020).

Media for isolation of *Campylobacter* species incorporate several selective agents in the culture media to inhibit competition by other bacteria and fungi in the effective recovery of campylobacteria during enrichment (Figure 2). The selective agents include the antibiotics sodium cefoperazone, trimethoprim, vancomycin, cycloheximide, and amphotericin B, which are commonly incorporated in the enrichment media, Bolton broth and modified charcoal cefoperazone deoxycholate agar (FDA 2017; ISO 2017; USDA 2020). Other studies have shown that addition of rifampicin is suitable for reducing false positives in the detection and selective recovery of *Campylobacter* in fresh produce and poultry (Jo et al. 2017). A concern is that the addition of antibiotics to the *Campylobacter* isolation media may contribute to select for resistant bacterial isolates, and consequently, result in biased antibiotic susceptibility data sets (Pumbwe and Piddock 2004). However, research demonstrated that the amounts of antibiotics employed in the selective media were significantly below the minimal inhibitory concentration of the antibiotic and do not induce overexpression of known efflux pump genes, which confer resistance to these antimicrobial agents in the selective media (Pumbwe and Piddock 2004).

Typical growth conditions for thermophilic campylobacters (*C. jejuni, C. coli* and *C. lari*) require an incubation temperature of 42°C for a period of at least two days under microaerobic conditions, consisting of 5% oxygen, enriched with 10% carbon dioxide and the balance with nitrogen (Wassenaar and Newell 2006; Vandamme et al. 2015). For the growth of non-thermophilic/emerging *Campylobacter* species, the incubation temperature is at 37°C, and the gas mixture to generate microaerobic conditions requires the addition of 10% hydrogen for emerging *Campylobacter* species (*C. concisus, C. curvus, C. gracilis, C. mucosalis, C. rectus, C. showae* and *C. hyointestinalis*) (Wassenaar and Newell 2006; Vandamme et al. 2015).

Selective broths are often supplemented with the enzyme Oxyrase to reduce the levels of oxygen and improving the isolation of *Campylobacter* species (Tran 1995). Current efforts by food safety laboratories are aimed at the development and optimization of a selective medium for growing campylobacteria under aerobic conditions. The supplementation of the basal media with dicarboxylates (fumarate, succinate, or malate) and monocarboxylates (pyruvate or lactate) and sodium bicarbonate promoted the growth of thermophilic *Campylobacter* species (Hinton and Cox 2018). Employing a method for the routine isolation of *Campylobacter* under aerobic growth conditions will thus significantly reduce equipment and supplies costs as well as simplify procedures associated with the generation of the microaerophilic atmospheres (Hinton and Cox 2018).

Enrichment mediums promote the growth of *Campylobacter* species present in low numbers and increases the motility of *Campylobacter* species when using subsequent methods for selecting the targeted species. A cellulose membrane filtration method with a pore size of 0.45 μm or 0.65 μm was initially developed in conjunction with selective agar media to improve the efficiency for isolation of campylobacters from stools since high levels of background microbial flora impede the detection of *Campylobacter* (Piersimoni et al. 1995; López et al. 1998; Engberg et al. 2000). By taking advantage of the increased motility of campylobacteria, the membrane filtration method has enabled the

recovery of these bacterial species from blood-based or blood-free agar in subsequent studies by testing a wide variety of samples, including wildlife, raw chicken carcasses, and fresh produce (Quiñones et al. 2007; Miller et al. 2017; Soto Beltrán et al. 2020; Chon et al. 2021). The presence of non-targeted/background microbial flora contributes significant challenges in culturing *Campylobacter* on selective and/or differential media and can likely influence the metabolism of *Campylobacter*. Temperature, nutrient media, incubation time and enrichment conditions all influence the ability to effectively recover *Campylobacter* isolates. As a result, the traditional culture-based techniques must be combined with detection methods that enable the confirmation of the recovered campylobacteria.

Methods for species identification and typing

Following enrichment and culturing, immunological, biochemical and molecular methods have been developed for the identification and confirmation of campylobacteria recovered from selective media (Table 1). For over 30 years, the 'gold-standard' immunological method for *C. jejuni* has been the Penner serotyping scheme method based on the heat-stable antigen, the capsular polysaccharide. Due to the high genomic variability in *C. jejuni*, many capsular polysaccharide moieties occur across serotypes, and a total of 47 *C. jejuni* Penner serotypes have been recognized, but due to cross-reactivity, the serotypes are categorized into 35 capsular polysaccharide serotypes, prompting the development of sequence-based methods for capsule characterization. As an improved and low-cost method when compared to Penner serotyping, a multiplex PCR assay, targeting the variable capsule region between *kpsC* and *kpsF* genes, enabled the characterization of the capsular polysaccharide in *C. jejuni* strains worldwide (Poly et al. 2015). Other immunological methods, latex agglutination and enzyme-linked immunosorbent assays employ monoclonal and polyclonal antibodies for detecting lipopolysaccharides, flagellin or other outer membrane antigens, allowing a fast and specific identification of thermophilic campylobacters on solid agar or liquid broths (Ricke et al. 2019). Several biochemical tests such as the catalase and oxidase tests, nitrate reduction, and lead acetate strip are commonly conducted by food regulatory agencies for phenotyping the recovered *Campylobacter* isolates (FDA 2017). Additional biochemical tests, measuring L-alanyl aminopeptidase activity, distinguish between campylobacteria from related genera and identify some isolates at the species levels (Hoosain and Lastovica 2009; Vandamme et al. 2015). Furthermore, the indoxyl acetate and hippurate hydrolase assays distinguish *C. jejuni* and *C. coli* strains from several other *Campylobacter* species (Nicholson and Patton 1995; Kaakoush et al. 2015). Growth with a hydrogen gas enriched atmosphere, an arylsulfatase test, and production of hydrogen sulfite are additional phenotypic tests that can aid in differentiation of emerging *Campylobacter* species (Vandamme et al. 2015).

As an alternative to biochemical testing, typing by employing high-throughput proteomics has becoming increasingly popular. In particular, bacterial colonies are used as samples for Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS), resulting in the spectra of the isolate's protein profile based on size and ionization charge. The spectra from the tested *Campylobacter* isolate can be quickly compared with those from reference strains using software for species identification (Bessède et al. 2011). In addition, the expression of virulence determinants and/or antimicrobial resistance factors can be identified if their characteristic biomarker spectra are known (Penny et al. 2016). Although MALDI-TOF MS has not always been able to determine phylogenetic relationships among *Campylobacter* at the subspecies level, improved software and reference spectra, incorporating complete allelic isoforms of core genes, has recently allowed proteotyping of clades within some *Campylobacter* species (*C. coli*, *C. fetus, C. jejuni*) (Emele et al. 2019b, 2019a; Feucherolles et al. 2021). Still standardization of MALDI-TOF MS protocols is necessary to allow comparison of mass spectrum data between laboratories (Penny et al. 2016). While the instrumentation and service maintenance are expensive, MALDI-

Table 1. Advantages and disadvantages of current detection methodologies for the detection and identification of *Campylobacter*.

Category	Method	Description of technique	Advantages	Disadvantages
Biochemical test	Enzymatic analysis	Target chemical reacts with reagents on disk or strip to determine phenotypes which differentiate genus and species.	Rapid and cost-effective technique.	Unable to distinguish among some *Campylobacter* species. Requires several days for culturing.
Immunological-based method	Serotyping	Characterization with the use of specific antisera to identify antigens.	Standard methods for species classification.	False positive results, time consuming, and immunological reagents can be limited in amount.
Mass spectrometry-based method	Matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry	Sample is mixed with energy absorbing matrix and ionized with a laser. Sizes of ionized peptides are measured by time of flight.	Rapid typing and virulence characterization technique with results in minutes. Low operating costs.	High initial capital costs. Standardization of technical parameters is necessary to compare data between different laboratories.
Nucleic acid-based methods	Single endpoint polymerase chain reaction (PCR)	Amplification of DNA target using sequence specific primers for detection of virulence genes and other DNA sequences of interest.	Simple, easy to perform and rapid genotyping method.	Sequence information on a single target gene. Inhibitors can affect amplification reaction. Requires analysis on gel agarose.
	Real-time PCR	Uses fluorescent dyes to improve time-to-result of PCR reaction and obtain quantification information.	Faster detection time than regular PCR, enabling quantification of amplified target with high sensitivity.	Expensive thermal cycler and reagents. Limited analysis of a single target amplification.
	Multiplex real-time PCR	Uses multiple primers for simultaneously target various species-specific sequences.	Detection of multiple targets per tested bacteria species.	Primer design is critical and may interfere with each other. Amplification can be inhibited in complex matrices. Expensive instrumentation.
	Multilocus sequence typing	Assigns alleles by performing DNA sequencing of multiple housekeeping genes.	Accurate and discriminatory power, readily available and reproducible, suitable for epidemiological studies.	By employing small subset of housekeeping genes, phylogenetic relationships may not be well examined. Expensive equipment for maintaining database.
	DNA microarrays	Array of short DNA oligonucleotide sequences spotted on a solid surface.	High-throughput and simultaneous analysis in a single assay of entire genomic sequences.	Expensive scanners for data analysis. Unable to detect novel sequences not previously spotted on array.
	Digital droplet PCR	Samples are split into many tiny droplets to improve quantification precision and accuracy of targeted sequences.	More precise quantification and robust with respect with PCR inhibitors.	Equipment is more expensive, and care must be taken to dilute samples to proper concentration to obtain best accuracy.
	Next generation sequencing	Sequence in parallel many fragments of target organism genomes and assemble the sequences to obtain complete genome information of the organism for superior typing and characterization information.	Higher resolution for strain typing and characterization with the entire genome.	Require large and expensive computational servers and standardization of in silico data. Typing analysis can be time consuming.

based applications provide fast results in just minutes and are less labor intensive, resulting in laboratory efficiency and reduced turnaround times so the cost per sample turns out to be low (Tran et al. 2015).

Several molecular methods have recently emerged for discriminating the isolates' relatedness and source of contamination (Table 1). Amplifying targeted genes with polymerase chain reaction (PCR) is the preferred assay for use by surveillance laboratories for routine analysis. The advantage of using PCR is the cost effectiveness and simplicity, and PCR-based assays provide an effective confirmation for the discrimination of those isolates that are indistinguishable by biochemical assays. A target in earlier studies for *Campylobacter* was a variable region of the 16S rRNA gene, indicative of *C. jejuni, C. coli*, and *C. lari* (Giesendorf et al. 1992). More recently, assays targeting other genes have been developed to increase specificity and sensitivity as well as virulence categorization. These targeted genes include the flagellin *flaA/flaB* (Bolton 2015), heat shock protein *hsp60* (Park et al. 2011), hippuricase *hipO* (Persson and Olsen 2005), cytolethal distending toxin *cdt* (Bolton 2015), and nitrate reductase (*nap*) (Miller et al. 2007). Real-time quantitative PCR (qPCR) assays have an improved speed for test results and provide quantitative information (Kralik and Ricchi 2017). Other assays employing qPCR allow multiplexing in the amplification of gene targets by using different fluorophores conjugated to the target probes, and a number of qPCR assays are available for the detection of *Campylobacter* species, which are capable of simultaneously detecting *C. jejuni, C. coli* and *C. lari* in a single test (Koziel et al. 2013). A limitation of qPCR is that the quantification can be significantly affected by enzymatic inhibitors present in complex organic matrixes, commonly present in some food products and fecal samples, and does not differentiate between live and dead cells when amplifying DNA (Kralik and Ricchi 2017). Therefore, several applications have been incorporated for differentiation which includes pre-enrichment to recover viable cells, targeting RNA, or using chemicals that penetrate dead cells membranes and bind DNA. Compounds such as ethidium monoazide and propidium monoazide have been proposed as DNA binding dyes, although in some cases excess of ethidium monoazide may affect viable genomic *Campylobacter* DNA, and low amounts of propidium monoazide may not sufficiently repress dead cell DNA (Krüger et al. 2014). A recently introduced platform, digital droplet PCR (ddPCR), divides the sample into tiny reactions in emulsions of oil, water and stabilizing chemicals forming droplets. The quantification of positive and negative reactions is determined with better precision and less susceptibility to enzymatic inhibitors than qPCR (Baker 2012). In comparison to qPCR, studies demonstrated that ddPCR more consistently detected *C. jejuni* from commercial poultry process water and accurately identified *Campylobacter* in meat and dairy samples (Rothrock et al. 2013; Cremonesi et al. 2016).

The development and implementation of additional sequence-based typing methods, including multilocus sequence typing (MLST), DNA microarrays and whole genome sequencing, have facilitated the detection and characterization of *Campylobacter*-related outbreaks and have enabled the improved differentiation of closely related isolates (Sabat et al. 2013). In particular, the MLST scheme for *C. jejuni* is based on the PCR amplification of seven highly conserved housekeeping genes, followed by the sequencing of the fragments and comparison of their nucleotide sequences using standard phylogenetic analysis. The advantage of MLST is that the data obtained is unambiguous and highly reproducible by using an internationally standardized nomenclature. As a high-throughput method, DNA microarrays technology has enabled the genotyping and profiling of genomic content in campylobacteria (Parker et al. 2006; Quiñones et al. 2007). This technology consists of a collection of DNA probes, attached in an orderly fashion to a solid surface, and the presence or absence of the complementary genome sequences in the tested isolate is detected after hybridization to the different probes on the array. Moreover, microarrays have allowed the detection of extra-genomic elements in *C. jejuni* (Parker et al. 2006), and pathogenic strains can also be simultaneously examined for their antimicrobial resistance and virulence potential (Quiñones et al. 2007, 2008; Sabat et al. 2013). One of the limitations of the microarray-based assays is that labeling of target DNA can be inconsistent and results in highly variable hybridization patterns. Another

disadvantage is that DNA microarrays allows the identification of only those known probe sequences previously attached to the array, making it difficult to identify emerging strains that are highly variable. Also, this technique is unable to distinguish highly clonal strains based on single nucleotide polymorphisms (Sabat et al. 2013).

Given the limitations of the previously discussed sequence-based typing methods, public and private health laboratories and governmental regulatory agencies worldwide have embraced the use of high-resolution/next-generation genome sequencing (NGS) for obtaining complete and simultaneous information about virulence, antimicrobial resistance and subtyping markers that are relevant to epidemiology and foodborne disease surveillance (Brown et al. 2019). By completing the sequence of the entire genome, NGS has gone beyond the identification of *Campylobacter* strains associated with an outbreak investigation. NGS has allowed the analysis of genomic rearrangements and recombination events for further analysis of the evolutionary characterization of the genus and has led to the development of diagnostic assays to improve food safety monitoring in clinical and agricultural settings (Ricke et al. 2019). Genome networks established by regulatory agencies, including GenomeTrakr (U.S. Food and Drug Administration), TraNet (China National Center for Food Safety Risk Assessment), and EpiPulse (European Centre for Disease Prevention and Control), have implemented pipelines for tracking foodborne pathogens to improve outbreak investigations and to provide more precise scientific traceback and environmental source data (Brown et al. 2019; Leitmeyer et al. 2020; Li et al. 2021). The decrease in reagents and supplies costs in conjunction with the improvements and simplification of the bioinformatic analyses and tools have allowed the use of NGS as the preferred diagnostic and surveillance platform for global food safety. Given that NGS technologies and analysis tools are rapidly evolving and improving, new platforms are now being deployed to diverse and remote field locations for enabling rapid pathogen detection in real time.

Colonization and virulence factors

Virulence in *Campylobacter* consists of multiple pathways, mainly attributed to flagella-mediated motility, bacterial adherence to intestinal mucosa, invasive capability, and the ability to produce toxins, and different sets of determinants are required for the successful colonization by *C. jejuni* of the host gastrointestinal tract (Table 2). Having arrived at the host's gastrointestinal epithelial cells, adherence to the cells is required for colonization (Bolton 2015). Important virulence determinants include CadF, a 37-kDa fibronectin-binding outer membrane protein, which is responsible for *Campylobacter* adhesion to fibronectin (Konkel et al. 1997). FlpA is also a fibronectin binding protein that may work together with CadF (Bolton 2015). Another factor is the CapA autotransporter that is also involved in adhesion. However, some strains use an alternate CapC protein, indicating that adhesion proteins vary between different *C. jejuni* strains due to diverse mechanisms of interaction and strategies of colonization (Mehat et al. 2020). Moreover, *Campylobacter* use a Type III protein secretion system (T3SS) for injecting and secreting putative virulence factors into host cells (Table 2), and this flagellar-based T3SS consists of FlhA, FlhB, FliO, FliP, FliQ and FliR (Bolton 2015). Among the various factors secreted from the flagellum are FlaC, CiaC, and CiaI, which are required for colonization, invasion, and intracellular survival (Carrillo et al. 2004; Konkel et al. 2004). Other virulence determinants include IamA and FspA, which are both required for invasion and colonization (Bolton 2015). The most characterized toxin produced by *Campylobacter* is the cytolethal distending toxin. The toxin consists of three subunits CdtA, CdtB, CdtC; CtdA and CdtC are responsible for the delivery of the active subunit CdtB, which enters the host cell nucleus and acts as a deoxyribonuclease to result in cell cycle arrest and death (Bolton 2015).

Carbohydrate structures on the surface of the *Campylobacter* cell can serve to facilitate host cell adhesion, invasion, immune evasion, and pathogenicity (Table 2). In particular, galactosyltransferases genes *cgtB* and *wlaN* are associated with modification to the

lipooligosaccharides on the surface of *Campylobacter* that trigger immune responses underpinning Guillain-Barré and Miller Fisher syndromes (Bolton 2015). Still, our knowledge of the interactions between the invading *Campylobacter* and the host cells has some gaps. In transcriptome experiments with almost 200 *C. jejuni* clinical isolates and a mammalian cell line (INT-407 cells), over 900 genes were upregulated compared to controls grown without a host (Kovács et al. 2020). In addition to the known virulence factors genes, other functional groups including transmembrane proteins, bacterial shape determinants, regulatory systems, energy systems, respiration, iron uptake, protein synthesis/modifications/secretion were also upregulated to support *Campylobacter* intracellular survival (Kovács et al. 2020). Future experiments involving gene inactivation, phenotypic assays, and transcriptomics with a diverse *Campylobacter* strain collection are thus needed to improve our understanding of *Campylobacter* pathogenesis.

Table 2. Characteristics and functions of genes commonly employed for the categorization of *Campylobacter* virulence potential.

Functional category	Gene	Characteristics and functions
Adhesion	*capA, capC*	Autotransporter and auxiliary adhesin.
	cadF, flpA	Outer membrane proteins required for adhesion to fibronectin.
	peb3	Periplasmic transporter and adhesin facilitator.
	virB11	Plasmid-encoded factor involved in host cell adhesion and invasion.
Bacterial shape determinant	*mreB*	Bacterial actin homologue defining cell morphogenesis
	mreC	Periplasmic space protein required for cell wall synthesis.
	pbpB	Required factor for bacterial growth and cell wall biosynthesis and beta-lactam antibiotics target site.
	pbpC	Major protein required for bacterial cell division and target site for beta-lactam antibiotics.
Chemotaxis	*cheA*	Histidine kinase for the relay of phosphorylation activity in chemotaxis.
	cheB, cheR	Adaptation proteins for the regulation of the chemosensory response.
	cheV, cheW, cheY	Cytoplasmic response regulators for sensory adaptation.
	cheZ	Phosphatase for flagellar rotational control to promote chemotaxis.
Iron uptake	*ceuE*	Lipoprotein involved in iron acquisition.
	cfrA	Factor responsible for high-affinity iron acquisition and sensing cues during colonization.
	cfrB	Receptor for the high affinity siderophore enterobactin for iron acquisition.
	chuA	Outer membrane receptor for hemin and hemoglobin.
	fur	Ferric uptake regulator.
Invasion	*iamA*	Factor required for mammalian host cell invasion.
	ciaB	Secreted protein required for internalization to human epithelial cells.
	ciaC	Secreted protein required for maximal host cell invasion and cytoskeletal rearrangement.
	ciaI	Secreted factor required for intracellular survival.
	flaC	Protein secreted into the host cells and essential for colonization and invasion.
	flhA	Membrane ion channel of type III secretion export system.
	flhB	Membrane protein regulating secretions through FlhA.
	fliO, fliP, fliQ, fliR	Membrane proteins serving as structural components around FlhA.
	fspA	Flagellum secreted protein vital for colonization and is a vaccine target for chicken immunizations.
	virK	Determinant essential for antimicrobial peptide resistance.
Motility	*flaA, flaB*	Major flagellin protein required for motility.
	flgE, flgK, flgL	Factors required for functional flagellar hook complex.
	fliF	Rod protein required for synthesis of the axial part of the flagellum.
	fliM, fliY	Proteins involved in switching motor of flagella.
	motA, motB	Motor protein powering flagella.
Outer surface saccharide	*cgtB, wlaN*	1,3 galactosyltransferases required for synthesis of lipooligosaccharide that act as ganglioside mimics.
	kspE	Transport protein required for capsular polysaccharide biosynthesis.
	kspM	Required for biosynthesis of outer membrane capsule polysaccharide.
Toxin	*cdtA, cdtC*	Cytolethal distending toxin (CDT) subunits which bind to host membrane and deliver CdtB into the cell.
	cdtB	CDT catalytic subunit with deoxyribonuclease activity causing DNA damage and cell death.

Antimicrobial resistance

Most *Campylobacter* human infections are treated with body fluid replacement and maintenance of electrolyte balance, but antimicrobial treatment is required for severe cases, such as a febrile patient with bloody stool. Antibiotic treatment with macrolides is the predominant choice followed by fluoroquinolones (Whitehouse et al. 2018). Macrolides have been shown to inhibit protein synthesis by binding to the ribosome and changing its conformation. The most common mechanism of resistance is mutations of the 23S rRNA and modification of the target site by methylation through the *erm(B)* gene. However, resistance against macrolides in *C. jejuni* is not commonly observed since the resistant strains have a reduced fitness and colonization of the chicken host when compared to susceptible *C. jejuni* strains. Increased use of macrolides has been associated with a species-specific shift in chicken colonization by favoring colonization by *C. coli* when compared to *C. jejuni* (Whitehouse et al. 2018). Traditionally, fluoroquinolones have been the treatment of choice; however, increased resistance to these agents is associated with their extensive use reducing their effectiveness. These agents target the DNA gyrase to inhibit DNA synthesis, and modification of the quinolone target site, the quinolone resistance-determining region of gyrase A (*gyrA*), is the predominant resistance mechanism of fluoroquinolones in *Campylobacter*.

Broad spectrum tetracycline and beta-lactams have been used for treating gastrointestinal infections. Resistance to tetracycline in *Campylobacter* is moderate to high and is generally mediated by the *tet(O)* gene, commonly found on the pTet plasmid but also on a genomic island. Beta-Lactam antibiotics act by binding to penicillin-binding proteins and disrupting peptidoglycan cross-linking during cell wall synthesis. Resistance through beta-lactamase, blaOXA-61, is widespread in *C. jejuni* and *C. coli*. The *Campylobacter* multidrug efflux pump CmeABC has also worked synergistically to provide resistance to beta-lactams as well as tetracyclines, macrolides and fluoroquinolones (Whitehouse et al. 2018). *Campylobacter* infections that are resistant to less toxic antibiotics may be treated with aminoglycosides, such as gentamicin (Fair and Tor 2014). Aminoglycosides bind to prokaryotic ribosomes impairing protein synthesis, and over 24 genes, encoding aminoglycoside-modifying enzymes, have been identified in *Campylobacter*. A gene cluster *aadE-sat4-aphA-3* confers multidrug resistance including aminoglycosides and has been found in *C. jejuni* and *C. coli*, recovered from food and human. This gene cluster has been detected on a plasmid and integrated in the chromosome (Zhao et al. 2016). Finally, an intrinsic resistance in some *C. jejuni* and *C. coli* isolates has been described against penicillin, older cephalosporins, trimethoprim, sulfamethoxazole, rifampicin, and vancomycin (Fitzgerald et al. 2008).

Even though campylobacteriosis is a zoonotic infection, it has been shown that the emergence of *Campylobacter* resistance in human clinical samples is connected to antimicrobial resistance found in animals. The inappropriate usages of antibiotics in the veterinary medicine and animal production contribute to the increased antimicrobial resistance and the emergence of multidrug resistance profiles. The indiscriminate use of antibiotics in food animal production has been indicated as a catalyst in the development of resistant foodborne or waterborne *Campylobacter* infecting humans (Wieczorek and Osek 2013; Whitehouse et al. 2018). Additionally, acquisition of genes by horizontal transfer can also provide resistance mechanisms including the enzymatic degradation, alteration of the antimicrobial compound, active efflux of the antimicrobial across the cell membrane, or alteration of the cell membrane to reduce the permeability to the antimicrobial.

Conclusions and perspectives

Campylobacter infections continue to be a leading cause of foodborne illness worldwide, accounting for a burden of millions of diarrheal illnesses per year with a billion-dollar cost in medical expenses and loss of productivity. Epidemiological studies have shown these foodborne pathogens are responsible for not only causing acute and self-limiting illness but also post-infectious

chronic symptoms (Kaakoush et al. 2015; Scharff 2020; EFSA 2021). The highly adaptable and dynamic genetic composition allows *Campylobacter* to persist ubiquitously in the environment and to acquire antimicrobial resistance and virulence factors via horizontal gene transfer (Wieczorek and Osek 2013; Whitehouse et al. 2018). Still additional research is needed to determine the association of the well-characterized and emerging pathogenic strains with disease outcome and prevalence in animal hosts and in the food chain. Analysis of the genetic content and virulence factor expression would provide a better understanding of mechanisms required for the development of severe human illness as well as environmental adaptability and persistence in various agricultural environments. The development of rapid, low-cost, and easy-to-perform methods for the accurate and sensitive monitoring of campylobacteria would thus provide food chain biosecurity and assist with source tracking of foodborne outbreaks in surveillance investigations. As the demand for more food products and faster line speeds increase worldwide (Fung et al. 2018), reliable automated methods that enable real-time and onsite decision making for producers will be needed for the surveillance of the entire process from sample-to-answer, reducing the time needed to systematically identify and confirm remediation and resolution of these pathogens.

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