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Review Article

Cultural and Immunological methods for the Detection of *Campylobacter jejuni*: A Review

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ABSTRACT

Campylobacter jejuni is Gram negative spiral-shaped foodborne pathogenic bacterium which belongs to the family Campylobacteriaceae. It is a microaerophillic and thermophillic microorganism that can grow well at the temperature range between 37°C and 42°C. *Campylobacter* can cause diseases in humans and animals including wild animals, pets and livestock species. Various cultural methods have been developed for the detection of the organism. However, isolation and identification of organism is a tedious and long task usually takes 5-7days. The tracking of the organism and source of the disease in real time are always challenging with these methods due to rapidity and robustness of available methods. Although a variety of immunoassays such as enzyme-linked immunosorbent assay (ELISA), fluorescent antibody technique (FAT) and PCR enzyme-linked immunosorbent assay (PCR-ELISA) have been developed for testing clinical and food samples for Campylobacter spp., including *Campylobacter jejuni*. However, it requires approval by regulatory bodies.

Key Words: *Campylobacter jejuni*; Foodborne pathogen; Public health; Cultural methods; ELISA; FAT.

Introduction

Campylobacter jejuni is Gram negative spiral-shaped foodborne pathogenic bacterium which belongs to the family Campylobacteriaceae. The bacterium is microaerophillic and thermophillic that can grow well at the temperature range between 37°C and 42°C. It can cause diseases in humans and animals including wild animals, pets and livestock species. *Campylobacter* infections are known as 'Campylobacteriosis' which can be recognized with typically muscle pain, headache and fever (known as the 'febrile prodrome') followed by watery or bloody diarrhoea, abdominal pain and nausea. Symptoms may last 1 day to 1 week or longer (usually 5 days). Excretion of the organism in stools occurs on average for 2 to 3 weeks and is mostly self-limiting. Hospitalisation has been reported in up to 13% of cases. The attack rate is around 45%. In long term effect Campylobacteriosis is cause of chronic sequelae in the form of Guillain-Barre syndrome (GBS). The frequency of GBS resulting from Campylobacteriosis has been estimated as 0.1% and usually occurs one to three weeks after enteritis. Approximately 20% of patients with GBS are left with some form of disability and approximately 5% die. Campylobacteriosis is also associated with Reiter's syndrome, a reactive arthropathy. The frequency of this illness has been estimated as 1% of all Campylobacteriosis cases (Silva et al., 2011).

Campylobacters were observed in stools of diarrhoeic infants in 1880 in Germany for the first time. However, in 1913 first recognized identification of the organism was made which was associated with abortion in sheep whereas confirmatory tests were carried out in 1918 when similar organisms were isolated from aborted bovine fetuses. Due to spiral appearance the organisms were

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originally assigned to the genus Vibrio and named as *Vibrio fetus*. However, it was not until 1947 that the human infection was first associated with the microaerophillic vibrios, which was associated with a pregnancy related infection, where the fetus died. Later, the bacterium causing enterocolitis in calves named as *Vibrio jejuni*. In 1944 enterocolitis in Swine was reported and named the organisms as Vibrio coli. In 1963, genus Campylobacter was proposed due to inability of utilizing sugars and had a different G+C content to that of Vibrio spp. In 1973, Veron and Chatelain named *C. jejuni* and *C. coli* for organisms recovered from seterocolitis case (Khan, 2012). Now the genus Campylobacter is comprising 25 species, two provisional species and eight subspecies which are morphologically different including spiral, curved or rod shaped. These are nutritionally fastidious bacteria that require complex nutritional environments. They grow under strictly anaerobic or microaerophillic conditions. These species of genus Campylobacter naturally colonize humans, other mammals, birds, reptiles and shellfish. *C. jejuni* is most well-known member causes bacterial gastroenteritis in humans worldwide whereas *C. coli*, which is closely related to *C. jejuni*, accounts for 1–25% of all Campylobacter-related diarrheal diseases. In last decade, few new Campylobacter species other than *C. jejuni* and C. coli have been also recognized as important pathogens in humans and animals such as *C. concisus*, *C. lari*, *C. upsaliensis* and *C. ureolyticus* (Man, 2011).

It has been noticed that *C. jejuni* may lead to serious outbreaks irrespective of the region. This leads to the spread of disease, more so in infants and aged individuals. Hence, rapid detection becomes important to contain the spread of the pathogen before it leads to a serious outbreak. Various techniques have been evolved to detect the *C. jejuni*.

Cultural media and methods

Initially a number of media formulations have been proposed for the isolation of the Campylobacters by various workers, especially thermophilic Campylobacter, and their number probably exceeds that for any other group of bacteria, especially if one considers that all have been published since 1972 and almost all since 1977. Before the medium of Dekeyser and his co-workers in 1972 was available, isolation of "related Vibrio" (as Campylobacter were then known) depended on the use of membrane filtration followed by subculture onto nutritionally rich blood agar. The method of Dekeyser and co-workers used a combination of centrifugation, filtration through a 0.65 um membrane filter and the pating of the filtrate onto a selective agar. In a pilot study was conducted by Kumar and co-workers to compare Blaserwang, Butzler and Skirrow's media for isolation of *Campylobacter* from poultry and found that Skirrow's medium gave maximum recovery of the organisms. In 1972, Clark and Dufty used enrichment medium for transport and enrichment of samples of preputial liquid for *C. fetus*. The medium consisted of 300µg/ml of 5-flourouracial, 100 IU/ml of polymyxin-B sulphate, 50µg/ml of brilliant green, 3 µg/ml of nalidixic acid and 100 µg/ml of cycloheximide as an antifungal agent. In a trial in which 89 samples were collected from 5 infected bulls and transported and cultured in the medium, *C. fetus* subsp. fetus was demonstrated in 77 samples by culture.

Approximately after one-decade Rothenberg and his co-workers in 1984 compared Doyle and Roman enrichment broth, Park and Stankiewiez enrichment broth and newly developed enrichment broth for the isolation of *C. jejuni* from raw chicken and found that Doyle and Roman enrichment broth showed greatest selectivity. DREB and Park enrichment broths were compared for recovery of *C. jejuni* from food No significant differences were found between the results obtained with the two broths. In another study in 1985 by Beuchat compared several enrichment and direct isolation media to evaluate their suitability for detection and enumeration of five strains of *C. jejuni* in refrigerated chicken meat, it was found that Campy Brucella agar (CBAP), blood free Campylobacter medium (BFCM) are superior than Modified Butzler agar (MBA) and Doyle and Roman Enrichment broth. Later in 1990, Kakkar and Dogra used candle jar for creating microaerophillic conditions for growth of Campylobacter from rectal swabs and successfully isolated *C. jejuni*, *C. coli* and *C. lari*. After two years of this in 1992, a modified internal gas generating system for creating microaerophillic conditions for isolation of *Campylobacter* spp. by using sodium borohydride, sodium bicarbonate and citric acid developed by

Shobha and their co-workers. They further compared its results by using gas pack for providing microaerophillic conditions and reported that Campylobacter was efficiently isolated by using both methods. United States Food and Drug Administration (USFDA) recommended the isolation of *C. jejuni* from raw meat and raw milk in Bacteriological Analytical Manual (BAM) online described by Hunt and co-workers in 1998. In the method sample was homogenized in Buffered Peptone Water (0.1%) and enriched in Campylobacter enrichment broth under microaerophillic conditions (5% O2, 10% CO2 and 85% N2). The sample was then streaked onto modified Campy blood free agar (mCCDA). Typical *C. jejuni* colonies showed a thick translucent white growth to spreading, film like transparent growth, round to irregular in shape with smooth edges (2). In our laboratory, isolation of *C. jejuni* using USFDA method was adopted. The schematic representation is depicted in Figure 1.

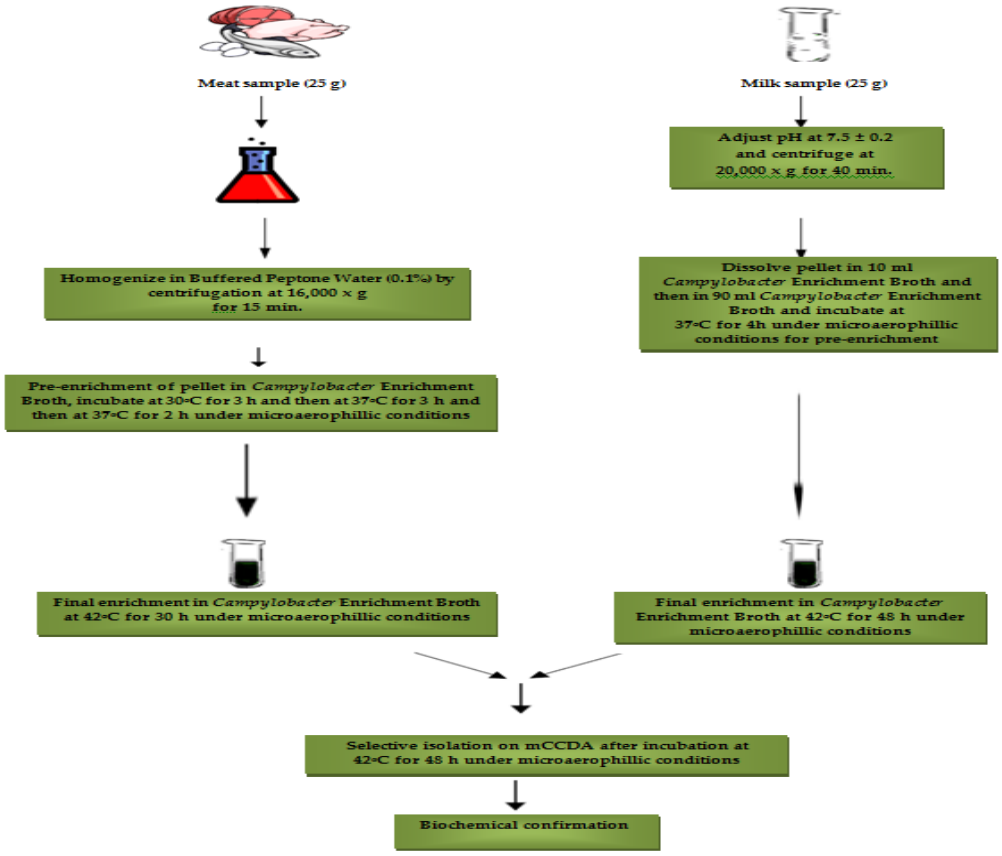

Figure 1 Isolation of *C. jejuni* from raw meat and raw milk

The recovered typical colonies of *C. jejuni* from pure culture are shown in Figure 2. A decade before, in our laboratory in 2006, a modified selective enrichment broth and blood free selective agar medium was prepared and compared it with Preston and Park and Sanders media for the isolation of *C. jejuni* by Barua and Rathore. They reported that isolation rates of *C. jejuni* were higher than that of Park and Sanders and Preston media, which was 57.4%, 50.0% and 39.8% respectively (5,6,7,8). Few years back, Chon and his colleagues compared Bolton enrichment broth supplemented with antibiotic triclosan (T-Bolton broth) and normal Bolton broth for the isolation of *C. jejuni* and *C. coli* from chicken carcass rinse samples. Whole chickens were rinsed with buffered peptone water before enrichment into the normal Bolton broth or T-Bolton broth. The samples were then inoculated onto modified Charcoal-Cefoperazone-Deoxycholate Agar (mCCDA) for the isolation of *Campylobacter* colonies. A significantly higher number of *C. jejuni* or *C. coli*-positive samples in the T-Bolton

broth (71.3%) than in the normal Bolton broth (27.5%) ($p < 0.05$) was observed after statistical analysis. Furthermore, the number of contaminated mCCDA plates was lower after enrichment in T-Bolton broth (3.8%) than in the normal Bolton broth (75%) ($p < 0.05$), indicated that T-Bolton broth has higher selectivity (Chon et al., 2016).

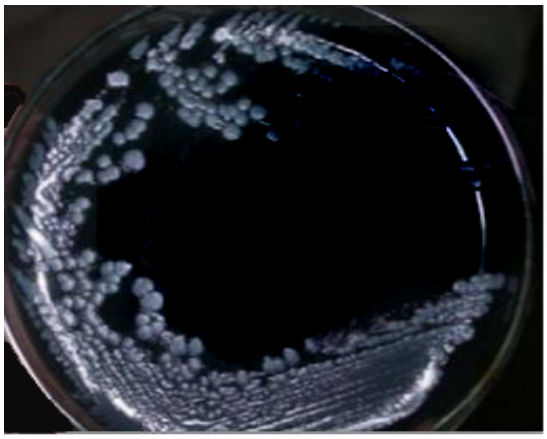

Figure 2 *C. jejuni* on modified Charcoal-Cefoperazone-Deoxycholate Agar (mCCDA)

Immunological methods

The investigation for alternative methods to replace radioactive methods for identifying biological entities led the development of enzyme-linked immunosorbent assay (ELISA) during 1960 to 1970. The development of immunoassays for the identification of bacterial pathogens came into existence with the development of hybridoma technology which made possible synthesis of monoclonal antibodies (MAbs) in mid-1970s. Since then, various immunoassays have been developed for the detection of foodborne pathogens including Campylobacter spp. The simplest is latex agglutination assay involves antibody-coated colored latex beads or colloidal gold particles for rapid confirmation of culture results or serotyping of culture isolates. Among all immunoassay, ELISA, is most popular immunoassay that has been used for pathogen detection (Ovarzabal and Battie, 2012).

ELISAs are typically performed in polystyrene plates containing 96-well (or 384-well), which passively bind antibodies and proteins. These polystyrene plates are termed as 'ELISA' or 'Microtitre plates'. An unknown amount of antigen affix onto a polystyrene surface and specific antibodies apply over the surface. So antibodies can bind to the antigen. Usually these antibodies are linked to an enzyme. In the final step, an enzyme specific substrate is added and subsequent reaction produces a detectable signal in form of colour change. ELISA involves at least one antibody specific to a particular antigen. The sample containing an unknown amount of antigen that can be immobilized onto a solid support 'specifically' captured by another antibody, specific to the same antigen. It is termed as "sandwich ELISA". The antigen can be immobilized onto a solid support 'non-specifically' also by adsorption onto the surface. After the immobilization of antigen, add the detection antibody that result a complex formation with the antigen. The detection antibody can be covalently linked to an enzyme, or can itself be detected by a secondary antibody which is linked to an enzyme through bio-conjugation. The microtitre plate typically washed with a mild detergent solution at each step. Hence removal of non- specifically bounded proteins or antibodies can be done. After the final wash step, add an enzymatic substrate into the plate to produce a visible signal indicates the quantity of antigen in the sample. A detection enzyme or other tag can be linked directly to the primary antibodies or introduced through secondary antibodies. It can also be linked to a protein such as Streptavidin if the primary antibody is biotin labelled. The most commonly used enzyme labels are Horse Radish Peroxidase (HRPO) and Alkaline Phosphatase (AP). Other enzymes have been used as well, but they have not gained widespread acceptance because of limited substrate options. These include β-galactosidase, acetylcholine esterase and catalase. Now days, a number of modifications to the basic procedure of ELISA has been done. The key steps involve the immobilization of the antigen of interest that can be accomplished by direct adsorption to the microtitre plate or indirectly through a capture antibody that has been attached to the plate. The antigen is then detected either directly (labelled primary antibody) or indirectly (labelled secondary antibody). The most useful ELISA assay format is the sandwich assay due to its sensitivity and robustness. A diagrammatic representation of various type ELISAs has shown in Figure 3.

Another method is lateral flow immune chromatographic method which is a modified ELISA, packaged in a simple device (dipstick or within a plastic casing) and used for rapid pathogen detection.

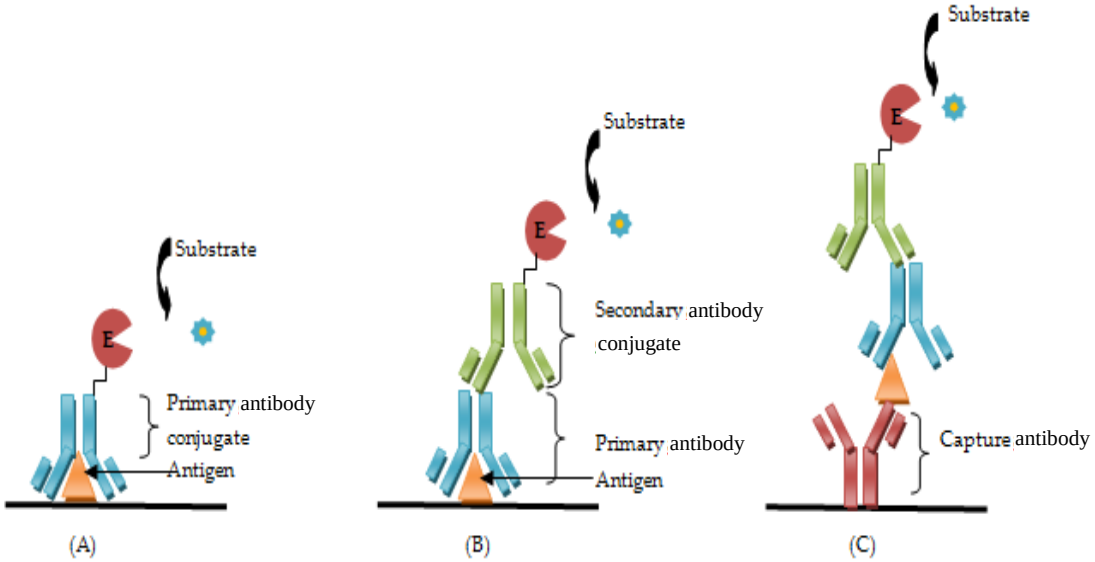

Figure 3 A diagrammatic representation of various type ELISA (**A**) Direct (**B**) Indirect (**C**) Sandwich

Typically, sandwich method used for the detection of large analytes such as bacteria. Samples migrate from the sample pad through a conjugate pad. In this conjugate pad the target analyte binds to the antibody conjugated to coloured particles. The sample is drawn across the membrane to the capture zone where the target/conjugate complex binds to immobilized antibodies. It produces a visible line on the membrane. To ensure a working test, the sample migrates further until it reaches the control zone, where excess conjugate is bound to produce a second visible line on the membrane. Two clear lines on the membrane indicate positive result while a single line in the control zone indicates negative result. Lateral flow immunoassays advantageous in terms of simplicity, result obtaining time (usually within 15 minutes), stability with a long shelf life and the low cost of the assay (Oyarzabal and Battie, 2012).

The immunofluorescent assays including fluorescent antibody technique (FAT), nanoparticles-based immunoassay, multiplexed sandwich chemiluminescent enzyme immunoassay and fluorescent nanoparticles probe immunoassay and PCR enzyme-linked immunosorbent assay (PCR-ELISA) have been described (Hong et al., 2003; Chemburu et al., 2005; Watson and Galan, 2008; Mortensen et al., 2011). Atwa and his co-workers in 2016 reported *C. jejuni* detection in raw and under cooked barbeque chickens. A total of 684 from chicken visceral contents, eviscerated raw, barbeque chickens and handlers were tested. The samples were subjected to standard phenotypic identification of *C. jejuni*, and subsequently Immune Fluorescent Technique (IFT) identification. The genetic identification was performed by PCR amplification using specific primers of hip'O' gene. The overall positive ratio of *C. jejuni* in chicken was 59.2%, where the higher and the lower values were recorded with intestinal contents and barbeque tissues (72.1 and 32.1) respectively. The total positive ratio in contact personals was 51%. Wherever, the higher and lower values were 75.9% and 40.3% corresponding to symptomatic consumers and handlers employees (Atwa et al., 2016).

Conclusion and future perspectives

C. jejuni is one of the most common causes of foodborne bacterial disease in humans worldwide. Typically, specimens such as blood, saliva, urine, or food sample containing possible pathogen can be analysed using cultural, immunological and molecular methods. Conventional cultural based detection and identification methods for *C. jejuni* typically tedious task and require long processing time. The development of cultural media and methods is very difficult for *C. jejuni* because the bacterium is fastidious in nature and does not grow in air in fact require microaerophillic (5% O2 is optimal) and capnophillic (3 to 5% CO2) conditions for growth. The sensitivity to drying, temperature, ambient oxygen concentration, lower temperature and resistant to a wide range of antibiotics are also typical constrains during cultural media development. The differentiation between *Campylobacter* species through conventional methods

is very difficult due to very few differences in biochemical characteristics. It is also well known that under unfavourable growth conditions, the bacteria may transform viable but non-culturable cells (VBNC). Moreover, the cultural methods have limited sensitivity and specificity. They require specialized equipment and trained users also. Therefore, these methods are costly and not available at all places for real time detection of the pathogen. The rapid and accurate methods including immunoassays are required to identify Campylobacters species including *C. jejuni*. Immunological methods such as ELISAs are usually employed to detect particular surface epitopes. These all methods are time consuming and costly due to the equipment required and specialist technical staff (Silva et al., 201; Donnison, 2003). In USA, investigations of outbreaks and more recently for screening processed poultry new standards are established. However, Yet, the development of antibody-based methods for Campylobacter spp. has not received enough attention. At present, none of the commercially-available EIAs are recommended for separate identification of Campylobacters more especially in stool sample due to limited and conflicting findings regarding their performances. Some of the commercial immunoassays show promising results for identification of Campylobacters in food however it includes broth enrichment for up to 48 hours strictly (Oyarzabal and Battie, 2012). More studies of these assays with naturally-contaminated samples from a variety of foods are required to validate performances of these assays. Areas of research to improve antibody based methods should include antibody/antigen characterization, biosensors platforms, and the dovetailing of concentration methods to improve sensitivity.

Conflict of Interest

The authors declare that they have no conflict of interest.

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