First report of Brucella suis *biovar 2 in a semi free-range pig farm, Italy*

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Brucella suis biovar 2,
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Summary
This communication describes the isolation of *Brucella suis* (*B. suis*) biovar 2 in semi-free-range pigs located in the province of Rome, Italy. Sera of 28 pigs from a herd with reproductive problems were tested for brucellosis. Twenty-five sera (89%) were found positive to Rose Bengal Test (RBT), while 22 (79%) were positive to Complement Fixation Test (CFT). Two positive pigs were slaughtered, organs were collected and tested for the presence of bacteria. *Brucella* spp. was isolated from the spleens and the abdominal lymph nodes of the 2 subjects. The isolates were identified as *B. suis* biovar 2 by biochemical and Polymerase Chain Reaction (PCR) tests. The frequent infringement in the fences of the premises and the birth of striped piglets provided evidence that sows mated with wild boar, the major reservoir of *B. suis* biovar 2. Conversely, the isolation of *B. suis* biovar 2 from spleens and lymphnodes of seropositive slaughtered animals only, as well as the constant negative results from all vaginal swabs and the abortion materials tested, raise doubts on the implication of *B. suis* biovar 2 in the infertility of the holding.

Prima segnalazione di Brucella suis *biovar 2 in allevamento semibrado di suini in Italia*

Parole chiave
Brucella suis biovar 2,
Cinghiale,
Italia,
PCR,
Suino.

Riassunto
Il presente studio riporta il primo isolamento di *Brucella suis* (*B. suis*) biovar 2 in un allevamento semibrado di suini in provincia di Roma, Italia. I sieri di 28 suini di un allevamento con problemi riproduttivi sono stati saggiati per brucellosi. Venticinque sieri (89%) sono risultati positivi al test Rosa Bengala (TRB) e 22 (79%) alla Fissazione del Complemento (FDC). Due soggetti sono stati abbattuti e sottoposti ad esami colturali. *Brucella* spp. è stata isolata dalla milza e dai linfonodi addominali di entrambi i soggetti. Gli isolati sono stati identificati come *B. suis* biovar 2 mediante prove biochimiche e biomolecolari. La nascita di suinetti striati ed il rilievo di infrazioni nei recinti dell'allevamento dimostrano l'avvenuto contatto con il cinghiale, serbatoio più importante della malattia. L'isolamento di *B. suis* biovar 2 dalla milza e dai linfonodi dei due animali sieropositivi abbattuti e la sua costante assenza in tutti i tamponi vaginali o aborti esaminati non chiarisce la sua implicazione come causa di infertilità nell'allevamento.

Porcine brucellosis is mainly supported by *Brucella suis* (*B. suis*), rarely by *B. abortus* or *B. melitensis*. *B. suis* includes 5 biovariants. Pigs are the main reservoir for variants 1, 2, and 3. In Europe, the brown hare (*Lepus europaeus*) and the wild boar (*Sus scrofa*) are the natural reservoirs of *B. suis* biovar 2 (EFSA 2009, OIE 2013)[1]. Biovariants 1 and 3 are important human pathogens (Godfroid *et al.* 2005, OIE 2013), while biovar 2 is rarely zoonotic (Institut de Veille Sanitaire 2007, Paton *et al.* 2001, Teyssou *et al.* 1989).

In Italy *B. suis* biovar 2 was first reported in brown hares in the 1990s (Quaranta *et al.* 1995) and recently, it was isolated in wild boars in North-Western Italy, Abruzzo and Latium (Bergagna *et al.* 2009, De Massis *et al.* 2012, Battisti, personal communication). However, no data about its isolation in domestic pig have been published. In this study, we describe the isolation of *B. suis* biovar 2 in pigs of semi free-range farm in Italy.

In 2009, we received samples (28 sera, 24 vaginal swabs, 2 aborted fetuses, and 9 stillborn piglets) from a breeding farm of out-reared pigs with reproductive problems. The farm, located in the province of Rome, counted about 100 Casertana and Large White breed pigs. The farm is surrounded by a wooded area abounding in wildlife, including wild boar.

The sera were tested by Rose Bengal (RBT) and Complement Fixation (CFT) tests, according to the methods described in the OIE Manual (OIE 2013). Of the 28 sera, 25 (89%) were positive to RBT and 22 (79%) to CFT, with titres ranging from 20 to 640 ICFTU/ml. The results were confirmed by the National Reference Laboratory for Brucellosis (Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise, Teramo). Pigs were then slaughtered and the organs of the 2 subjects with the highest CFT titres were further processed by culture tests.

Culture tests for *Brucella* spp. were performed in accordance with the International Standards (OIE 2013), using enrichment cultures on Trypticase-soy broth + 5% equine serum with amphotericin B, polymixin B, bacitracin and vancomycin, on Farrell's and on Modified Thayer Martin's media with incubation at 10% CO_2 and aerobically.

Brucella spp. was isolated through direct and enrichment cultures from spleens and abdominal lymph nodes of both examined subjects, 1 of which was an hybrid boar. The isolates readily grew under both aerobic and microaerophilic conditions. The isolates were confirmed by bcsp31 Polymerase Chain Reaction (PCR) assay as belonging to the *Brucella* genus (Elfaki *et al.* 2005).

[1] Iowa State University, Center for Food Security & Public Health, Iowa State University. 2007. Porcine and rangiferine brucellosis: *Brucella suis*, 1-6. http://www.cfsph.iastate.edu/Factsheets/pdfs/brucellosis_suis.pdf.

Vaginal swabs, fetuses, and stillborn piglets were also collected to be examined for the presence of Bacteria. They were negative for *Brucella* spp. as well as for major infectious abortion agents.

Species and biovar molecular identification were performed according to the AMOS-PCR (Abortus Melitensis Ovis Suis-PCR) protocol using the primers described in literature (Bricker *et al.* 1994, Bricker and Halling 1995, Félix *et al.* 1994, Vemulapalli *et al.* 1999). The omp2a and omp31 PCR products were submitted to Restriction Fragment Lenght Polymorphism (RFLP) by digestion with NcoI (omp2a) and AvaII (omp31) restriction endonuclease (Vizcaino *et al.* 1997). The identification at biovar level was also performed using additional tests (CO_2 demand, H_2S production, agglutination with anti-A, anti-M anti-R -CVL, Addlestone-mono specific antisera and growth in Thionine agar and fuchsin agar), according to the OIE Manual (OIE 2013). The identification at biovar and species level demonstrated the presence of *B. suis* biovar 2.

In areas where *B. suis* biovar 2 is reported, the presence of wild boar and hare infected populations is considered the most important risk factor for pigs reared 'outdoor' (EFSA 2009). In wild boar *B. suis* biovar 2 is often isolated without any macroscopic lesions in target organs (Godfroid 2002) and its role in abortion is not well defined by the available literature, even when data on biovars detected in the same areas are retrospectively compared (Cvetnić *et al.* 2003, Cvetnić *et al.* 2009). Brucellosis in domestic and wild suids is not subject to official eradication or control plans, its real diffusion is therefore still unknown. Additionally, the available serological tests do not assure high sensitivity and specificity, so that they may not be reliable for the routine diagnosis of swine brucellosis (Praud *et al.* 2012).

In recent years, several studies have been conducted to evaluate the performances of some alternative serological tests as Fluorescence Polarization Assay (FPA), Indirect Enzyme-Linked Immunosorbent Assay (I-ELISA), Competitive Enzyme-Linked Immunosorbent Assay (c-ELISA), Dissociation-Enhanced Lanthanide Fluorescent Immunoassay (DELFIA). These tests, while having comparable or better performances than traditional tests (RBT, CFT), are not sufficient for the individual diagnosis, although they represent a valuable tool to enhance the overall sensitivity, if used in parallel testing (Di Febo *et al.* 2012, Praud *et al.* 2012, Silva *et al.* 2000). Even in our study, we suspected that the introduction of the disease could have occurred following the mating with wild boars from the adjacent wooded area. The hypothesis was confirmed by the frequent finding of infringement in the farm fences and the birth of striped piglets.

The disease could have spread by the use of hybrid boars as production stock, as confirmed by farmers, and through fomites. Despite these observations, it is still difficult to consider *B. suis* biovar 2 as the possible cause of infertility and abortions in the breeding farm, since, in this farm, *B. suis* biovar 2 has never been isolated from fetuses, stillborn piglets or vaginal swabs. Indeed, the role of *B. suis* biovar 2 as a cause of abortion or infertility remains elusive in literature, in contrast with the role played by biovar 1 (Lord *et al.* 1998) or biovar 3 (Cornell *et al.* 1989).

The correct characterization at biovar level has important implications both for animal and public health. In fact, the zoonotic role and impact of *B. suis* biovar 2 in public health is considered minor in comparison with *B. suis* biovars 1 and 3 (Institut de Veille Sanitaire 2007, Paton *et al.* 2001, Teyssou *et al.* 1989).

Brucellosis is one of the most important endemic agents of wild boars (*Sus scrofa*) in Europe (Al Dahouk *et al.* 2005, Bergagna *et al.* 2009, Gennero *et al.* 2006, Godfroid *et al.* 1994, Grégoire *et al.* 2012, Hars *et al.* 2004, Leuenberger *et al.* 2007, Melzer *et al.* 2007, Ruiz-Fons *et al.* 2006, Vengust *et al.* 2006). Control measures should be implemented for domestic and wild species to limit the mutual transmission of pathogens. In light of this study, it appears appropriate to adopt risk-based surveillance measures, applying a proper strategy of diagnostic testing, including free-range or outdoor breeders for semen screening, either employed for natural mating or artificial insemination, in conformity with the current laws[2].

Further studies would be needed to estimate the prevalence in domestic and wild swine populations, to isolate the biovars in the study areas and explore patterns involving wildlife and domestic animals. It is important to highlight that the evaluations related to the ecology of the diseases in wild animal populations should involve different professionals and pursue a population-based approach (Lanfranchi *et al.* 2003). The aim of the disease surveillance in wild animals is to preserve the health of wild populations and to ensure the health status of humans (*i.e.* zoonotic diseases) and farmed species in the areas under surveillance, therefore, in that sense, the etiologic agents of swine brucellosis are no exception.

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[2] European Union (EU). 2012. Commission Implementing Regulation (EU) No 176/2012 of 1 March 2012 amending Annexes B, C and D to Council Directive 90/429/EEC as regards animal health requirements for brucellosis and Aujeszky's disease. *Off J*, **L 61**, 02/03/2012.

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