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First report of *Brucella suis* biovar 2 in outdoor reared pigs (*Sus scrofa domesticus*) in Serbia

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Abstract: The objective of this work was to present the first case of *Brucella suis* biovar 2 isolation in outdoor reared pigs in Serbia. *B. suis* biovar 2 has not previously been detected in Serbia, from either wild boar or outdoor reared pigs. In our case, brucellosis was discovered in the region of Srem, which geographically constitutes a part of northwestern Serbia. Outdoor reared pigs in the Srem region are part of an extensive breeding system in the field and woods. In the course of a random visit to a herd in the above-mentioned area, the animals' owner discovered two aborted fetuses, thereafter presented for laboratory examinations. *B. suis* biovar 2 was isolated from both fetuses. Isolated strains were identified using both classical and molecular techniques, including genomic sequencing. Based on epizootiological data, we were unable to establish the source of infection.

Key words: *Brucella suis* biovar 2, outdoor reared pigs, isolation, polymerase chain reaction, Serbia

1. Introduction

Brucellosis is primarily a disease of domestic and wild animals caused by facultative intracellular bacteria of the genus *Brucella*. In addition to a series of different animal species, brucellosis also occurs in pigs, causing complex health problems and, consequently, significant economic losses in swine production. Brucellosis in pigs is caused by *Brucella suis* and less often by other species, such as *Brucella abortus* and *Brucella melitensis* (1). *B. suis* is divided into 5 biovars (2). Biovars 1, 2, and 3 cause brucellosis in pigs, and they differ among themselves according to their affinity toward different hosts, geographic distribution, and zoonotic potential (1). Of the others, biovar 4 has been established in reindeer and caribou, and biovar 5 has been found in rodents (3). In addition to the natural pig host, infections related to different *B. suis* biovars have also been recorded in nonnatural host animals, such as cattle (4), dogs (5), and horses (6). Biovar 2 can also infect hares (*Lepus europaeus*) and red foxes (*Vulpes vulpes*) (7,8). Biovars 1 and 3 are important in the occurrence of the disease in humans, while biovar 2 has a much smaller role and rarely expresses its zoonotic potential (9,10).

Brucellosis is most often manifested in pigs through abortions and sterility in sows and orchitis in boars (11). A more precise clinical picture of brucellosis depends on the site where the process is located, so that the symptoms will be in keeping with the function of the affected organ. *Brucella* sp. are shed from the infected organism through vaginal discharge, the placenta, aborted fetuses, urine, and sperm (9). In addition to the venereal route, environmental factors also have a significant role in the spread of brucellosis. High humidity and the absence of direct sunlight favor the survival of the bacteria in the environment (12). This enables infection through the ingestion of aborted fetuses, placentas, or contaminated food and water.

Sporadic infections with *B. suis* in domestic pigs have been recorded in Austria, Germany, France, Croatia, Spain, and Portugal (13). Results of investigations among wild animals indicate that wild boars and hares are natural carriers of *B. suis* biovar 2 (13). The significance of the infection in wild boars is in the fact that the transmission of pathogens is most frequently from wild to domestic animals. The most exposed animals are outdoor herds of domestic pigs (14–16). This is why brucellosis often occurs in regions where pigs are traditionally often kept in pastures or in the woods.

The incidence of brucellosis in outdoor reared pigs has been recorded in Germany, France, and Croatia (14,17,18). In Italy, infections linked to *B. suis* biovar 2 were established in pigs originating from semifree-range pig farms (19).

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B. suis biovar 2 has not been previously detected in Serbia, from either wild boars or outdoor reared pigs. In our case, the appearance of brucellosis was discovered in the region of Srem, which is geographically a part of northwestern Serbia. It is situated between the Danube River in the northeast, the Sava River in the south, and the Bosut River in the west. Pigs are traditionally reared outdoors in these areas, on pasture in fields and forests. In these areas, wild boars are present and often inflict damage to agricultural crops, in particular to corn. Pig production in Srem has a long history. Certain intensive production farms were closed down 10–15 years ago, but a few of them have been restored with modern technology in recent years. The objective of this work is to present the first case of *B. suis* biovar 2 in outdoor reared pigs in Serbia.

2. Case history

In the course of a random visit to a herd on pasture in Srem, the animals' owner discovered two aborted fetuses. The case was reported to a veterinary service that submitted both fetuses to the Scientific Institute of Veterinary Medicine in Belgrade for laboratory analysis.

2.1. Bacteriological examination

The stomach contents of the aborted fetuses were inoculated on four media plates: two plates of Brucella Selective Medium and Brucella Selective Supplement (HiMedia, India) with 5% sheep erythrocytes and 5% inactivated horse serum, and two plates of MacConkey agar (HiMedia, India). Two media (1 plate blood agar and 1 plate of MacConkey agar) were incubated at 37 °C in aerobic conditions and the other two in conditions of 5%–10% CO_2 (Genbox CO_2, bioMérieux, France). The growth and morphology of the colonies were observed for 6 days. Isolates were identified on the basis of the morphology of the colonies and stained by the Gram method. Other characteristics were their growth in the presence of basic fuchsin and thionine at a final concentration of 20 µg/mL, H_2S production, CO_2 requirement, and catalase, oxidase, and urease tests. Agglutination with monospecific sera for A and M antigens was carried out (20). The final identification of biovars was done using molecular methods.

2.2. Polymerase chain reaction (PCR) detection of *Brucella suis*

Two individual colonies were suspended in 50 µL of DNA/RNA-free water and heated at 95 °C for 5 min. For the detection of the *Brucella* sp. genome, a commercial kit (TopTaq Master Mix kit, QIAGEN, Germany) and the thermal protocol and primers (forward primer (JPF) 5'-GCGCTCAGGCTGCCGACGCAA-3' and reverse primer (JPR) 5'-ACCAGCCATTGCGGTCGGTA-3') described by Leal-Klevezas et al. (21) were used. In order to differentiate the species within the genus *Brucella*, primers (*B. abortus*-specific primer 5'-GACGAACGGAATTTTTCCAATCCC-3', *B. melitensis*-specific primer 5'-AAATCGCGTCCTTGCTGGTCTGA-3', *B. ovis*-specific primer 5'-CGGGTTCTGGCACCATCGTCG-3', *B. suis*-specific primer 5'-GCGCGGTTTTCTGAAGGTTCAGG-3', IS711-specific primer 5'-TGCCGATCACTTAAGGGCCTTCAT-3'), the thermal profile for multiplex PCR described by Bricker and Halling (22), and a commercial kit (QIAGEN Multiplex PCR Kit, QIAGEN, Germany), according to manufacturer's instruction, were used.

The PCR products were analyzed by electrophoresis in 2% agarose gel, stained with ethidium bromide and visualized under UV transillumination. In order to determine the characteristic length of the amplified segment, we used a commercial molecular marker (GelPilot Ladder 100 bp Plus, QIAGEN, Germany).

2.3. Sequence analysis

The product of the multiplex PCR reaction was purified using a commercial kit for DNA purification (MiniElute PCR Purification Kit, QIAGEN, Germany) and sequenced using the Sanger sequencing method (Macrogen Inc., the Netherlands).

3. Results and discussion

The growth of clearly visible colonies was observed on both plates of blood agar on the 5th day. Colonies without hemolysis grew in pure culture and were small, round, convex, smooth (S form), and around 1 mm in diameter. The presence of individual gram-negative coccobacilli was confirmed by microscopy. There were positive reactions of catalase, oxidase, and urease at a very fast rate without the production of H_2S. Colonies developed on thionine, but there was no growth on basic fuchsin. The motility test was negative. There was positive agglutination with monospecific A antiserum. From both samples, identical cultures were isolated. These characteristics indicated the presence of *B. suis*. There was no growth on the MacConkey agar. The initial pure culture was kept in a refrigerator at a temperature of 6–8 °C and it remained viable for 42 days during the entire control period.

Both isolates yielded expected product sizes of 193 bp with *Brucella* sp. primers, while both isolates amplified products of approximately 500 bp by multiplex PCR reaction, as shown in the Figure. The amplified products were sequenced due to the lack of the expected band for *B. suis* biovar 1. Nucleotide sequences of our isolate of *B. suis* biovar 2 were deposited in GenBank under accession number KT309077 at the US National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov).

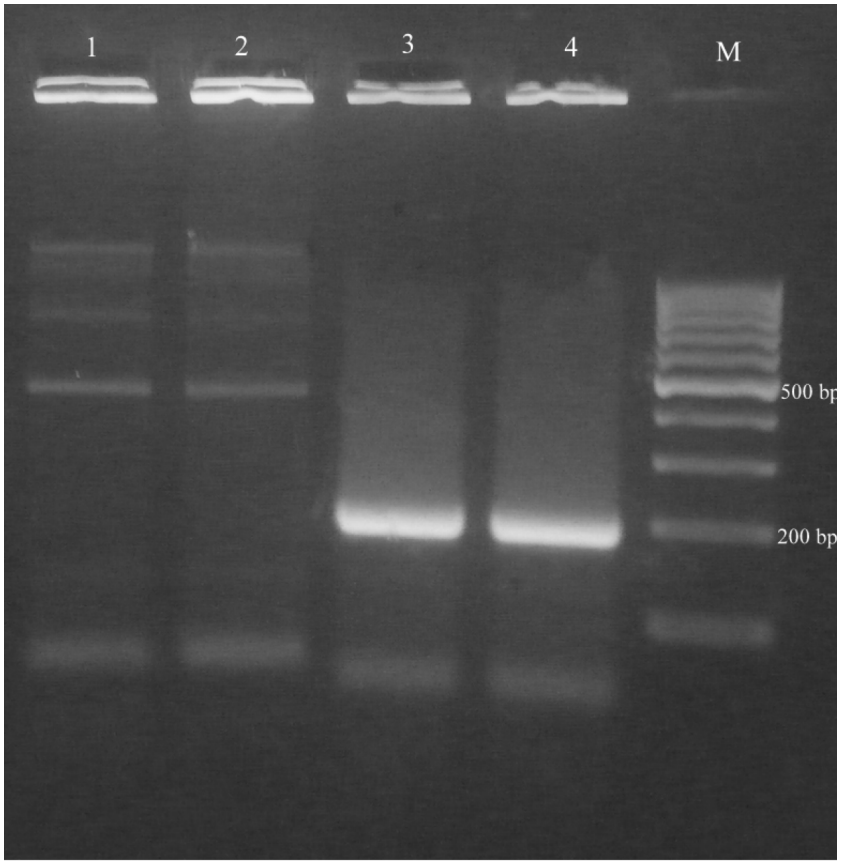

Figure. Detection of *Brucella* sp. DNA. Lanes 1 and 2: Amplified product (≈500 bp), both strains with multiplex PCR *Brucella* sp. primers; lanes 3 and 4: amplified product (≈193 bp), both strains with PCR *Brucella* sp. primers; M: molecular marker.

Through use of the *Brucella* sp. PCR protocol, we managed to confirm that both isolates belonged to the genus *Brucella*. After defining our isolates as *B. suis*, according to their phenotype, we applied the multiplex PCR protocol (22). Bearing in mind the fact that multiplex PCR for both isolates amplifies products (500 bp) that are not anticipated for *B. suis* biovar 1 according to the reference (285 bp), we decided to sequence our PCR products. The results of the sequence analysis revealed that our isolates were most similar to *B. suis* biovar 2. In order to investigate the possibility of detecting *B. suis* biovar 2 by multiplex PCR, we believe that it would be useful to test a larger number of *B. suis* biovar 2 isolates using the same protocol for multiplex PCR. As a possible reason for the inability of multiplex PCR to detect *B. suis* biovar 2, we recognized the absence of sequences of *B. suis* biovar 2. When the protocol was optimized, only a small number of *B. suis* isolates were tested. Moreover, the protocol was optimized only for cattle in the United States, where *B. suis* biovar 1 is the only *B. suis* biovar detected among cattle (23). For the time being, the protocol that we used is considered appropriate for the diagnosis of *B. suis* biovar 1 only, which was confirmed by original tests reported by Bricker and Halling (22).

The presence of brucellosis in outdoor herds of domestic pigs has been recorded in Germany and France, with wild boars being marked as the source of infection (14,17). In one outdoor farm in Switzerland, two out of ten serum samples showed a positive result in indirect ELISA (24). Additional tests showed a negative result for brucellosis on this particular farm. Brucellosis in outdoor pig production areas was also confirmed in Croatia, in the region of Turopolje. *B. suis* biovar 2 was isolated from the organs of 13 piglets out of a total of 30 that gave a positive reaction in previous serological tests (18). Barlozzari et al. (19) reported that 89% of the sera of the 28 examined sera of pigs originating from a semifree-range farm in Italy had a positive Rose Bengal test reaction. *B. suis* biovar 2 was isolated from two serologically positive piglets. The birth of striped piglets indicates that it is justified to suspect that the sow from the semifree herd mated with a wild boar. Pilo et al. (25) suggested that the presence of free-range

pigs can be a risk factor because they have contact with wild boars, and they represent a bridge between wild boars and domestic pigs.

In Serbia, there have not been any investigations so far of the presence of the disease in wild boars or in outdoor pigs; as a result, the current situation is still unknown. In accordance with the Program of Measures of Health Protection of Animals, adopted annually by the Ministry of Agriculture and Environment, all boars kept for artificial insemination are serologically tested in Serbia. In addition, every abortion in a sow must be serologically and bacteriologically examined for brucellosis. Brucellosis in Serbia has been sporadically determined in domestic pigs in the private sector, where the owners report reproductive disorders (26,27). In the course of a 5-year conducted serological survey, brucellosis was found in 88 domestic pigs (28).

However, in spite of the contact with potentially infected pigs, there have been no descriptions so far of the isolation of *B. suis* in humans, which could indicate that *B. suis* strains have a low zoonotic potential.

This report describes the first case of brucellosis in outdoor reared pigs in Serbia. The disease was established in northwestern Serbia, in the region of Srem. The region is predominantly a wide, flat land interspersed with wooded areas. Outdoor reared pigs in the region of Srem are part of an extensive breeding system both in the field and in forests. Based on epizootiological data, we were unable to establish the source of infection. We assume that infection occurred through direct or indirect contact with wild boars. We associate this supposition with the results of investigations of other authors who point out that wild boars present the most frequent source of infection of outdoor pigs (15,16,29). The genetic similarity of strains originating from domestic and wild pigs from Hungary and strains from Germany and Croatia point to the fact that state borders are not an obstacle to the spread of pathogens (30). The same authors pointed out the existence of identical genotypes in strains isolated from Hungarian wild boars and Croatian domestic pigs. Transfer of the disease from wild to domestic animals is conditioned upon the prevalence of brucellosis in wild animals, the susceptibility of the host, the survival of the agent in the environment and, certainly, the possibility of contact between wild and domestic animals (12).

In conclusion, knowledge of the characteristics of *B. suis* isolates is very important for the understanding of the epizootiology of the disease and the application of measures for the control of brucellosis. The lack of control of wild animals and the presence of an outdoor rearing system, as well as several cases of uncontrolled animal trade, are potential critical points that can affect the spread of brucellosis in Serbia. The appearance of brucellosis in pigs in the Srem region indicates the need for serological screening of outdoor pigs and wild boars. It is also necessary to examine the characteristics of the isolated strains. Programs for brucellosis surveillance must focus on the risk points and also emphasize the preservation of animal health and, consequently, human health. They should also expand cooperation among countries in the region in order to gain knowledge about the epizootic situation and to obtain a better understanding of the biology of the pathogen.

References

1. World Organisation for Animal Health. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Paris, France: OIE; 2016.
2. Corbel MJ, Brinley-Morgan WJ. Genus *Brucella*. In: Krieg NR, Holt JC, editors. Bergey's Manual of Systematic Bacteriology. Baltimore, MD, USA: Williams & Wilkins; 1984. pp. 377-388.
3. Godfroid J, Nielsen K, Saegerman C. Diagnosis of brucellosis in livestock and wildlife. Croat Med J 2010; 51: 296-305.
4. Fretin D, Mori M, Czaplicki G, Quinet C, Maquet B, Godfroid J. Saegerman C. Unexpected *Brucella suis* biovar 2 infection in a dairy cow, Belgium. Emerg Infect Dis 2013; 19: 2053-2054.
5. Ramamoorthy S, Woldemeskel M, Ligett A, Snider R, Cobb R, Rajeev S. *Brucella suis* infection in dogs, Georgia, USA. Emerg Infect Dis 2011; 12: 2386-2387.
6. Cvetnic Z, Spicic S, Curi S, Jukic B, Lojkic M, Albert D, Thiebaud M, Garin-Bastuji B. Isolation of *Brucella suis* biovar 3 from horses in Croatia. Vet Rec 2005; 156: 584-585.
7. Szulowski K, Iwaniak W, Pilastek J, Truszczynski M, Chrobocinska M. The ELISA for the examination of hare sera for anti-*Brucella* antibodies. Comp Immunol Microb 1999; 22: 33-40.
8. Hofer E, Reisp K, Revilla-Fernandez S, Plicka H, Romanek G, Bago Z, Winter P, Köfer J. Isolation of *Francisella tularensis* and *Brucella suis* from red foxes (*Vulpes vulpes*). Tieraerztl Umschau 2010; 65: 229-232.
9. World Organisation for Animal Health. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Paris, France: OIE; 2009.
10. Kutlu M, Cevahir N, Erdenliğ-Gürbilek S, Akalın Ş, Uçar M, Sayın-Kutlu S. The first report of *Brucella suis* biovar 1 isolation in human in Turkey. Journal of Infection and Public Health 2016; 9: 675-678.
11. Megid J, Mathias LA, Robles CA. Clinical manifestations of Brucellosis in domestic animals and humans. Open Veterinary Science Journal 2010; 4: 119-126.

12. Godfroid J. Wildlife brucellosis: spillover or sustainable infection? In: Brucellosis 2014 International Research Conference: 67th Annual Brucellosis Research Meeting. Berlin, Germany: BFR; 2014. pp. 205-208.

13. European Food Safety Agency. Porcine brucellosis (*Brucella suis*). Scientific Opinion of the Panel on Animal Health and Welfare. EFSA Journal 2009; 1144: 1-112.

14. Melzer F, Lohse R, Nieper H, Liebert M, Sachse K. A serological study on brucellosis in wild boars in Germany. Eur J Wildlife Res 2007; 53: 153-157.

15. Cvetnic Z, Spicic S, Toncic J, Majnaric D, Benic M, Albert M, Thiebaud M, Garin-Bastuji B. *Brucella suis* infection in domestic pigs and wild boar in Croatia. Rev Sci Tech OIE 2009; 28: 1057-1067.

16. Wu N, Abril C, Thomann A, Grosclaude E, Doher MG, Boujon P, Ryser-Degiorgis MP. Risk factors for contacts between wild boar and outdoor pigs in Switzerland and investigations on potential *Brucella suis* spill-over. BMC Vet Res 2012; 8: 2-12.

17. Garin-Bastuji B, Hars J, Calvez D, Thiebaud M, Artois M. Brucellose du porc domestique et du sanglier sauvage due a *Brucella suis* biovar 2 en France. Rev Epid San Anim 2000; 38: 1-5 (in French).

18. Spicic S, Benic M, Zdelar-Turk M, Duvnjak S, Karakas A, Kis T, Sucec I, Cvetnic Z. Brucellosis in Turopolje pig breeding in the 2008-2011 period: an overview of laboratory diagnostics and eradication system. Vet Arhiv 2016; 86: 243-252.

19. Barlozzari G, Franco A, Macri G, Lorenetti S, Maggiori F, Dottarelli S, Maurelli M, DiGiannatale E, Tittarelli M, Battisti A, Gamberale F. First report of *Brucella suis* biovar 2 in a semi free-range pig farm, Italy. Veter Ital Ser 2015; 51: 151-154.

20. Alton GG, Jones LM, Angus RD, Verger JM. Techniques for the Brucellosis Laboratory. 1st ed. National Institute of Agricultural Research; 1988.

21. Leal-Klevezas DS, Martinez-Vasquez IO, Lopez-Merino A, Martinez-Soriano JP. Single-step PCR for detection of *Brucella* spp. from blood and milk of infected animals. J Clin Microbiol 1995; 33: 3087-3090.

22. Bricker BJ, Halling SM. Differentiation of *Brucella abortus* bv. 1, 2, and 4, *Brucella melitensis*, *Brucella ovis* and *Brucella suis* bv. 1 by PCR. J Clin Microbiol 1994; 32: 2660-2666.

23. Ewalt DR, Payeur JB, Rhyan JC, Geer PL. *Brucella suis* biovar 1 in naturally infected cattle: a bacteriological, serological, and histological study. J Vet Diagn Invest 1997; 9: 417-420.

24. Köppel C, Knopf L, Ryser MP, Miserez R, Thür B, Stärk KDC. Serosurveillance for selected infectious disease agents in boars (*Sus scrofa*) and outdoor pigs in Switzerland. Eur J Wildlife Res 2007; 53: 212-220.

25. Pilo C, Tedde MT, Orru G, Addis G, Liciardi M. *Brucella suis* infection in domestic pigs in Sardinia (Italy). Epidemiol Infect 2014; 143: 1-21.

26. Rogozarski D, Potkonjak A, Gagrcin M, Lako B, Plavsic B. Isolation and identification of *Brucella suis* biotype 2 from epididymal puncture performed on a boar affected with brucellosis. Veterinarski Glasnik 2009; 63: 153-161 (article in Serbian with an English abstract).

27. Zutic J, Stanojevic S, Plavsic B, Vojinovic D. Prevalence and significance of brucellosis in the Republic of Serbia. In: Book of Abstracts of the Symposium Brucellosis in Croatia and Neighbouring Countries. Zagreb, Croatia; 2013. pp. 1-2.

28. Zutic J, Vojinovic D, Stanojevic S, Cvetojevic DJ, Plavsic B. Seroepizootiological situation of brucellosis in Republic of Serbia. In: Proceedings of the 2nd International Symposium of Veterinary Medicine. Belgrade, Serbia; 2016. pp. 30-35.

29. Duvnjak S, Racic I, Spicic S, Zdelar-Turk M, Reil I, Cvetnic Z. Characterisation of *Brucella suis* isolates from Southeast Europe by multi-locus variable-number tandem repeat analysis. Vet Microbiol 2015; 180: 146-150.

30. Kreizinger Z, Foster JT, Ronai Z, Sulyok KM, Wehmann E, Gyuranecz M. Genetic relatedness of *Brucella suis* biovar 2 isolates from hares, wild boars and domestic pigs. Vet Microbiol 2014; 172: 492-498.