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Cultural and molecular techniques for the detection of *Yersinia enterocolitica* in food: An update

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Abstract
Yersinia enterocolitica, a Gram-negative, rod-shaped zoonotic bacterium, is an important cause of foodborne infections. It is the principal etiological agent of yersiniosis, a disease that can affect people of all ages and both sexes. The bacterium is transmitted through the fecal-oral route by ingesting contaminated food or water or contacting infected animals or people. It can be found most commonly in cooler climates. Yersiniosis can cause symptoms, such as fever, abdominal pain, diarrhea, and vomiting in humans. Some cases of yersiniosis can lead to serious pathologies, such as self-limiting enteritis, acute mesenteric lymphadenitis, and septicemia. The foodborne yersiniosis mostly occurs in sporadic form but outbreaks are also reported from many countries including Australia, Brazil, China, Finland, Japan, Norway, and the United States. Livestock are not known to develop clinical signs on their own, except for decreased thriftiness and sudden mortality. Identification of the agent could be made by recovery facilitated by the use of Cefsulodin-Irgasan-Novobiocin (CIN) selective agar or *Y. enterocolitica* chromogenic agar (YECA); if possible, isolates should be serotyped and/or bio-typed via biochemical assays, multiplex polymerase chain reaction (PCR), pulsed-field gel electrophoresis (PFGE) and antigen capture enzyme-linked immunosorbent assay (ELISA). Many selective enrichment and plating media for isolating *Yersinia enterocolitica* from foods have been described. Cold enrichment in phosphate-buffered saline plus 1% sorbitol and 0.15% bile salts (PBSSB) and two-step enrichment with try tone soy broth (TSB) and bile oxalate sorbose (BOS) broth are very efficient methods for the recovery of a wide spectrum of *Y. enterocolitica* serotypes. Cefsulodin irgasan novobiocin (CIN) and Salmonella-Shigella deoxycholate calcium chloride (SSDC) agar is the most frequently used plating media. DNA-based methods like PCR assays are becoming more popular and sensitive than conventional culture methods.

Keywords: Animals, isolation, food, humans, PCR, *Yersinia enterocolitica*

Introduction
Many zoonotic pathogens can be transmitted through food and water. *Yersinia enterocolitica* is one of these agents, an emerging foodborne pathogen of global public health significance (Pal, 2007) [34]. The infection caused by *Yersinia enterocolitica* is reported many countries of the world including India (Pal, 2007) [34]. *Yersinia enterocolitica* is a member of the genus *Yersinia* of the family *Enterobacteriaceae*. It is a rod-shaped, Gram-negative, non-spore-forming, non-lactose-fermenting, oxidase-negative facultative anaerobe that is motile at 25 °C and non-motile at 37°C (Khan *et al*., 2018; Pal *et al*., 2013) [24, 35]. The organism can survive and multiply at refrigerator temperatures (Annamalai and Venkitanarayanan, 2005; Pal *et al*., 2013) [3, 35]. The bacterium was first reported by Mclver and Picke in 1934 (Bottone, 1977) [8]. *Yersinia enterocolitica* is subdivided into six biotypes based on biochemical tests, and there are more than 70 serotypes (Le Guern *et al*., 2016; Wauters, 1988) [27, 44]. The serotypes most clearly pathogenic to humans include O:3, O:5, 27, O:8, O:9, and O:13 (Khan *et al*., 2018) [24].
Although *Y. enterocolitica* is found in many parts of the world, its prevalence is more common in temperate countries (Khan *et al*., 2018) [24]. In Europe, *Y. enterocolitica* infections are the fourth most frequently reported bacterial foodborne zoonosis (EFSA and ECDC, 2019) [14]. It is most often transmitted by consumption of contaminated food, inadequately or unpasteurized milk, untreated water, or by direct or indirect contact with animals (Huovinen *et al*., 2010; Sabina *et al*., 2011) [22, 38]. Person-to-person transmission is rare. However, there are reports of food contamination by infected food-handler and nosocomial infections (Moriki *et al*., 2010) [31].

Outbreaks of *Y. enterocolitica* infections have been linked to eating raw vegetables (Khan *et al*., 2018) [24], which might get contaminated by the soil, water, fertilizers, and other objects.

Yersinia enterocolitica is associated with a wide range of diseases, including intestinal diseases, such as enterocolitis (inflammatory diarrhea), acute terminal ileitis (a severe illness of the large intestine), and mesenteric lymphadenitis (swelling of the mesenteric lymph nodes), etc. It can also cause rare extraintestinal manifestations, such as urinary tract and respiratory tract infections (empyema), osteoarticular infection (reactive arthritis), erythema nodosum, infected mycotic aneurysm, axillary abscesses, and endocarditis (Bottone, 1977; Kwaga *et al*., 1992; Pal *et al*.,2013; Mancini *et al*., 2022; Miller *et al*., 1989) [8, 25, 35, 29, 30,].

Yersinia enterocolitica can be challenging to isolate even on selective media, and recommended enrichment steps are time-consuming. No single procedure is currently available which will recover all pathogenic serotypes. The low isolation rates of pathogenic *Y. enterocolitica* in natural samples may be due to the limited sensitivity of culture methods. DNA-based methods, including PCR and DNA colony hybridization, can identify the pathogen *Y. enterocolitica* more quickly and with greater sensitivity (Hoorfar and Holmvig, 1999) [22]. The objective of this paper is to review the possible detection methods of *Y. enterocolitica* in different types of foods.

Cultural Methods of Isolation

The method of isolation of *Y. enterocolitica* is deeply affected by the type and source of the sample. In clinical samples obtained from patients with acute gastroenteritis or organ abscesses, pathogenic strains of *Yersinia* are often the dominant bacteria, thus making their isolation straightforward. However, they are rare in asymptomatic carriers, foods, or environmental samples of complex metrics. Due to the small number of pathogenic strains of *Yersinia* in asymptomatic carriers and a large number of background flora in food and environmental samples, direct isolation, even on selective media, is seldom successful. To increase the number of pathogenic strains of *Yersinia* in samples, enrichment in liquid media must be done before isolating them on solid media (De Boer, 1992) [9].

Cold Enrichment

Although the presence of many background bacteria is the main problem for detecting *Y. enterocolitica* in complex metrics samples, an enrichment step can increase the probability of detecting this bacterium (Sirghani *et al*., 2018) [41]. *Yersinia enterocolitica* can grow at 4°C, and enrichment at this temperature for 2-4 weeks is widely used as a specific enrichment step for this bacterium. This temperature will reduce the growth rate of other competitive bacteria on the plating media. Only supporting the growth of *Y. enterocolitica*, which can be isolated easily (De Boer, 2003) [10]. Cold enrichment media is used to detect *Y. enterocolitica* in food and water samples. Several phosphate-buffered saline (PBS) based media are used, such as PBS with 1% mannitol and 0.15% bile salts (PMB), PBS with 0.5% peptone and 1% sorbitol, 0.15% bile salts (PSB), PBS with 0.25% peptone and 0.25% mannitol (PMP), or buffered peptone water (BPW). Sometimes, treating cold enrichment media with potassium hydroxide (KOH) reduces the background flora, thus making the selection of *Y. enterocolitica* colonies less laborious (Schiemann, 1982) [40].

Selective Enrichment

Selective enrichment uses media that contain antimicrobial agents. Several selective enrichment media for isolating *Y. enterocolitica* at higher temperatures have been developed (Fredriksson-Ahomaa and Korkeala, 2003) [15]. The most common is modified Rappaport broth (MRB) which was developed by Wauters and co-investigators (1997) [45]. It contains magnesium chloride, malachite green, and carbenicillin, and the inoculated sample is incubated at 25°C for 2 to 4 days. Next, developed an enrichment broth derived from the modified Rappaport base, with irgasan, ticarcillin, and potassium chlorate (ITC) supplements. Both media have been helpful in the recovery of bio-serotype 4/O:3 strains. Earlier, Schiemann (1979) [39] developed a bile-oxalate-sorbose (BOS) medium for the isolation of *Y. enterocolitica*, particularly for the bio-serotype 1B/O:8 strains. Two lesser-known enrichment procedures based on tryptic soy broth (TSB) were proposed later. In another study, Landgraf *et al*. (1997) [26] used TSB with polymyxin and novobiocin and incubated at 18°C for three days to isolate *Y. enterocolitica* in milk.

The recommended agar media to isolate presumptive strains of *Y. enterocolitica* are the ITC broth and Cefsulodin-Irgasan-Novobiocin (CIN) media. CIN is the most frequently used (Schiemann, 1979) [39]. It is more specific than other conventional selective agars, such as Salmonella-Shigella (SS), MacConkey (MAC), Cellobiose-Arginine-Lysine (CAL), pectin agars, and other lactose-containing media, for the differentiation of *Yersinia* spp. from contaminating bacteria (Aleksic and Bockemühl, 1999; Head *et al*., 1982) [2, 20]. To identify *Yersinia*, at least two tests are needed: the Kligler iron and Christensen urea tests. These tests help to differentiate the bacteria of similar colony morphology on CIN agar (Devenish and Schiemann, 1981) [12]. Although several selective media are identified for better isolation of Yersinia, cold enrichment yields higher recovery rates of pathogenic *Y. enterocolitica* than selective enrichment (Fukushima *et al*., 2011) [18].

Biochemical Tests

The recommended approach to isolate *Y. enterocolitica* is by using ISO 10273–2003 method [23]. Here, a pure culture from a suspected colony is tested for oxidase, urease production, hydrogen sulfide production, sugar (glucose and lactose) fermentation, and gas formation. Presumptive colonies positive for urease and glucose fermentation and negative for oxidase, lactose fermentation, hydrogen sulfide production, and gas formation from glucose are then selected and further tested for another series of tests. These are lysine decarboxylase and ornithine decarboxylase, sucrose, rhamnose, xylose and trehalose fermentation, and citrate test.

There is a commercially available kit Enterotest24 which identifies *Y. enterocolitica* based on biochemical reactions (Indole, hydrogen sulfide, lysine, ornithine, urease, arginine, Simmons' citrate, malonate, phenylalanine, β-galactosidase, inositol, adonitol, cellobiose, sucrose, trehalose, mannitol, acetoin, esculin, sorbitol, rhamnose, melibiose, raffinose, dulcitol, glucose). A miniaturized identification kit like the API 20E (bioM6rieux) or the Crystal E/NF system (Becton Dickinson) can be valuable for the rapid identification of *Y.*

enterocolitica strains (Peele *et al*., 1997; Neubauer *et al*., 1998) [36,32]. An advanced alternative to time-consuming and biochemical-based techniques is MALDI TOF MS (Matrix-assisted Laser Desorption Ionization-Time of flight Mass Spectrometry), a suitable, rapid, and powerful tool for the identification of the *Y. enterocolitica (*Bardon, 2015; Deng *et al*., 2014) [5, 11].

PCR-Based Molecular Methods

DNA-based molecular methods for identifying *Y. enterocolitica* strains, such as PCR and DNA sequencing, have been introduced (Bhaduri *et al*., 1997) [6]. The procedure uses the 16S rDNA-specific PCR designed to accurately and rapidly confirm *Yersinia* at the species level. The principle is based on detecting polymorphisms (differences in sequence) in the hypervariable regions of the 16S rRNA gene which exist in all bacteria. Other PCR methods detect particular species-specific virulence genes associated with *Y. enterocolitica* (Naubber *et al*., 2000; Syczyło *et al*., 2016) [33, 42].

Most Common Target Genes

Several PCR assays have been developed to detect virulence plasmid, i.e., pYV-positive *Y. enterocolitica* in clinical, food, and environmental samples. Many of these methods use primers targeting the *virF* or *yadA* gene located on the virulence plasmid (pYV). Viitanen *et al*. (1996) [43] applied primers specific for the virulence plasmid coding the *yopN* (*lcrE*) gene of *Y. enterocolitica* O:3. The *yopN* is involved in the control of *Yop* (Yersinia outer proteins) release in pathogenic *Y. enterocolitica* to resists the primary immune response of the host. Arnold, *et al*. (2001) [4] developed a test that uses primers to target the *yopT* gene, which codes for an effector protein that induces a cytotoxic effect in macrophages. Because of possible plasmid loss on subculture and storage, PCR methods targeting chromosomal virulence genes have also been generated for natural samples. The attachment invasion locus (*ail*) gene responsible for adhesion, located in the chromosome of pathogenic *Y. enterocolitica* strains, is the most frequently used target. In addition, some PCR assays have been designed to detect the *inv* and *yst* genes related to invasive and enterotoxigenic properties of bacterium (Neubauer *et al*., 2000) [33].

Multiplex PCR methods can simultaneously detect more than one gene from Y. enterocolitica. Different primer sets designed for different genes are used together to identify and differentiate the bacteria. The most common combinations in these multiplex PCR assays have been *virF* and *ail* genes. This helps in differentiating positive samples from negative ones. By detecting multiple genes, it is possible to determine which isolate of the bacteria is responsible for a particular infection (Blaise and Philippe, 1995) [7]. Two multiplex PCR methods using a combination of primers against *inv*, *ail*, and *virF* have been designed to detect *Y. enterocolitica* and *Y. pseudotuberculosis* in food and water. One method, developed by Harnett *et al*. in 1996 [19], uses primers to simultaneously detect the *yst*, *ail*, and *virF* genes of *Y. enterocolitica*. Weynants *et al*.1996 [46] developed a different multiplex PCR assay that uses the *rfbC*, *inv*, *ail*, and *virF* genes to differentiate between pathogenic *Y. pseudotuberculosis* and *Y. enterocolitica*.

Sample Preparation

PCR is a technique that can be very effective at detecting the presence of specific types of bacteria or viruses. However, because it works by copying the selected part of the DNA of the organisms, it is less effective when applied to samples containing many other contaminants. One reason is that proteins can break down the DNA polymerase structure, blocking the PCR process from working. For instance, bile salts in fecal samples can cause a PCR-inhibitory effect. This effect is also seen in blood samples which can be controlled by adding bovine serum albumin. Similarly, the PCR-inhibitory effect of pork samples is caused mainly by heat-stable molecules able to pass through a 0.2-µm filter (Lentz *et al*., 1999) [28]. Therefore, a step is needed for DNA purification before running PCR.

DNA Extraction

DNA can be extracted by either lysing its walls to release the DNA from cells or using more complicated methods. After or during extraction, DNA purification is done, which is a process to remove unwanted substances from the target DNA. Heat can be used to inactivate harmful PCR inhibitors, but it is not always enough to extract DNA from all microbes. Thus, Proteinase K treatment is widely done before heat treatment to detect *Y. enterocolitica* in natural samples by PCR directly. Proteinase K is used to break down cell wall proteins and other PCR inhibitors in a sample, preventing the possibility of heat-stable DNase contamination (Fukushima *et al*., 2003) [16]. Dickinson *et al*. (1995) [13] have shown that by increasing the amount of proteinase K from 0.2 to 1 mg per ml and using isopropanol precipitation of DNA, *Y. enterocolitica* can efficiently be detected directly in raw chicken and cheese samples. Of several methods available, the most traditional method is phenol-chloroform extraction and ethanol precipitation. However, this process is time-consuming, therefore, not suitable for large numbers of samples. Many commercial DNA purification kits are now available, making DNA isolation faster and easier (Fukushima *et al*., 2010) [17].

Detection of PCR Products

During PCR, millions of copies of small pieces of DNA are produced. Agar gel electrophoresis is performed to visualize the amplification of the gene of interest. It displays the type of isolates (positive or negative) and size of products with a rough estimate of the concentration of the bacteria. However, it is not always possible to be sure that the product contains the correct sequence between primers and using a carcinogen like ethidium bromide that is used to stain the agarose gel and may not be safe for routine use in food-monitoring laboratories (Fredriksson-Ahomaa and Korkeala, 2003) [15]. To overcome these problems, Rasmussen *et al*. (1995) [37] used a technique to detect the amplified products of *Y. enterocolitica* by capturing the products using a hybridization technique to an immobilized oligonucleotide. The immobilized PCR products in microtiter wells were detected with fluorescence. Recently, the 5′-nuclease PCR (TaqMan) assay, which no longer requires agar gel-based detection, has been used to detect *Y. enterocolitica* directly in blood and food samples. An online rapid-cycling real-time PCR assay was developed by Aarts and co-workers (2001) [1] for detecting pathogenic *Y. enterocolitica* in pure culture. Here, the double-stranded DNA of the PCR product binds the SYBR Green I dye, so internal probes are not needed anymore in this method.

Conclusion

Yersinia enterocolitica is a Gram-negative bacterium that causes a zoonotic disease called yersiniosis that is reported from many regions of the world. Cultural methods for the identification of *Y. enterocolitica* are complex and time-consuming. *Yersinia*-selective agar plates are not sensitive enough. Non-pathogenic *Y. enterocolitica* colonies have the same appearance as pathogenic ones, which makes it challenging to select appropriate colonies for further confirmation. While selective enrichment media are not selective enough, they also contain agents inhibiting the growth of some pathogenic strains. PCR assays have provided a better estimation of the occurrence of pathogenic *Y. enterocolitica* in clinical, food, and environmental samples than conventional culture methods. Real-time PCR assays are rapid and sensitive methods for identifying and enumerating pathogenic *Y. enterocolitica* in natural samples with complex metrics. It is emphasized that sincere attempts should be made to develop simple, sensitive, specific and cheap method that can be easily used for the detection of foodborne pathogens including *Y. enterocolitica* at the Primary Health Centers which are usually located in rural areas.

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Contribution of authors

During the writing of the manuscript, all of the authors contributed equally. They read the final manuscript and gave it their approval for publishing.

Conflict of interest

There are no conflicts of interest declared by the authors.

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