Isolation of *Yersinia pseudotuberculosis* in bovine mastitis: A potential milk-borne hazard

Andrea Lorusso, Luciana Addante, Loredana Capozzi, Angelica Bianco, Laura Del Sambro, Maria Ester Gallitelli, Antonio Parisi

Istituto Zooprofilattico Sperimentale di Puglia e Basilicata, Foggia, Italy

Abstract

This paper describes the first confirmed case of a subclinical mastitis caused by *Yersinia pseudotuberculosis* in a dairy cow from Italy. Milk samples from an adult cow of the Bruna breed were analyzed accordingly to standard milk cultivation protocols. Bacteriological examinations allowed to isolate atypical Gram-negative rods identified as *Y. pseudotuberculosis* using biochemical tests. The isolate was subjected to Whole Genome Sequencing (WGS) and the species identification was confirmed using rMLST. Moreover, the virulence and antibacterial susceptibility of the isolate have been also determined. The most common virulence genes were screened through WGS, showing the presence of inv, ail, pil and HPI genes. No antibiotic resistance was found. Even though scarcely described as causal agent of subclinical mastitis, the detection of *Y. pseudotubercolusosis* suggests that this pathogen could be spread to humans through raw milk, representing a potential food safety hazard

Case Report

Yersinia pseudotuberculosis is one of the eighteen species belonging to the genus *Yersinia*, family Enterobaceriaceae. Enteropathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* together with *Y. pestis*, the plague bacillus, are the three virulent species for humans and animals (McNally, Thomson, Reuter, and Wren, 2016). *Y. pseudotuberculosis* is a small Gram–negative, facultative anaerobe, rod-shaped bacterium which affects wild and domestic animals as well as birds (Giannitti *et al.*, 2014). This pathogen is responsible for foodborne infections being transmitted by the consumption of raw or undercooked foods [e.g., milk products, undercooked or raw meat (pork), fresh vegetables (carrot, lettuce), vegetable juices and sandwiches] (Giannitti *et al.*, 2014). Alternative transmission pathways include the direct contact

with infected animal or human (Vincent *et al.*, 2008). Raw milk and raw milk products can represent an important source of infection as recently reported in a milk-associated outbreak of *Y. pseudotuberculosis* in Finland (Pärn *et al.*, 2015). Furthermore, *Y. pseudotuberculosis* can grow at low temperatures (*i.e.* 0-4°C) reaching infectious concentrations in food (e.g., raw vegetables, processed pork) stored within refrigerators (Palonen, Lindstrom, and Korkeala, 2010).

In humans, *Y. pseudotuberculosis* causes an intestinal disease called *yersiniosis*. The most common clinical signs of this bacterial illness include mesenteric lymphadenitis, diarrhea (even bloody), abdominal pain, fever with symptoms similar to appendicitis especially in young adults, leading to septicemia and, rarely, arthritis (Fredriksson-Ahomaa, Lindström, and Korkeala, 2009). Lethal systemic infections have been reported in populations at risk (e.g. diabetic patients) (Prober, Tune, and Hoder, 1979). In cattle, *Y. pseudotuberculosis* is associated with a variety of clinical manifestation such as enteritis, pneumonia, abortion and, rarely, mastitis (Shwimmer *et al.*, 2007). Indeed, the presence of *Y. pseudotubercolusis* in bovine mastitic milk has been reported in single cases (Bleul, Buhler, Stephan, Pospischil, and Braun 2002). Recently, Shwimmer *et al.* (2007) described the isolation of this pathogen from nine dairy cows with mastitis. Only in one case, *Y. pseudotuberculosis* has been isolated in a cow with subclinical mastitis (Sampimon, Sol, Koene, van der Schaaf, and Kock, 2005).

This applied study describes, to the best of our knowledge, the first confirmed case of a subclinical mastitis caused by *Y. pseudotuberculosis* in a dairy cow from Italy. Furthermore, the aims of this study were to determine the duration of bacterial shedding in the milk and the capacity of *Y. pseudotuberculosis* to spread in the herd.

In July 2018, milk samples from dairy cows (n=10) of a small family-run farm located in the province of Bari were collected for routine laboratory analyses. Milk bacteriological test and Somatic Cell Count (SCC) were requested as a screening test for the presence of mastitis pathogens within the herd. Herd reported a bulk milk SCC of 136.000 cells/mL. Before sampling, teats were cleaned and dried with disposable paper towels and disinfected individually with ethyl alcohol 70%. After discarding the first milk jets, the milk was collected in sterile vials. Samples from the four quarters were submitted to a standard milk culture according to the National Mastitis Council guidelines (National Mastitis Council, 1999). Ten µL of each sample were inocu-

lated onto 5% sheep blood agar plates and incubates at 37°C; the samples were evaluated after 24 and 48 hours. The samples were also inoculated on Rabbit Plasma Fibrinogen (RPF) agar for the direct detection of coagulase-positive staphylococci and Edwards Medium (Modified) with 7% Defibrinated Sheep Blood for the isolation of streptococci, particularly *Streptococcus agalactiae*. The milk SCC was determined by Flow Cytometric analysis (Fossomatic FC). In one sample, collected from a cow in the eighth lactation and who gave birth four weeks before sampling, after 24 hours incubation, a pure growth of small grey colonies was observed on the blood agar plate and no growth was observed on RPF and Edwards agar. In addition, the SSC count on this sample showed a score >2 x 10^6 cells/mL. The isolated colonies were examined microscopically after doing Gram's stain showing the presence of Gram-negative coccobacilli. Furthermore, the isolate was positive to catalase test and negative to oxidase test. A suspect colony was streaked to Triple Sugar Iron (TSI) agar and incubated at 30°C for 24 hours. Cultures revealed a typical reaction of *Y. pseudotuberculosis* in the TSI medium: glucose positive, sucrose negative, and lactose negative, yellow slant and red agar column (Fukushima, Shimizu, and Inatsu, 2011). For an additional confirmation, colonies

were re-inoculated onto Cefsulodin Irgasan Novobiocin (CIN) agar and examined after 24 hours incubation at 30°C. Typical colonies of *Y. pseudotuberculosis* characterized by a red bull's-eye shape surrounded by a transparent border were well visible through stereomicroscope analysis. Biochemical examination using the API 20E (bioMérieux, Marcy l'Etoile, France) allowed a strain identification at the species level as *Y. pseudotuberculosis* with a percentage of ≥99.9%.

The biotype of the isolate was determined by the examination of the metabolism of raffinose, melibiose and metabolic conversion of citrate. The biochemical reactions were performed by conventional methods (Fredriksson-Ahomaa *et al.*, 2009). The strain was melibiose positive and citrate and raffinose negative belonged to biotype 1.

Antimicrobial susceptibilities of the isolated strain was determined on Mueller Hinton agar (Oxoid Ltd, Cambrige, UK) by the standardized disk diffusion method according to the Clinical Laboratory Standards Institute (CLSI, 2016) slightly modified, incubating plates for 24 hours at 30°C instead of 37°C. *Escherichia coli* ATCC 25922 was included as quality control. In order to evaluate the antimicrobical resistance, antimicrobial test discs were used [*i.e.*, amoxicillin/clavulanic acid (20/10 μg), ampicillin (10 μg), cefotaxime (30 μg), cephalexin (30 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), gentamicin (10 μg), cephalotin (30 μg), kanamycin (30 μg), nalidixic acid (30 μg), streptomycin (10 μg) and trimethoprim/sulfamethoxazole (1.25/23.75 μg), tetracycline (30 μg)]. Results were recorded by measuring the inhibition zones and scored as sensitive, intermediate or resistant. The strain showed antibiotic sensitivity to all antibiotics tested.

Genomic DNA of the bacterial isolate was purified using the QIAamp DNA Mini and Blood kit (Qiagen, Italy) and the WGS was performed using an Illumina MiSeq Sequencing System (Illumina Inc., San Diego, CA, USA) with a paired-end run of 2 by 250 bp after Nextera XT paired-end library preparation. Sequencing reads were assembled using SPAdes genome (version 3.12).

The species identification was confirmed submitting the draft genome to rMLST database (https://pubmlst.org/rmlst/).

The *Y. pseudotuberculosis* isolate was analyzed for the presence of virulence genes, located on the chromosome or on plasmid pYV, by in silico analysis. The chromosomal virulence genes include *inv*

(YPO1793), which promotes the invasion into eukaryotic cells and *ail* (NP_406409.1) which allows the adhesion to host cells. Both *inv* and *ail* genes were detected in the isolate.

The PlasmidFinder-2.0 server (https://cge.cbs.dtu.dk/services/PlasmidFinder/) excluded the presence of virulence plasmid (pYV) in the draft genome of the isolate.

The tool identified the presence of the conjugative cryptic plasmid pYptb32953, that it is not related to any important virulence-associated characteristic of *Y. pseudotuberculosis*.

Additional virulence factors that have been tested include a superantigenic toxin, the *Y. Pseudotuberculosis*-derived Mitogen (YPM), an High-Pathogenicity island (HPI), which encodes the synthesis of the siderophore yersiniabactin, and adhesion pathogenicity island (YAPI) have been searched using Virulence Factor Database (http://www.mgc.ac.cn/VFs/search_VFs.htm).

The isolate carried a complete HPI (BX936398) and the YAPI (AJ627388).

Furthermore none of the three variants *ypm*A (AF335466), *ypm*B (AF418982) and *ypm*C (AF414083) of YPM was detected.

The Multilocus Sequence Types (MLST) of the isolate was determined using the web-based tools Enterobase (https://enterobase.warwick.ac.uk/). The isolate was classified as sequence type 42 (ST42) according to Achtman scheme, as ST 96 according to McNally scheme and one novel ST (3027) was identified according to wgMLST scheme.

Geneious® 10.2.6 (Biomatters Ltd.) was also used to determine the in silico serotype using the know O-antigen cluster sequences and O-unit structure (Kenyon, Cunneen, and Reeves 2017). The isolate belonged to serotype O:1a as confirmed by the perfect match of sequence with Genebank O:1a (AF461768).

The cow diagnosed as positive to mastitis by *Y. pseudotuberculosis* received one intramammary inoculation of a commercial trimethoprim/sulfamethoxazole preparation.

In order to evaluate the capacity of *Y. pseudotuberculosis* to spread among animals and the duration of bacterial shedding in the milk, samples were collected and analysed as previously described from the entire herd 8 months after the diagnosis. All tested animals showed to be negative to *Y. pseudotuberculosis* in the followed-up except for the previously infect cow. The microorganism had been isolated exclusively in the same infected quarter demonstrating that the infection does not spread to other

quarters and cows. Furthermore, the infected animal showed high values of SCC (>2 x 10^6 cells/mL) with no visible changes in the appearance of the milk confirming the presence of a subclinical mastitis.

Discussion

The present study describes the first report of *Y. pseudotuberculosis* as subclinical mastitis agent in Italy. The isolate observed in our study was typed as ST42 via MLST and serotype O:1a using WGS. Strains of ST42 have been associated with outbreaks of human yersiniosis as in New Zealand where 87 out of the 93 outbreak-associated isolates belonged to this strain (Williamson *et al.*, 2016). Moreover, serotypes O:1 and O:3 represent the two serotypes most commonly found in affected humans in Europe (Fredriksson-Ahomaa *et al.,* 2009). Furthermore, the Y. *pseudotuberculosis* O:1a strains have been isolated worldwide from human and non-human sources and are classified as major gastroenteric pathogens.

Among the virulence factors, the pYV has a key role in the pathogenesis of *Y. pseudotuberculosis* infection and it is usually used to determine the pathogenicity of the strains. In this study, the isolate lacks pYV. Indeed, it should be consider that this unstable plasmid can be easily lost during cultivation in the laboratory (Bhaduri and Smith, 2011) and pYV-negative *Y. pseudotuberculosis* strains have been isolated from infected human patients (Fukushima, Sato, and Nagasako, and Takeda 1991). For instance, in Galindo and collaborators (2011) pYV was absent in one – fourth of the known virulent serotypes, which harbored HPI and/or YPMa. This circumstance may cause false negative results in tests based only on presence of this plasmid. While the absence of the pYV, the isolated strain of *Y. pseudotuberculosis* harbored HPI. As in our case, this virulence factor is usually found only in strains of serotype O1 and it is commonly associated to a high-virulence profile (Galindo *et al.*, 2011).

After the first isolation and the treatment with an intramammary solution of trimethoprim/sulfamethoxazole preparation, the bacteria was still detected only in the same infected quarter at 8 months postdiagnosis. While *Y. pseudotuberculosis* shown to be sensitive to the tested antibiotics *in vitro*, the treatment does not hinder the shedding of the pathogen in the milk. Therefore, it can be speculated that *Y. pseudotuberculosis* could be not contagious for the other quarters or animals and that the treatment is not resolutive potentially caus-

ing management problem of the mastitis case. Furthermore, the persistent excretion of *Y. pseudotuberculosis* from the infected quarters as also described in Sampimon et al. (2005) may favor the maintenance in the environment and in the milking system representing a potential source of contamination for raw milk and raw milk products. Indeed, the milk from dairy cows with subclinical mastitis generally shows no significant modifications, and it is not discarded favoring possible infections.

No visual defects in the milk and no clinical signs were observed in the cows during this study although the high count of somatic cells indicated an evident non–specific udder inflammation. The presence of persistent high levels of SCC should be taken into account from veterinarians as useful predictor of intramammary infection caused by uncommon pathogens as *Y. pseudotuberculosis*.

Cleaning water and cattle faeces containing *Y. pseudotuberculosis* could represent alternative transmission sources for the milk contamination (Castro *et al.*, 2019).

Y. pseudotuberculosis is a psychrotrophic pathogen which can multiply in refrigerated milk or in products stored at refrigerator temperature reaching the infectious concentration in several days. Moreover, raw milk represents a good growth substrate for bacteria considering the nutrient composition and the neutral pH. For these reasons, *Y. pseudotuberculosis* should be considered as a microbiological hazard potentially transmissible to humans through raw milk. Indeed, *Y. pseudotuberculosis* is included in the European Food Safety Authority's shortlist of microbiological hazards associated to raw milk, but at the moment is not regarded as main hazard especially given the limited data available (EFSA BIOHAZ Panel, 2015). Importantly, the enteropathogenic *Yersinia* is the third most common cause of bacterial enteritis in European countries among human patients, mainly reported in northeast Europe, north America, Japan and Russia. In Italy, producers who sell raw milk directly to consumers and through vending machines are required to test their milk for pathogens as *Staphylococcus aureus*, *Listeria monocytogenes*, *Salmonella* spp, *Escherichia coli* O157 and *Campylobacter* but not for *Y. pseudotuberculosis* or *Yersinia* spp. in according to SRA 25/01/2007 (Italian Regulation, 2007). Furthermore, the absence of a mandatory notification system of the presence of *Y. pseudotuberculois* and *Yersinia* spp. in human patients showing mild clinical symptoms (e.g., diarrhea), food (e.g. raw milk) and infected animals may result in a dangerous underestimation of the number of cases of yersiniosis.

Conclusions

In conclusion further studies should be conducted to better understand the role of *Y. pseudotuberculosis* as mastitis agent and to ascertain the role of milk as a transmission vehicle of this infectious biological agent.

Moreover, WGS approach used in this study to characterize an isolate detected by conventional culturing techniques has enabled to determine higher resolution information (*i.e.*, species identification, serotyping, presence of known virulence factors and sequence typing) in a single process work. The interrogation of genomic data publicly released into the NCBI can be a valid tool to improve the monitoring of the zoonotic transmission of these pathogens.

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