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Research paper

Brucella suis biovar 2 multi locus sequence type ST16 in wild boars (*Sus scrofa*) from Abruzzi region, Italy. Introduction from Central-Eastern Europe?

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ABSTRACT

Porcine brucellosis occurs in many countries where pigs are farmed, often representing an underrated problem. *B. suis* biovar 2 is the most common isolate in Europe, with high prevalence reported in wild boars in which it is generally isolated in the absence of gross lesions. In the last five years, we tested for *Brucella* spp. 389 lymph nodes of wild boars collected during hunting seasons or during necropsy procedures.

In this paper, we describe the first case of isolation of *B. suis* biovar 2 from a wild boar aborted foetus, and we analyse the genomic relationships with *B.suis* biovar 2 strains isolated in the past five years in Abruzzi Region, Central Italy. The genetic fingerprint revealed that the isolates under study belong to the MLST ST16 and to the MLVA11 Gt 57, similar to the Central-Eastern European strains. Massive restocking (for hunting purpose) of wild boars from Eastern Europe have been done since 1950 in Italy contributing to the increasing of population size and distribution, as well as to the interbreeding between these foreign breeds and the local population. The contamination of pastures with infected material such as aborted wild boars foetuses can increase the risk of transmission of *Brucella* among wild and domestic animals. The contact of *B. suis* with domestic ruminants may also cause serological reactions to brucellosis serological testing, and even unapparent infection, thus hampering the efforts made in the brucellosis eradication campaign.

1. Introduction

Brucellosis in pigs is primarily caused by biovars 1, 2 or 3 of *Brucella suis*. Sporadic infections caused by *B. abortus* or *B. melitensis* have also been observed in pigs in areas where brucellosis is enzootic in ruminants (EFSA, 2009). Porcine brucellosis occurs in many countries where pigs are farmed. In some countries, it may be a serious, but presently unrecognised problem. Diseases caused by biovars 1 and 3 are similar, while the one caused by biovar 2 differs in host range specificity and geographical distribution (EFSA, 2009).

The most common *B. suis* biovar isolated in animals in Europe is biovar 2, with high prevalence reported in wild boars throughout continental Europe (EFSA, 2009). Wild boar is considered as the main wild reservoir for this infection and in some ecological system, and it has been reported as the source of transmission of biovar 2 to outdoor reared pigs (Hars et al., 2004; Melzer et al., 2007). *B.suis* biovar 2 has the ability of infecting the European hare (*Lepus capensis*), which may act as reservoir of the disease and may infect domestic animals (grazing pigs and cows), even in the absence of a wild boar population (Godfroid et al., 2005).

Although biovars 1 and 3 are highly pathogenic for humans, causing severe disease, *B. suis* biovar 2 is very rarely a human pathogen (Godfroid et al., 2002). However, several human cases caused by *B. suis* biovar 2 have been reported in Europe, mainly in immuno–compromised hunters (INVS, 2007).

Brucellosis in pigs is a chronic disease manifesting most often by infertility and abortion in sows and by orchitis in boars. *B. suis* biovar 2 can also cause miliary lesions, that often become purulent, particularly in reproductive tissues.

The most important routes of spreading are genital and digestive systems. Infected pigs excrete *Brucella* in urine, sperm, vaginal discharge, milk, and also trough placenta, lochial secretion, aborted foetuses and the content of subcutaneous abscesses (MacMillan, 1999; Cvetnic, 2002). In wild boar, *B. suis* biovar 2 has been isolated from lymph nodes, testes, uterus, spleen (Cvetnic et al., 2003; Bergagna et al., 2009; Hinić et al., 2009; Muñoz et al., 2010), generally in the

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absence of gross lesions (De Massis et al., 2012; Gennero et al., 2004; Godfroid, 2002). However, to the best of our knowledge, there are no reports of direct isolation of *B. suis* biovar 2 from aborted foetuses and, consequently, of the possible implication of this route of excretion as potential source of environmental contamination and infection for domestic ruminants sharing the same habitat.

B. suis biovar 2 does not persist in cattle (Godfroid et al., 2005), however, they sometimes acquire unapparent infections which cause them to react positively to routine brucellosis testing in the context of bovine eradication campaigns (Alton, 1990). Spillover from wild boar to cattle has been already reported in Europe in the past (Andersen and Pedersen, 1995; Godfroid et al., 1994; Godfroid et al., 2002), but the pathogenicity of *B. suis* biovar 2 in cattle is actually unknown (Godfroid et al., 2002). Even if rarely, sheep may be infected by *B. suis* (Paolicchi et al., 1993). Sheep and goats experimentally vaccinated with *B. suis* biovar 2 vaccine develop antibodies detectable with routine brucellosis testing (Blasco et al., 1993; Verger et al., 1995). Therefore, the contamination of domestic ruminants by direct or indirect contact with infected wild boar materials might be possible and may lead to the production of antibodies detectable with the serological tests used in the framework of the brucellosis eradication campaign in domestic ruminants.

Brucella spp., compared with its host specificity, has relatively minor genetic variation between species. As a matter of fact, most molecular methods have shown the inability to discriminate greatly and/or reliably at the sub-species level. On the contrary, genome-based approaches like MLST and MLVA are able to give meaningful information at phylogenetic and epidemiological level (Whatmore, 2009). MLST has been used to depict phylogenetic roots of the genus demonstrating the different genetic entity of the classical *Brucella* species with their relatedness (Whatmore et al., 2007). MLVA continues to demonstrate its importance as a tool in the field of molecular epidemiology, being able to help in identifying the ways of introduction, reintroduction or reactivation of brucellosis outbreaks (Garofolo et al., 2016).

Aim of this paper is to describe the first case of isolation of *B. suis* biovar 2 from a wild boar aborted foetus, to compare the genetic relatedness with other isolates from the same Region, and to discuss the possible implications in the brucellosis eradication programme of domestic ruminants.

2. Material and methods

2.1. Samples collected

From years 2011 to 2015, samples from 389 wild boars were gathered in Abruzzi and Molise regions (Central Italy) from hunted wild boars and from wild boar carcases delivered to the Brucellosis National Reference Laboratory (NRL) for necropsy procedures.

Submandibular and retropharyngeal lymph nodes were collected for brucellosis investigation and submitted for bacteriological examination. It is noteworthy that a wild boar foetus and its placenta, found in the protected area named “Lecceta Torino di Sangro”, were delivered by a hunter to the laboratory on the 29th December 2014.

2.2. Bacterial isolates

The samples, after homogenization, were spread directly onto modified Thayer-Martin and blood agar plates and enriched on a Thayer-Martin broth. Then they were incubated in aerobiotic and microaerophilic atmosphere conditions (5%–10% [*v*/v] CO_2) at 37 °C ± 1 °C. Weekly subcultures onto solid Thayer-Martin medium were performed for up to 6 weeks. Plates were observed after 3 days, and then daily, to identify the presence of bacterial colonies. Suspected colonies were subcultured and examined microscopically using the Gram stain, biochemical tests (urease, oxidase and catalase), and motility tests. Colonies confirmed as *Brucella* spp. were submitted to species and biovar identification (OIE, 2009).

2.3. Species identification and genotyping

Primary isolates were subcultured in pure culture before the DNA extraction (Maxwell® 16 Blood DNA Purification Kit). Species identity for all isolates was confirmed by AMOS PCR and RFLP (Bricker and Halling, 1994; Bricker and Halling, 1995; Cloeckaert et al., 1995). The six isolates were subjected to multilocus sequence typing (MLST) as described by Whatmore et al. (2007), followed by a multilocus VNTR analysis (MLVA) using a 16 loci panel as previously described by Garofolo et al. (2013a). MLST sequences by Whatmore et al. (2007) (accession number from AM694191 through AM695630) were downloaded from GeneBank database. Each distinct allele at each of the nine loci was retrieved giving a numerical designation according to Whatmore et al. (2007). Our raw sequences were analysed and assigned to the nomenclature accordingly. The MLVA was used to test: (i) the phylogenetic hypothesis using the 11 loci panel (MLVA11) with the 450 public *B. suis* biovar 2 MLVA profiles available in the MLVA repository on the related website (http://mlva.u-psud.fr/); (ii) the epidemiological relationships among the *B. suis* biovar2 from Abruzzi using the16 loci panel (MLVA16) (Garofolo et al., 2013b). Cluster analysis was performed using goeBurst algorithm implemented in Phyloviz; clonal complex was assigned using the most stringent definition, where all members assigned to the same group differ only at one locus with at least one other member of the group.

3. Results

3.1. Samples collected

The foetus was about 4–5 cm in length and in initial colliquative phase. The PCR for parvovirus, Aujeszky's disease virus, and porcine reproductive and respiratory syndrome (PRRS) virus gave negative results.

3.2. Bacterial isolates

Two weeks after the initial incubation (*i.e.* one week of incubation of the first subculture from Thayer-Martin broth), direct observation of cultures prepared from brain revealed the presence of small translucent colonies of a pale honey colour that were circular and convex in shape and were suspected to be *Brucella* spp. colonies. Microscopic examination showed the presence of Gram negative coccobacilli. Urease, oxidase and catalase tests gave positive results, while the motility test was negative. According to the methods described in the material and methods section, all the isolates were identified as *Brucella suis* biovar 2. Out of 389 samples tested in the period 2011–2015, *Brucella suis* biovar 2 was isolated from the retropharyngeal lymph nodes of five wild boars, sampled in L'Aquila province respectively in years 2011, 2014, 2015, and in a wild boar aborted foetus sampled in Chieti Province in 2015. No *Brucella* spp. strains were isolated from Molise Region. The geographical localisation of isolates is shown in Fig. 1.

3.3. Species identification and genotyping

Species identity for all isolates was confirmed by AMOS PCR and RFLP. Among the 6 isolates studied only the ST16 was defined, neither new alleles nor new combination of alleles were found (Table 1). ST16 has one point mutation for the *gap* (glyceraldehydes 3-phosphate dehydrogenase) gene and is a single-locus variant of the most prevalent ST15 that identify the reference strain Thomsen (Whatmore et al., 2007).

The MLVA showed no mutations among the isolates from Abruzzi for the first 11 loci featuring all the strains with the same genotype 57

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