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Detection, seroprevalence and antimicrobial resistance of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* in pig tonsils in Northern Italy

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ABSTRACT

Yersiniosis is the third most common reported zoonoses in Europe, with *Y. enterocolitica* and *Y. pseudotuberculosis* responsible for 98.66% and 0.94% of the confirmed human cases in 2013. From June 2013 to October 2014, 201 pigs at slaughter belonging to 67 batches were tested for *Y. enterocolitica* and *Y. pseudotuberculosis* in tonsils. Diaphragm muscle samples were tested for antibodies against *Yersinia* by a commercially available ELISA test. *Y. enterocolitica* 4/O:3 was detected in 55/201 pig tonsils (27.4%; 95% CI 23.1–37.1). The positive pigs came from 38/67 batches (56.7%) and were reared in 36/61 farms (59.0%). There was no statistical difference between farrow-to-finish and finishing farms. The mean count of Y. enterocolitica was 3.56 ± 0.85 $\log_{10}$ CFU/g with a minimum of 2.0 $\log_{10}$ CFU/g and a maximum of 4.78 $\log_{10}$ CFU/g. Y. pseudotuberculosis was isolated from 4/201 pig tonsils (2.0%; 95% CI 0.0–4.5). Three isolates belonged to serotype O:3 and one to serotype O:1. The positive pigs belonged to 4/67 batches (6.0%) and came from finishing farms only. Y. pseudotuberculosis could be enumerated in one sample only (4.27 $\log_{10}$ CFU/g). The ELISA test demonstrated that 56.1% of the meat juice samples were positive for *Yersinia* antibodies. Serological positivity was found in 67.9% (36/53) of the *Y. enterocolitica*- and 75.0% (3/4) of the *Y. pseudotuberculosis* positive pigs. A significant association was found between serological results and the presence of *Y. enterocolitica* in tonsils (OR = 1.97, $p = 0.044$).

All the *Y. enterocolitica* 4/O:3 isolates were susceptible to amoxicillin-clavulanic acid, gentamicin, ceftazidime, ertapenem and meropenem, 94.5% to cefotaxime, 89.1% to kanamycin and 78.2% to tetracycline. The highest resistance rates were observed for ampicillin (100%), sulphonamides (98.2%) and streptomycin (78.2%). Y. pseudotuberculosis strains were sensitive to all the antimicrobials tested, *i.e.* amoxicillin, amoxicillin/clavulanic acid, azithromycin, cephalothin, cefoxitin, ceftriaxone, ciprofloxacin, nalidixic acid, sulphonamide, tetracycline and ticarcillin.

The study shows that Italian fattening pigs are frequently infected with human pathogenic *Y. enterocolitica* 4/O:3. Although the isolation rate is slightly lower than in other European countries, the serological test demonstrates that the infection is widespread among pig population. In fact, seroprevalence is similar to other EU countries. The detection of *Y. pseudotuberculosis* serotypes O:1 and O:3 in pig tonsils is of concern.

Since tonsils may represent a contamination source for pig meat at slaughter, further studies regarding human infections by both microbial species are strongly recommended.

1. Introduction

Yersinia enterocolitica and *Yersinia pseudotuberculosis* are food-borne pathogens that can cause serious disease in humans (Bottone, 2015; Jalava et al., 2006). They belong to the family *Enterobacteriaceae*. The genus *Yersinia* comprises three pathogenic species which share tropism for lymphatic tissue: *Y. enterocolitica*, *Y. pseudotuberculosis* and *Y. pestis*, whose genomes are 97% identical. Nevertheless, severity of the diseases

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they cause in humans is vastly different (Bergsbaken and Cookson, 2009). *Y. enterocolitica* is responsible for acute gastroenteritis in humans, but more invasive syndromes as terminal ileitis, mesenteric lymphadenitis mimicking appendicitis and septicemia may occur (Bottone, 1999; Bottone, 2015). *Y. pseudotuberculosis* causes acute gastroenteritis and mesenteric lymphadenitis, whose symptoms of fever and acute abdominal pain are clinically indistinguishable from those of an acute appendicitis (Long et al., 2010; Tertti et al., 1984; Tertti et al., 1989). *Y. enterocolitica* and *Y. pseudotuberculosis* postinfection sequelae include reactive arthritis, erythema nodosum and glomerulonephritis (Bottone, 1999; Hannu et al., 2003; Jalava et al., 2006).

Yersiniosis is the third most common reported zoonoses in the European Union (EU) and European Economic Area (EEA) with 6471 confirmed cases in 2013 and a notification rate of 1.92 cases per 100,000 population. *Y. enterocolitica* was isolated from 98.66% and *Y. pseudotuberculosis* from the 0.94% of the confirmed human cases (EFSA and ECDC, 2015).

Y. enterocolitica shows large heterogeneity, as it is characterized by six biotypes (1 A, 1B, 2, 3, 4, 5) (Skurnik et al., 2009) and different serotypes, with the invasive strains predominantly belonging to serotypes O:3, O:9, O:5,27 and O:8 (Kirjavainen et al., 2008). The biotypes are characterized according to their pathogenicity: nonpathogenic biotype 1A, weakly pathogenic biotypes 2–5 and highly pathogenic biotype 1B. Only 1B and 2–5 carry the *Yersinia* virulence plasmid (pYV) (Cornelis et al., 1989) and several chromosomal genes that have been implicated in virulence, such as *ail* (adhesion and invasion locus), *inv* (invasion), and *ystA* (*Yersinia* stable toxin A), which allow the bacteria to penetrate the epithelial layer, elicit an inflammatory response and evade phagocytosis by neutrophils and macrophages (Revell and Miller, 2001). Only the biotype 1B harbors the chromosomal high-pathogenicity island (HPI), as do almost all European isolates of *Y. pseudotuberculosis* serotype O:1 (Carniel, 1999). The biotype 1A lacks the pYV plasmid and is generally regarded as avirulent, even if there is some clinical evidence that some strains can cause food-borne gastroenteritis (Tennant et al., 2003). Further, *Y. enterocolitica* biotype 1A may represent more than one genetic group, with different pathogenic potential (Sihvonen et al., 2012). Most strains causing yersiniosis in Europe belong to the bio-serotypes 4/O:3 and 2/O:9 (EFSA, 2007). *Y. enterocolitica* is recovered from farm animals and domestic pets to wild animals (Wang et al., 2009). Among livestock animals, pigs are regarded to be the main reservoir of the microorganism, which finds a niche in the lymphatic tissue and may be shed by the faecal route (Bonardi et al., 2013; Fredriksson-Ahomaa et al., 2007). Since pig carcass contamination at slaughter occurs, *Yersinia*-carrier pigs and *Yersinia*-positive farms can be identified before slaughter by serological monitoring of pigs, which could be a useful, simple and time-saving tool compared to microbiological examinations (Van Damme et al., 2014).

Y. pseudotuberculosis can be classified into 15 serotypes (O:1–O:15) and 10 subtypes (O:1a–O:1c, O:2a–O2:c, O:4a–O:4b, O:5a–O:5b) (Bogdanovich et al., 2003). In Europe most strains belong to the serotypes O:1–O:3, while serotypes O:4–O:15 are common in Asia (Niskanen et al., 2009). Most strains are considered pathogenic, containing the 70 kb virulence plasmid (pYV) and chromosomal genes encoding virulence factors. Among them, the invasion protein (Inv) encoded by the *inv* gene is the most important factor in the early phases of intestinal infection (Marra and Isberg, 1997), promoting binding to and invasion of the intestinal epithelial cells (Simonet and Falkow, 1992).

Y. pseudotuberculosis has a worldwide distribution and is primarily an animal pathogen which infects humans rarely (Bergman et al., 2010; Schiemann, 1989). It can infect many animal species, including pigs, sheep, goats (Slee and Button, 1990; Toma, 1986), cattle (Toma, 1986; Welsh and Stair, 1993), deer (Toma, 1986), horses (Czernomysy-Furowicz, 1997), buffalos (Hum et al., 1997), birds (Wallner-Pendleton and Cooper, 1983), rabbits and hares (Percy and Barthold, 2007), hamsters, guinea pigs and beavers (Percy and Barthold, 2007; Toma, 1986), bats (Childs-Sanford et al., 2009) and primates (Buhles et al., 1981).

The aims of this study were to estimate the prevalence of *Y. enterocolitica* and *Y. pseudotuberculosis* in tonsils of pigs at slaughter and to determine the association between cultural detection and positive *Yersinia* serology. The ISO 10273:2003 method was compared to direct culture and cold enrichment to evaluate its performance. Virulence of the isolates was studied, in order to identify pathogenicity of the strains circulating among pigs in Italy. The antimicrobial resistance of the isolates was tested in accordance with Directive 2003/99/EC on zoonosis and zoonotic agents (Anonymous, 2003).

2. Materials and methods

From June 2013 to October 2014, 201 pigs were randomly selected at slaughter to be tested for *Y. enterocolitica* and *Y. pseudotuberculosis* in tonsils. Tonsils were aseptically excised after carcass evisceration and placed in sterile bags. From each carcass, a 10 g sample of diaphragm muscle was collected and placed in sterile containers. The samples were collected from 67 batches of pigs (three pigs per batch) at one slaughter plant processing 280 pigs/h during 33 sampling visits. The batch definition used was a group of pigs coming from a single farm in a given day. The pigs were reared in 61 farms located in four regions of Northern Italy (Lombardy, Emilia-Romagna, Piedmont and Veneto regions); 12 farms were farrow-to-finish and 49 were finishing farms. Sampled pigs were minimum nine months old with an average live weight of 160 kg (Landrace, Large White, Duroc and their hybrids) as required by DOP (Protected Designation of Origin) Italian pork products, such as Parma Ham and traditional dry-cured salami. All samples were transported to the laboratory at refrigeration conditions and tested on the day of collection.

2.1. Yersinia enterocolitica detection, enumeration and typing

Tonsils were tested for *Y. enterocolitica* following the ISO 10273:2003 (International Organization for Standardization, 2003) method and by direct plating. Enumeration of the microorganism was performed by the direct plating method.

Tonsils were washed by pouring sterile water before being aseptically cut into small pieces. A10 g aliquot of tonsils was suspended 1:10 in Phosphate Buffered Saline added with 2% sorbitol and 1.5% bile salts (PSB; Biolife Italiana, Milan, Italy) and homogenized for 4 min in a Stomacher blender (Van Damme et al., 2010). From the 1:10 PSB initial suspension, testing was carried out as follows.

i) Enrichment following ISO 10273:2003: *a*) The 1:10 PSB suspension was incubated at 25 ± 1 °C for 2 days. Thereafter, 10 μl were streaked onto CIN agar plates, which were incubated at 30 ± 1 °C for 48 h. In parallel, the enriched PSB cultures were treated with alkali before plating onto CIN agar, mixing 0.5 ml of the broth culture with 4.5 ml of 0.5% potassium hydroxide (KOH) solution for 20 s. After mixing, 10 μl of the alkali treated cultures were plated onto CIN agar plates, incubated at 30 ± 1 °C for 48 h. *b*) 10 ml of the initial 1:10 PSB suspension were transferred to 90 ml of Irgasan-Ticarcillin-Potassium Chlorate broth (ITC broth; Biolife) and incubated at 25 ± 1 °C for 48 h. Thereafter, 10 μl were streaked onto CIN agar plates, which were incubated at 30 ± 1 °C for 48 h. CIN agar was the medium of choice, instead of *Salmonella – Shigella* agar with sodium desoxicholate and calcium chloride (SSDC) recommended by the ISO method, because of its higher sensitivity (Bonardi et al., 2014). Flat, not mould colonies with entire edge having a red centre ("bull's-eye") surrounded by a translucent, transparent or milk-white zone were considered suspect *Y. enterocolitica* colonies. Five to ten characteristic colonies were selected for biochemical confirmation, or all colonies if less than five were present.