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Short Review

Challenges in Detection and Sub typing of Yersinia Enterocolitica by Conventional Culture Methods

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ABSTRACT

Yersinia enterocolitica is an important food borne pathogen, but the incidence of infected people is underestimated since clinical samples are not routinely tested for *Yersinia*. Problems emerge also from the similarities with other *Enterobacteriaceae* and Y. *enterocolitica-like* species and the heterogeneity of Y. *enterocolitica* as it comprises both pathogenic and non-pathogenic isolates. This review focuses on the methods for Y. *enterocolitica* detection and sub typing by culture methods as well as the difficulties that are met.

INTRODUCTION

Yersinia enterocolitica is a Gram negative bacterium that belongs to the order Enterobacteriales and the family Yersiniaceae [1]. It is highly heterogonous species which according to biochemical activity and Lipopolysaccharide (LPS) O antigens is divided into six biotypes and in about 70 O-serotypes, respectively. Biotypes includethe non-pathogenic 1A and the pathogenic biotypes 1B, 2, 3, 4 and 5.Additionally, the Presence Of The Virulence Plasmid (pYV) is a strong indication of pathogenicity that is essential for the bacterium to survive and multiple in lymphoid tissues 2,3]. The most prevalent bio serotypes associated with human infections are 1B/O:8, 2/O:5,27, 2/O:9 and 4/O:3. *Yersinia enterocolitica* is responsible for human gastrointestinal yersinios iscaused most often by eating raw or undercooked pork, since pigs are considered the major reservoir for *Y. enterocolitica* (EFSA, 2007) [4].Y. enterocolitica is psychrotrophic having the unusual ability among pathogenic enterobacteria to develop in the contaminated food stored in the refrigerator before being ingested and can also survive for extended periods of time in frozen food [5].

Detection and isolation of a Y. enterocolitica isolate from clinical samples is essential in order to assess the pathogenicity and antimicrobial susceptibility and its study for epidemiological and phylogenetic purposes. However, many laboratories do not routinely test stool of patients for *Yersinia*. Moreover, *Yersinia* isolation is difficult since strains grow slowly and pathogenic strains are in low number among the background flora. Additionally, numerous tests are required in order to evaluate the virulence potential of an isolate (bio-serotyping, pathogenicity tests). Consequently, difficulties in detection and isolation of pathogenic Y. enterocoliticastrainsby culture based methods leading to an underestimated prevalence [2,5].

DETECTION METHODS

A variety of cultural methods has been used by different researchers in the detection of *Yersinia enterocolitica*. In clinical specimens - stool, blood, bile, wound, throat swab,

mesenteric lymph node, cerebrospinal fluid, or peritoneal fluid – where pathogenic strains of Y. enterocolitica are often the dominant bacteria, it is easier to isolate the microorganism by direct plating (without enrichment) on selective media. If yersiniosis is suspected, in order to increase the likelihood of a positive culture, enrichment procedures are required. These procedures are especially recommended for isolating *Yersinia* from food, water, and environmental samples. There are several culture methods used for these samples, relying on standard enrichment and selective plating protocols. For the detection in food samples standardized reference methods include the International Standard Organization method for the detection of presumptive pathogenic Y. *Enterocolitica* [3], the NMKL method No. 117 "*Yersinia enterocolitica* Detection in foods" by the Nordic Committee on Food Analysis (NCFA) [4] and the Bacteriological Analytical Manual (U.S. FDA) method for the isolation of Y. enterocolitica [6]. Due to the heterogeneous nature of Y. enterocolitica, no single isolation procedure appears to be optimal for the recovery of all pathogenic serotypes or biotypes [2].

Cold Enrichment

As a psychrotrophic bacterium *Yersinia* can outgrow mesophilic *Enterobacteriaceae* when cultivated at low temperatures. The incubation time depends on temperature: at 4°C, incubation for 8–22days [3], or at 10°C, incubation for 10 days [6]. Cold enrichment media are non-selective broths such as peptone, with 1% sorbitol and 0.15% bile salts (PSB) broth or Cold Enrichment Broth (CEB) [3,6]. Drawbacks of the enrichment are the prolonged incubation period that can also increase the recovery of non-pathogenic Y. *enterocolitica* 1A and Y. *enterocolitica* – like strains and other psychrotrophic bacteria [5].

Selective Enrichment

Selective enrichment methods utilize broths with supplements that are in favor of Y. *enterocolitica* and include Modified Rappaport Medium (MRB) containing magnesium chloride, malachite green, and carbenicillin, Irgasan-Ticarcillin-Potassium Chlorate Medium (ITC), Bile Oxalate Sorbose Broth (BOS) and modified selenite medium. These media are highly selective for some strains of Y. *enterocolitica* but are also quite inhibitory for others. BOS is most suited for recovery of serotype O:8, while MRB and ITC are more suited for isolation of serotype O:3 strains. The most frequently used media are ITC broth and MRB [2]. Advantages of these methods are higher selectivity and shorter incubation time.MRB and ITC broths are inoculated either directly from sample homogenates or from cold-enriched cultures at ratios of 1 volume inoculum to 100 volumes broth. Incubation of the selective broths occurs typically at 25 °C for 2days [3]. Both media have been useful in recovery of strains of bio serotype 4/O:3, which is the most common clinical serotype of Y. *enterocolitica* in Europe [7].

Post enrichment alkali treatment

Alkali tolerance is an additional property that facilitates the isolation of Y. *enterocolitica* due to population reduction of competing microorganisms. In potassium hydroxide treatment, 0.5 ml of enrichment medium or diluted sample (direct, cold, selective isolation) is added to weak (0.25–0.5%) KOH solution with 0.5% NaCl, usually for 20–30s and then plating the inoculums on selective media. Alkali treatment has been found to increase the yield of Yersinia spp. fourfold [8].

Selective media

Many different selective agar plating media have been used for isolation of Y. *enterocolitica*. MacConkey (MAC) agar has been used widely to isolate Y. *enterocolitica* that produces color less colonies since the organism does not ferment lactose, but selection of isolates is difficult among the background flora. *Salmonella-Shigella*-Deoxycholate Calcium Chloride Agar (SSDC), a modification of *Salmonella-Shigella* (SS) agar in which Y. enterocolitica forms grey coloured colonies differentiation of Yersinia from microbial flora such as Morganella, Proteus, Serratia and Aeromonas is often difficult [9].

Cefsulodin–Irgasan–Novobiocin (CIN) agar has been the most widely accepted selective medium and contains cefsulodin, irgasan, and novobiocinas selective antimicrobials that inhibit the growth of other Enterobacteriaceae. On CIN, Y. *enterocolitica* forms small and smooth characteristic colonies with a deep red centre (due to mannitol fermentation) surrounded by a translucent zone, which are called `bull's-eye' colonies. This characteristic colony morphology provides better recovery rates than MAC and SSDC agar plates. The optimal temperature of 25-30°C for Y. *enterocolitica* is recommended for the incubation [5].

However, CIN has limited specificity since *Serratia* and some *Enterobacteriaceae* species such as *Citrobacter*, *Enterobacter, Aeromonas, Morganella, Pantoea, Providencia,Stenotrohomonas, Klebsiella* and *Proteus*, as well as other *Yersinia* spp. form colonies on CIN agar quite similar in appearance to Y. *enterocolitica*, limiting the possibility of picking presumptive colonies for identification. Moreover, as MAC agar CIN lacks the ability to differentiate among virulent Y. *enterocolitica*, non-pathogenic strains and other *Yersinias*. Despite the reported limitations of CIN it can be currently considered the optimal medium for detection of Y. enterocolitica particularly when it is used after cold enrichment and careful selection of presumptive colonies with a stereomicroscope [5].

New chromogenic media have been designed to facilitate the detection and isolation of virulent Y. *enterocolitica*. The Y. *enterocolitica* Chromogenic Medium (YeCM) that was developed by Weagant (2008) [10] for isolation of potentially virulent Y. enterocolitica contains cellobiose as a fermentable sugar, a chromogenic substrate, and cefsulodin and vancomycin to suppress microflora. Y. enterocolitica biotype 1A and Yersinia enterocolitica-like species (non-pathogenic) form blue or purple colonies on YeCM, while only virulent Y. *enterocolitica* biotypes 1B and 2-5 form similar to CIN agar red bulls-eye colonies.

Y. *enterocolitica* agar - Selective Chromogenic Medium for pathogenic Y. *enterocolitica* Screening (YECA developed by AES Chemunex, Combourg, France) was tested to detect pathogenic Y. *enterocolitica* from pig tonsils. YECA favored the growth of the pathogenic Y. *enterocolitica* (Biotype 2, 3, and 4) producing typical small, red fuchsia colonies, while non-pathogenic strains produced small, violet colonies. Growth of biotype 1A was significantly reduced as well as strong inhibition was observed on the growth of the Yersinia-like and non-Yersinia strains [9].

A chromogenic medium CHROMagar*Yersinia* (CAY) (CHROMagar, Paris, France) was developed for the detection of Y. enterocolitica in stool samples. CAY was found to be as sensitive as the CIN, but was significantly more specific. Pathogenic Y. *enterocolitica* produced colorless colonies after 24-h incubation and mauve colonies with a diameter of 1-2 mm after 48-h incubation whereas non-pathogenic Y. *enterocolitica* produced metallic blue colonies, 1-3 mm in diameter after 24 and 48-h incubation. The growth of Y. *pseudotuberculosis, Y. kristensenii, Y. aldovae, Y. intermedia, and Y. mollaretii* was inhibited on CAY. Y. *frederiksenii* produced metallic blue colonies resembling non-pathogenic Y. enterocolitica and only Y. *bercovieri* had a growth similar to Y. enterocolitica [11].

BIOCHEMICAL IDENTIFICATION

Presumptive colonies are roughly identified as Yersinia by biochemical tests, such as the widely used Kligler iron agar, oxidase and Christensen urea tests. *Yersinia* is oxidase negative, urease positive and ferments glucose, but most strains do not ferment lactose. Biochemical differentiation within the genus *Yersinia* is typically based on the following tests: indole production, Voges-Proskauer, citrate utilization, L-ornithine, sucrose,cellobiose, L-rhamnose, melibiose and L-sorbose. Y. *enterocolitica* can be identified by fermentation of sucrose, L-rhamnose and melibiose, as metabolizes sucrose but not rhamnose and melibiose. Biochemical analysis should be performed at 25-30°C optimal temperature for Y. *enterocolitica* growth [5].

Commercial rapid identification tests are suitable alternatives to tube tests. The API 20E system has been accurate in identifying Y. *enterocolitica* [12]. Compared to the available identification systems API 20E, API Rapid32 IDE and Micronaut E, API 20E was found to be the most sensitive and cost-efficient method for Y. *enterocolitica* identification at the species level [12]. Incubation of API 20E at 28°C instead of the 37°C temperature as suggested by the manufacturer, has been shown to yield better identification rates [13]. Identification by the Viteksystem (BioMerieux, France) is not as effective since sometimes cannot differentiate between *Y. enterocolitica* and Y. *frederiksenii* and additional tests are needed [14].

Fourier Transform Infrared Spectroscopy (FT-IR) and Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF-MS) are new techniques applied for the identification of *Y. enterocolitica*. FT-IR provides information about the total biochemical composition of the bacterial strain and has successfully identified the bio-serotypes 1A, 2/O:9, 2/O:5 and 4/O:3. It has been proven better than the API 20E system and could differentiate strains according to biotypes and serotypes with >98 % and >92 % accuracy, respectively [15]. MALDI-TOF based on identification of a specific protein profile of each bacterial strain has been used as an accurate

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and rapid method for identifying *Y. enterocolitica* strains and sub typing to the biotype level [16,17]. Still, problems with separating BT 1B from BT 1A by MALDI-TOF have been reported [18]. High speed, easy applicability, and high-throughput analysis makes these techniques superior to other identification methods available for the detection of *Yersinia* spp.

PATHOGENICITY CHARACTERIZATION

Determination of pathogenicity of an isolate includes the detection of the pYV and bio-serotyping. PCR tests that target the plasmid-located genes *yadA(Yersiniaadhesion)* and *virFare* used to detect pYV positive Y. *enterocolitica* [5]. There are also described conventional tests that detect the phenotypic characteristics associated with the presence of pYV. These tests include calcium dependence, measured by growth restriction on magnesium oxalate agar, Congo red binding and auto agglutination at 35-37°C in certain media [5]. Congo-red magnesium oxalate (CR-MOX) agar combines Congo red uptake and calcium dependent growth at 37°C. Strains harboring the pYV grow as pinpoint (calcium dependent growth at 37°C) sharp orange-red (Congo-red binding) colonies on CR-MOX agar, while strains that have lost their plasmid or non-pathogenic virulence plasmid free strains, produce large white or colorless colonies [3]. Nevertheless, these tests targeting the pYV may still give false negative results because of plasmid loss during laboratory manipulation. Therefore, the CR-MOX test shall be carried out at an early stage of identification [3,5].

Bio typing and O-serotyping are both used to type isolates associated with human disease and a number of strong associations between specific biotypes and serotypes have been identified [5]. Complete bio typing scheme includes tests for esculin hydrolysis, production of pyrazinamidase, acid production from salicin, xylose and trehalose, lipase (Tween-esterase) andindole production. Pathogenic isolates can mainly be differentiated from non-pathogenic isolates with the pyrazinamidase test [3,19]. Bio typing is recommended because biotypes 1B, 2–5 are associated with human and animal disease. Strains belonging to biotype 1A are usually regarded as non-pathogenic, however, some strains belonging to this group may act as opportunistic pathogens.

The most common serotypes that involve in human infection are O:3, O:5, O:8 and O:9 and commercial antisera targeting these antigens have been used extensively. However, these antigens can sometimes be found in non-pathogenic Y. enterocolitica strains and in other *Yersiniaspp* [19]. In addition to that, cross-reactions occasionally occur between Yersinia and members of the family of *Enterobacteriaceae*, e.g., Morganellamorganii and Salmonella spp., or between serogroup O: 9Y. *Enterocolitica* and *Brucella abortus* and *serogroup* O:3 and *Rickettsia* spp. Thus, the pathogenic potential of an isolate should be based on both serotype and biotype [5].

CONCLUDING REMARKS

The heterogeneity of Y. *enterocolitica* which includes pathogenic and non-pathogenic strains, along with the phenotypic similarities within the genus itself and with *Enterobacteriaceae*, makes the detection and identification of pathogenic Y. *enterocolitica* a challenging task. Furthermore, pathogenicity characterization of an isolate and bio-serotyping is time-consuming, labour-intensive and involves numerous biochemical tests and reagents. Utilization of new chromogenic media for the direct isolation of pathogenic Y. *enterocolitica* is imperative to overcome most of these challenges [5].

Use of molecular assays offers higher detection rates compared to conventional culture methods. PCR tests target virulence genes (plasmid- and chromosome-located) of pathogenic Y. enterocolitica in clinical, food, water, and environmental samples and are rapid, highly specific and sensitive. Should an isolate be positive for Y. *enterocolitica*, detection of pyrazinamidase confirms pathogenicity since some virulence genes may be present in non-pathogenic 1A strains. Loop-Mediated Isothermal Amplification (LAMP) and DNA microarray technology is novel molecular diagnostic tool for infections with Y. *enterocolitica*. Nevertheless, molecular methods should be combined with conventional techniques, since the isolation is crucial for the antimicrobial susceptibility testing and epidemiological study in case of food borne outbreaks [5]. Genotypic sub typing methods have been applied for Y. *enterocolitica* strains in epidemiological and phylogenetic studies. Recently, Multi Locus Sequence Typing (MLST) that has been previously used for phylogenetic analysis of Y. *enterocolitica*, a new on Core-Genome Multi Locus Sequence

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Typing (cgMLST) scheme identified successfully *Yersinia* at species and bio-serotype levels. This scheme remains to be evaluated whether it could be a useful tool in clinical microbiology for epidemiological investigations of *Yersinia* out breaks [20].

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