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Development of a rapid method for the detection of *Yersinia enterocolitica* serotype O:8 from food

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ABSTRACT

In this study, a new and alternative method based on monoclonal antibodies (MAbs) for the rapid detection of *Yersinia enterocolitica* O:8 was developed. This microorganism is an emerging foodborne pathogen causing gastrointestinal disease in humans. The transmission can occur through contaminated food such as raw or undercooked meat, milk and dairy products, water and fresh vegetables. Nine MAbs (46F7, 54B11, 54C11, 62D10, 64C7, 64C10, 72E8, 72E10, 72G6) were characterized and selected *versus Y. enterocolitica* O:8, and only 2 of them showed also a weak cross-reaction with *Campylobacter jejuni*. The MAb 54B11 was used for the development of *Y. enterocolitica* capture-ELISA in food matrices, i.e. meat and dairy products (n = 132). The method was validated by ISO 16140:2003 and compared with the official method for the detection of presumptive pathogenic *Y. enterocolitica* (ISO 10273:2003). Relative accuracy, sensitivity and specificity corresponded to 100%. The selectivity was evaluated on other food samples (n = 126) showing a lower confidence limit of 90.3% and an upper confidence limit of 100%. The results from this study demonstrated that the developed method was rapid and cheap, specific and sensitive for the screening of the pathogen in food.

1. Introduction

The genus *Yersinia*, belonging to the family *Enterobacteriaceae*, includes three zoonotic pathogens, *Yersinia pestis*, *Yersinia pseudotuberculosis* and some strains of *Yersinia enterocolitica* (Bursová et al., 2017). The last species is divided into six biotypes (1A, non-pathogenic, 1B, and 2–5 causing human and/or animal infections), and more than 70 serotypes based on their O lipopolysaccharide determinants O:3, O:8, O:9 and O:5,27 (Peruzy et al., 2017; Platt-Samoraj et al., 2017; Saraka et al., 2017). According to report of the EFSA (2015) 4/O:3 and 2/O:9 are the primary bio/serotypes revealed in humans in Europe, while biotype 1B and serotype O:8 have been called for many years the American serotypes due to the occurrence area, and both food import and export and transfer of people spread it also to the European countries. However, Bottone (2015) reported that *Y. enterocolitica* O:8 has been isolated also in Japan and Europe, and it was considered the second most common serotype isolated from clinical material in Poland (Zadernowska and Chajęcka-Wierzchowska, 2017).

Yersinia enterocolitica causes a human infection known as yersiniosis that is the third most commonly reported zoonosis in Europe with 7,202 confirmed cases in 2015, after campylobacteriosis and salmonellosis. The highest specific total cases reported in the Member States were in the following decreasing order: Germany (2,752), Czech Republic (678), France (624) and Finland (582) (EFSA and ECDC, 2016; Rohde et al., 2017). Moreover, outbreaks were described in other countries in the world, such as Finland, Japan, India and United States (Gnanasekaran et al., 2017). The symptoms range from mild and self-limiting gastroenteritis to acute enteritis with diarrhea and abdominal pain, mesenteric lymphadenitis and pseudo-appendicular syndromes indistinguishable from an acute appendicitis particularly in children older than 5 years, or septicemia in elderly and immuno-compromised individuals (Petsios et al., 2016; Saraka et al., 2017). Besides post-infection events, *Y. enterocolitica* can cause also primary cutaneous infections, endocarditis, pneumonia and other nosocomial infections, such as meningitis, septicemia, osteomyelitis, pharyngitis and conjunctivitis (Bonardi et al., 2016; Bottone, 2015). Some

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sequelae such as reactive arthritis or erythema nodosum have also been described (Bozcal et al., 2017).

The principal source of yersiniosis in humans is the contaminated food and pigs can be considered a key reservoir, due to the high prevalence of strains with high virulence (Ye et al., 2016). More specifically, pigs can asymptomatically carry the microorganism in lymph nodes, tonsils and/or intestinal tract and during the slaughtering process it can spread to the carcass, near head and sternum (Van Damme et al., 2017). Carcass refrigeration can facilitate its proliferation because *Y. enterocolitica* is a typically psychrophile microorganism (Bancerz-Kisiel et al., 2016). With regards to temperature, it can grow in a wide range from −2 to 42 °C and therefore it can be found in food stored at refrigeration temperature (Peruzy et al., 2017). In addition, the pathogen can hardly survive cooking treatment due to its heat sensitivity (Wang et al., 2016).

Yersinia enterocolitica can be isolated also from livestock (horses, sheep, cattle, rabbits), pets (dogs and cats) and rodents (Zhang et al., 2015). Besides raw pork meat, milk and dairy products, fruits, vegetables and water have been often involved in human outbreaks (Gensberger and Kostić, 2017). Yersiniosis has been reported in Norway and United States after consumption of ready-to-eat salad mix and pasteurised milk, respectively (Longerberger et al., 2014; MacDonald et al., 2016). The presence of *Y. enterocolitica* in the latter product can be due to different conditions such as pasteurization failure, post-pasteurization contamination and/or post-pasteurization addition of raw milk or other ingredients (Bursová et al., 2017).

Both conventional and molecular methods have been described for the detection of *Y. enterocolitica* in food samples. The first are culture-based methods through cold and/or selective enrichment followed by phenotypic identification. They are laborious, time consuming and sometimes they do not provide the possibility to precisely determine the pathogenicity of the isolates. Moreover, they are characterized by a low sensitivity when the pathogen is present at low concentrations and therefore it is difficult to detect amongst high background microbiota (Petsios et al., 2016). On the contrary the molecular methods, such as hybridization or polymerase chain reaction (PCR) including multiplex PCR and real-time PCR, that are capable to detect also a variety of specific genes (i.e. *ail*, *inv*, *yadA*, *yops*, *yst* or *virF*) are highly sensitive, rapid and specific (Bancerz-Kisiel and Szweda, 2015; Petsios et al., 2016). However, most of molecular methods as well as immunological assays cannot distinguish between living and dead bacteria, unlike other tests for live/dead differentiation, such as the viable count method (Rohde et al., 2017).

Some authors reported other methods for the enumeration of *Y. enterocolitica* such as an impedance method also reliable to enumerate the cells growing in biofilms in a more convenient way with regards to time and effort if compared with a standard plate count (Wang et al., 2016). Zhang et al. (2015) described a new isothermal and cross-priming amplification assay combined with immunoblotting analysis for the rapid detection of the pathogen in milk powders, that could be achieved in 90 min after pre-enrichment. This method is highly specific and sensitive and constitutes an alternative to PCR assay.

Other immunological methods for the detection of *Yersinia* spp. using monoclonal and polyclonal antibodies were developed (Hochel and Škvor, 2007; Balakrishna et al., 2012; Rütera et al., 2014). These methods are rapid and require only few hours to detect the pathogen. Generally antibody based tests are based on simple, quick and cheap detection system (Wangman et al., 2017). However, the prevalence of this pathogen in food is often underestimated due to different factors, such as the low concentration, the similarities with other *Y. enterocolitica*-like species as well as the heterogeneity of isolates, but not least some hurdles in the detection methods (Petsios et al., 2016). So, the aim of this study was the development of a rapid and easy method for its detection in food matrices of animal origin, based on the use of monoclonal antibodies (MAbs) specific for the lipopolysaccharide of *Y. enterocolitica* serotype O:8. Moreover, the developed method was compared with the official qualitative microbiological assay of ISO 10273:2003.

2. Materials and methods

2.1. Bacterial strains

A total of 20 strains, distinguished in 7 *Y. enterocolitica* strains and 13 belonging to other microorganisms were obtained from the American Type Culture Collection (ATCC). *Escherichia coli* O14 was provided from the Bundesinstitut für Gesundheitlichen Verbraucherschutz und Veterinärmedizin (BGVV), one strain of *E. coli* O157:H7 was obtained from the Istituto Superiore di Sanità (ISS); 14, 5 and 1 strains of *Brucella* genus were provided from the National Collection of Type Cultures (NCTC), the Central Veterinary Laboratory (CVL, Weybridge) and from the Agence Française de Sécurité Sanitaire des Aliments (AFSSA), respectively. *Ochrobactrum anthropi* was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) and *Vibrio cholerae* El Tor from the Istituto Sieroterapico of Milano. Other 37 bacterial strains and 6 *Y. enterocolitica* field strains belonging to the collection of the Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise "G. Caporale" (IZSAM) were also used (Table 1).

2.2. Preparation of bacterial antigens

The bacterial strains were grown in Brain Heart Infusion (BHI) Broth (Oxoid Ltd., London, UK) at 37 °C for 14–16 h to obtain a final concentration of 2×10^8 cfu/ml and then inactivated at 60 °C for 1 h. Then they were centrifugated at 5500 *g* for 30 min, washed 3 times with 0.01 M phosphate buffered saline (PBS) pH 7.2 and resuspended in PBS. The cell suspensions were stored at −20 °C until use.

These antigens were used for the MAbs characterization and for the determination of cross-reaction by capture-ELISA. *Yersinia enterocolitica* O:8 ATCC 23715, used for mice and rabbit immunization and hybridoma screening, was sonicated on ice 2 times for 2.5 s and between the 2 rounds of sonication, the sample was put on ice for 5 min. The total protein concentration of the sonicated antigen was determined using the Bicinchoninic Acid (BCA) Protein Assay Kit (Thermo Scientific, Rockford, IL, USA).

2.3. Preparation of Y. enterocolitica lipopolysaccharide

Yersinia enterocolitica lipopolysaccharide (LPS) was prepared using the phenolic extraction method with some modifications (Portanti et al., 2006). *Yersinia enterocolitica* O:8 ATCC 23715 was grown in BHI broth and centrifugated at 4000 *g* for 20 min; the pellet was resuspended in sterile deionized water and added with 85% phenol. Then the mixture was heated at 66–70 °C for 20 min and centrifugated at 20000 *g* for 20 min at 4 °C. The phenol extract was diluted 1:10 with deionized water and dialyzed against NaCl physiological saline solution. The LPS was precipitated overnight at −20 °C with three volumes of methanol/sodium acetate; the pellet was then re-suspended in sterile deionized water and stored at −80 °C. The quantity of LPS was determined by 2-keto-3-deoxyoctonate (KDO) assay (Karkharis et al., 1978).