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Short communication

Atypical *Yersinia pseudotuberculosis* serotype O:3 isolated from hunted wild boars in Italy

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ABSTRACT

Atypical *Yersinia pseudotuberculosis* serotype O:3 was isolated from rectal contents of two wild boars hunted in Italy within a regional wildlife management program. No outbreak of yersiniosis was reported in this area in the same period and no lesions were found by the veterinarian at post-mortem inspection. Nevertheless, after histological examination, granulomatous lesions were detected in submandibular lymph nodes of one of the two wild boars. Microbiological and bio molecular characterization of the isolates revealed a melibiose-negative, biotype 2, wbyK + O:3 genotype, carrying *inv*, *yop* (*yopH* and *yopB*), *virF*, and R-HPI. Strains showing the same profile, matching to the criteria of genetic group 5, have been recently reported in fatal cases of yersiniosis in cynomolgus macaques and in farmed deer and atypical O:3 serotype has been suggested as a pathogenic subtype of O:3. This is the third report of an atypical O:3 *Y. pseudotuberculosis* strain, the first outside the American continent and the first one not associated to fatal yersiniosis. Wild boars could be a possible reservoir of this emerging pathogen.

1. Introduction

Yersiniosis, caused by *Yersinia enterocolitica* and, less commonly, *Y. pseudotuberculosis*, was the fourth most frequently reported zoonosis in the EU in 2011, with an overall notification rate of 1.63 cases per 100,000 population (EFSA, 2013). In Europe, *Y. pseudotuberculosis* infections typically cause ileocolitis and mesenteric lymphadenitis, often mistaken for appendicitis, while in the Far East a variety of systemic manifestations, such as scarlatiniform rash and erythema nodosum, are also described (Galindo et al., 2011). Moreover, *Y. pseudotuberculosis* is an agent of disease in animals, causing enterocolitis, abortion, mastitis, pneumonia and septicaemia in several domestic and wild species, such as birds, sheep, cattle, pigs, deer and hare (Shwimmer et al., 2007; Zhang et al., 2008; Wuthe et al., 1995). *Y. pseudotuberculosis* has been divided into 15 O-serotypes (O:1-O:15) and ten subtypes (O:1a-c; O:2a-c; O:4a-b and O:5a-b) on the basis of variable lipopolysaccharides O-side chains (Laukkanen-Ninios et al., 2011). The distribution and pathogenicity of *Y. pseudotuberculosis* strains vary according to the serotype: in Europe, O1a and O1b are usually identified in cases of gastroenteritis in humans, whereas in the Far East, serotypes O1b, 2b, 4b and 5b are dominant in human cases. The pathogenicity of *Y. pseudotuberculosis* is determined by several virulence factors; invasion (Inv) and *Y. pseudotuberculosis* derived mitogen (YPM) are chromosomial (Galindo et al., 2011; Yoshino et al., 1995). A plasmid, pYV, encodes Yersinia adhesion (Yad A) and Yersinia outer proteins (Yops), and it is considered essential for virulence (Galindo et al., 2011).

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Moreover, a chromosomal segment, designated as High Pathogenicity Island (HPI), is associated with highly pathogenic *Y. pseudotuberculosis* strains; HPI is only found in strains of serotype O:1, or, in its truncated form (R-HPI), in isolates belonging to serotype O:3 (Galindo et al., 2011; Carniel, 1999). The different geographical distribution described for serotypes has also been reported for virulence factors (Fukushima et al., 2001; Yoshino et al., 1995). YPM-producing strains are isolated from systemic forms in the Far East, while yersiniosis in Europe is usually associated with HPI + YPMa strains (Fukushima et al., 2001).

Recently, an atypical O:3 serotype, carrying an additional gene, *wbyK*, has been reported in fatal cases of yersiniosis in farmed deer and cynomolgus macaques in North America. This atypical O:3 serotype carried several virulence genes, in both cases *inv*, *yopB*, and *yopH* were detected; *ypmC, irp1, ybtP-ybtQ, yadA* and *lcrF* were found in isolates from cynomolgus macaques, which were also reported as melibiose-negative (Zhang et al., 2008; Zao et al., 2013). To the best of our knowledge, this atypical O:3 strain has never been reported in healthy animals or outside North America.

Here we report the isolation of two atypical *Y. pseudotuberculosis* serotype O:3 strains from rectal contents of hunted wild boars in Italy.

2. Materials and methods

The wild boars originated from two different areas of the Umbria region and were hunted in December, 2009, within a regional wildlife management program. Rectal contents, submandibular lymph nodes and tonsils were sampled and sent to the laboratory within 24 h.

For histological examinations, representative portions of submandibular lymph nodes and tonsils were fixed in 10% neutral buffered formalin (pH 7) and then dehydrated and embedded in paraffin. Three to 4-μ-thick consecutive sections were cut, stained with haematoxylin and eosin (HE). The image was digitalized using a video camera connected to a microscope (DMR Fluo HC, Leica, USA).

2.1. Microbiology

The two *Y. pseudotuberculosis* isolates were cultured from rectal contents following a cold enrichment procedure as described by Laukkanen et al. (2009). Briefly, 5 g of rectal content were put in 45 ml phosphate-buffered saline with 0 5% peptone, 1% mannitol and 0 15% bile salts (PMB), incubated at 4 °C for 7–14 days and then subcultured onto a CIN agar plate and SSI Enteric Medium (34121, Statens Serum Institut of Copenhagen). *Y. pseudotuberculosis* suspect colonies were presumptively identified using oxidase tests, urea slants and indole. Oxidase-negative, urea-positive and indole negative isolates were then confirmed by ID 32 E (bioMérieux Italia Spa, Bagno a Ripoli, FI, Italia), incubated at 28 °C as suggested by Neubauer et al. (1998).

The isolates were further characterized by serotyping, by the slide agglutination method using commercial antisera O:1-O:6 (Denka Seiken, Tokyo, Japan). Antimicrobial susceptibility was assessed by the disc diffusion method, following the CLSI M31-A2 (2002) procedure, except for incubation, which was performed at 30 °C for 24 h. The following commercially available antimicrobial discs (Oxoid Ltd., Cambridge, UK) were used: amoxycillin/clavulanic acid (30 μg), ampicillin (10 μg), cefotaxime (30 μg), ceftazidime (30 μg), cephalexin (30 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), erythromycin (15 μg), gentamicin (10 μg), kanamycin (30 μg), nalidixic acid (30 μg), streptomycin (10 μg), sulphamethoxazole/trimethoprim (25 μg) and tetracycline (30 μg). Autoagglutination test to detect the presence of pYV was carried out in trypticase soy broth, incubated at 25 °C and 37 °C, as already described (Fukushima et al., 2011). Finally, the isolates were biotyped as described by Tsubokura and Aleksic (1995), using citrate utilization, and melibiose and rhaffinose fermentation.

2.2. Extraction of bacterial DNA

DNA was extracted from bacterial cells by the boiling method. Briefly, 2–3 colonies grown on Tripticase Soy Agar plates were suspended in 100 μl of DNase/RNase free water, vortexed and boiled for 10 min. The tube was again vortexed and cooled on ice for five minutes before being centrifuged at 12.000 × *g* for 5 min at 4 °C. Five microliters of the resulting supernatant were used as template for PCR.

2.3. PCR detection of virulence genes

Four sets of primers were used for detection of the virulence genes *inv, virF, yopH, yopB* in a multiplex PCR (Thoerner et al., 2003; Zhang et al., 2008). The primers used for analysing YPMa, YPMb, YPMc and HPI genes were the same as those described previously (Fukushima et al., 2001). Separation was performed using the AM320 method (100 ng/μl sample was injected at voltage 5 kV for 10 s, and separation voltage was 6 kV for 320 s). A DNA ladder (100.0 bp–2.5 Kb) was used to estimate PCR products size in base pairs (bp).

2.4. O-antigen genotyping

A multiplex PCR based O-genotyping was performed using HotStartTaq® Master Mix Kit (Qiagen GmbH, D-40724 Hilden) and nine sets of primers targeting different regions of the O-antigen gene cluster of *Y. pseudotuberculosis*. Primer sequence and concentration were the same as those described previously (Bogdanovich et al., 2003).

Bacterial strains, ranging from O:1 to O:15, were kindly provided from Skurnik Laboratory (Haartman Institute, University of Helsinki) and included, as positive control, with each test run.

2.5. Sequencing

wbyK and *ypm* PCR products were purified using the QIAquick PCR purification kit following the recommended protocol (Qiagen). DNA sequencing was carried out using BigDye Terminator Cycle Sequencing kit v1.1 on an ABI PRISM310 Genetic Analyzer (Applied Biosystems – Life Technologies Italia, Monza MB, Italy). The resulting