Review of current methodologies to isolate and identify *Campylobacter* spp. from foods

Gregory Gharst\(^a\), Omar A. Oyarzabal\(^{b,*}\), Syeda K. Hussain\(^c\)

\(^a\) U.S. Food and Drug Administration, Bedford Park, IL, USA
\(^b\) IHB Laboratories and Consulting Group, Lake Forest Park, WA, USA
\(^c\) Department of Biological Sciences, Alabama State University, Montgomery, AL, USA

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**A B S T R A C T**

This article summarizes the most effective protocols to isolate *Campylobacter* spp. (mainly *Campylobacter jejuni* and *Campylobacter coli*) from food, primarily poultry products, and includes a summary of the current methods recommended by the Food and Drug Administration and the U.S. Department of Agriculture in the USA, and ISO in Europe. The recommended temperature for incubation of the samples throughout the isolation procedure is 42 °C. The enrichment of the samples for 48 h, which can be performed under aerobic conditions, is recommended to achieve a detectable number of *Campylobacter* colonies. Bolton broth or buffered peptone water supplemented with ceftiofur and amphotericin B is commonly used enrichment broths. The transfer of the enriched samples to plate media using membrane filters helps to obtain pure *Campylobacter* colonies. Charcoal cefoperazone deoxycholate (CCDA) is the best choice among all plate media. There is no need to add oxygen quenching substances or blood to enrichment broth for the isolation of *Campylobacter* spp. However, the addition of blood to plate media aids in differential identification of presumptive colonies. Phase contrast microscopy and latex agglutination tests are confirmatory tests for presumptive *Campylobacter* isolates. The use of multiplex polymerase chain reaction (mPCR) assays is the simplest and most rapid method to identify isolates to the species level. mPCR assays, or other methods assessing DNA sequence variations, will probably become the confirmation procedure of choice in the future. Recent work with retail broiler meat has revealed that the rinsing of meat is more sensitive for the recovery of naturally contaminated retail broiler meat than current reference methods and requires less time for preparation and processing of the samples. This protocol could be coupled with DNA-based methods for a fast screening of positive samples.

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1. Introduction

*Campylobacteriosis* is a highly prevalent foodborne disease in industrialized nations. In the US, *Campylobacteriosis* is the third most frequent bacterial foodborne disease, with 0.8 million estimated cases that represent 8% of the overall estimated foodborne disease illnesses (Scallan et al., 2011). Besides the typical diarrheal syndrome, *Campylobacter* infection has been linked to Guillain–Barré syndrome, which is an autoimmune-mediated disorder of the peripheral nervous system that results in acute demyelinating polyneuropathy (Kaldor and Speed, 1984; Speed et al., 1984).

Although there are more than 20 *Campylobacter* spp., *Campylobacter jejuni* and *Campylobacter coli* are the two most important species associated to human disease (Man, 2011). *C. jejuni* accounts for 80–90% of the infections reported in the US (Gilliss et al., 2013). In the USA, a mean of 28 outbreaks per year has been reported for years 2003–2008 (Taylor et al., 2013). The consumption of undercooked poultry is considered a major risk factor for sporadic infections (Friedman et al., 2004). However, the analysis of outbreak data in the last 10 years identifies raw milk as the main vehicle of transmission of *campylobacteriosis*, with 29% of the outbreaks associated with dairy products versus 11% associated with poultry and 5% associated with produce (Paintner et al., 2013; Taylor et al., 2013).

*C. jejuni* has a relatively low infectious dose based on experimental infection studies. Robinson (1981) reported that approximately 500 organisms were enough to produce infection after the consumption of a *C. jejuni* strain isolated from a raw milk outbreak in 1979 (Robinson et al., 1979). A previous study reported an infectious dose of 10⁶ *Campylobacter* cells (Steele and McDermott, 1984), but the origin of the strain used in that study is unknown. The last report on experimental infections in humans was published in 1988, when disease was induced in volunteers who were given doses as low as 800 *Campylobacter* cells of a *C. jejuni* strain originated from a milk outbreak. These experiments corroborated that a low number of cells (less than 1000) can produce disease in humans (Black et al., 1988). It is accepted that all *C. jejuni* strains from all food sources are equally pathogenic, although none of...
the strains isolated from poultry products or other foods have ever been tested for infectivity in humans. There is a great deal of data uncertainty when only the single dose–response model is used to calculate infectious doses, and in the future it is expected that the calculation of confidence intervals will provide a better understanding of the variability of infectious doses for Campylobacter spp. and other bacterial foodborne pathogens (Moon et al., 2013).

Campylobacter spp. colonize domestic animals (Friedman et al., 2004) and pets, especially puppies (Damborg et al., 2004; Tenkate and Stafford, 2001), and can be found in recreational waters. In the intestine of avian species, such as commercial chickens and turkeys, Campylobacter spp. establish a commensal relationship with the host that results in their occurrence in high numbers in colonized birds (Potturi-Venkata et al., 2007). Molecular techniques have helped the scientific community understand that C. jejuni and C. coli are the only species found in commercial chicken production worldwide (He et al., 2010; Oyarzabal et al., 1997; Suzuki and Yamamoto, 2009). Although Campylobacter lari strains have been isolated from live commercial turkeys (Smith et al., 2004), these strains have not survived storage and no further confirmation of the presence of C. lari in turkey flocks has been reported (S. Kathariou, personal communication, March 18, 2013). Therefore, it appears that turkey isolates are also primarily C. coli or C. jejuni, but C. coli is the most predominant species.

Contrary to sporadic cases associated with the consumption of chicken, the consumption of raw, unpasteurized milk has been the most important source of campylobacteriosis outbreaks in the USA, Canada and Europe in the last 15 years (Heuvelink et al., 2009; Jay-Russell et al., 2013; Lejeune and Rajala-Schultz, 2009; Oliver et al., 2006; Schildt et al., 2006). Feces from animals or infected humans may also contaminate waters, which in turn may become another source for campylobacteriosis. Overflow of sewage, polluted water run-off, and agriculture run-off are some of the ways water becomes contaminated with Campylobacter spp.

The protocols for isolation of Campylobacter spp. from foods were adapted originally from clinical microbiology protocols, and the methodology for isolation of Campylobacter from milk remains unchanged. However, the methods for isolation of Campylobacter spp. from poultry products have undergone several modifications. This review summarizes the protocols that are currently available for the isolation of Campylobacter spp. from poultry products, with emphasis on the improvements made in the last 10 years to the isolation and identification protocols targeting C. jejuni and C. coli.

2. Isolation of Campylobacter from foods

Campylobacter spp. are microaerobic bacteria with some species being thermotolerant. King (1957) noticed that Campylobacter strains isolated from humans grew well under microaerobic conditions at 42 °C and were serologically different from animal strains. In addition, the animal strains did not grow at 42 °C, suggesting that these strains were Campylobacter fetus. Soon after these findings were published, microaerobic conditions (5% O₂, 10% CO₂, and 85% N₂) and 42 °C became the standard incubation conditions to isolate Campylobacter spp. from clinical samples. Eventually, the use of microaerobic environments and a higher incubation temperature became the protocol of choice to isolate Campylobacter spp. from poultry and milk products. This higher incubation temperature inhibits some of the competing microflora present in food samples and allows for the isolation of C. jejuni, C. coli, C. lari and C. upsaliensis, which are sometimes referred to as the “thermotolerant” Campylobacter species.

2.1. Enrichment conditions for isolation

During isolation from food samples, the presence of other bacteria with faster generation time always poses a limitation for the isolation of low number of Campylobacter cells. Therefore, the enrichment step plays an essential role in facilitating the growth of low numbers of Campylobacter cells for later detection. It is unclear as to which condition selectively is in favor of the multiplication of Campylobacter cells, and not of the competing bacteria, during enrichment. All attempts to reduce the enrichment time from 48 to 24 h during the isolation of Campylobacter spp. from chicken meat have been unsuccessful (Oyarzabal et al., 2007; Liu et al, 2009). Therefore, the low numbers of cells (0.6–0.8 CFU per g) present in retail broiler meat, for instance (Oyarzabal et al., 2007), require incubation at 42 °C for 48 h.

Several types of enrichment broths have been developed for isolation of Campylobacter from foods (Corry et al., 1995). Usually, enrichment broths consist of a basal medium, such as Brucella broth or nutrient broth (Bolton and Robertson, 1982; Corry et al., 1995), supplemented with antimicrobials. Originally most enrichment media were supplemented with lysed horse or sheep blood, but it has been found that blood is not required to isolate Campylobacter spp. from poultry meat (Liu et al, 2009). Furthermore, formulations without blood may be more amenable to the coupling with molecular methods for faster detection and identification. The basal medium does not need to be rich for the isolation of Campylobacter spp. Bolton broth continues to be one of the best alternatives for enrichment (Baylis et al., 2000) but buffered peptone water, which has a similar composition as the basal component of Bolton broth, is equivalent to Bolton broth for the isolation of Campylobacter spp. from retail broiler meat (Oyarzabal et al., 2007). The enrichment protocols for the isolation of Campylobacter spp. from retail broiler meat are based on a ratio of 25 g of meat in 225 ml of enrichment broth. However, an enrichment ratio of 1:9 (meat:broth) has been shown to perform similarly to the 1:9 ratio and reduces the amount of broth used during the isolation process (Oyarzabal et al., 2007).

2.2. Microaerobic environment

Campylobacter spp. have been traditionally isolated under microaerobic conditions. Various methods have been developed to create a microaerobic environment that would allow Campylobacter spp. to grow. A simple system is the use of sachets (Oxoid BR56, Oxoid, UK) that generate carbon dioxide from sodium bicarbonate and citric acid, or hydrogen from sodium borohydride with the use of palladium as a catalyst that converts hydrogen and oxygen to water (Sails et al., 1998). These sachets are used in a contained, sealable environment provided by jars traditionally used to create anaerobiosis. The sachets have been improved over the years and it is no longer necessary to add water to generate the microaerobic conditions. Newer sachets, for instance, generate carbon dioxide without the production of hydrogen (CampyGen, Oxoid).

Another system for generation of microaerobic conditions is the evacuation-replacement system, which uses a pump to evacuate the air within a jar and then replaces the air with a microaerobic mix. Microaerobic cylinders are easy to obtain from a gas manufacturer and the industrial microaerobic mix is adequate in providing the conditions necessary for the growth of Campylobacter spp. All the above systems can be employed in conjunction with plastic bags, such as Ziploc bags.

Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Positive</th>
<th>Negative</th>
</tr>
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<tbody>
<tr>
<td>Bolton + Oxylase</td>
<td>34</td>
<td>16</td>
</tr>
<tr>
<td>Bolton</td>
<td>19</td>
<td>62</td>
</tr>
</tbody>
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Comparison of Bolton broth with and without the addition of Oxylase. Chi-square (as defined by McNemar) = 0.46. Statistical differences are found when the chi square values are ≥ 3.8.