

Brucella suis biovar 2 isolations from cattle in Poland

Krzysztof Szulowski, Wojciech Iwaniak, Marcin Weiner, Jolanta Złotnicka

Department of Microbiology, National Veterinary Research Institute, Pulawy, Poland

Szulowski K, Iwaniak W, Weiner M, Złotnicka J. *Brucella suis* biovar 2 isolations from cattle in Poland. Ann Agric Environ Med. 2013; 20(4): 672–675.

Abstract

Bovine brucellosis is an infectious disease caused by bacteria of the *Brucella* genus, primarily by *B. abortus*, less frequently by *B. melitensis*, and occasionally by *B. suis*. In the European Union, brucellosis in cattle has been eradicated in most of the Member States, which are recognized as 'officially free from bovine brucellosis'. Nevertheless, cattle herds continue to be serologically monitored for the potential re-emergence of the disease. The aim of the presented study was to show the results of bacteriological investigations of cattle slaughtered in Poland in years 2002–2011 on account of positive serological reactions for brucellosis. Specimens (sera and tissues) from 176 cows were examined. Sera from the animals were tested using RBT (rose bengal test), SAT (serum agglutination test), CFT (complement fixation test), 2-ME (2-mercaptoethanol test), Coombs (Coombs antiglobulin test) and ELISA (enzyme linked immunosorbant assay). Tissue samples were cultured for *Brucella*, according to official protocols. All sera were RBT and SAT-positive, 170 of them were CFT-positive, whereas 6 other samples were CFT negative while positive in Coombs and ELISA. In bacteriological examination, *B. abortus* was not isolated. On the other hand, *B. suis* biovar 2 was isolated from 5 cows, which had never been reported previously in Poland. Three cows came from the same herd. Conventional, as well as, molecular investigations based on PCR methods, confirmed that the bacteria isolated belong to the *B. suis* biovar 2. In Poland, as in many other European countries, wildlife (wild boars and hares) constitutes a huge reservoir of the said biovar. The results of the presented research indicate that *B. suis* biovar 2 can easily infect cattle, and undoubtedly plays a role in the epidemiology and control of bovine brucellosis.

Key words

brucellosis, cattle, *Brucella suis* biovar 2, Poland

INTRODUCTION

Bovine brucellosis is a chronic infectious disease caused by bacteria of the *Brucella* genus, primarily by *B. abortus*, less frequently by *B. melitensis*, and occasionally by *B. suis*. In the European Union (EU), brucellosis in cattle has been eradicated in most of the Member States which, according to EU Directive 64/432/EEC, are recognized as 'officially free from bovine brucellosis' [1]. Nevertheless, cattle herds continue to be serologically monitored for the potential re-emergence of the disease.

In Poland, bovine brucellosis (*B. abortus*) was eradicated in 1980, but due to EU regulations, the country obtained its official 'Brucellosis-free' status as late as 2009 [2]. On the other hand, wildlife constitutes a huge reservoir of *B. suis*. Surveys revealed that 12.3% of the sera from wild boars [3] and 0.9% of the hare sera [4], collected from various part of the country, reacted positively in ELISA. The serology was confirmed by culture, which showed the occurrence of *B. suis* biovar 2, both in hares and wild boars [3, 5].

The diagnosis of bovine brucellosis in Poland is primarily based on serological tests. All animals monitored for brucellosis and positive in the Rose Bengal test (RBT) undergo a further examination with the use of a serum agglutination test (SAT) and a complement fixation test (CFT) in accordance with the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals [6], and Annex C to EU Directive 64/432/EEC – No 535/2002 [7]. 2-mercaptoethanol

test (2-ME), Coombs antiglobulin test (Coombs), were carried out according to official protocols [8, 9]. An indirect-ELISA commercial test (Pourquier, France), was also used in the National Reference Laboratory for Brucellosis (NRL) as a panel of confirmatory tests for further analysis. All serum samples from the positive in RBT and SAT, which are positive in CFT and/or in one or more of additional tests (2-ME, Coombs, ELISA), in accordance with the Polish regulations, are classified as finally positive. In such cases, the animals are obligatorily slaughtered and subjected to bacteriological examination.

This paper presents the outcomes of bacteriological and molecular investigations of slaughtered cattle for the presence of *Brucella* in Poland.

MATERIALS AND METHOD

Examined samples. The specimens from 176 cows slaughtered on account of the results of serological examinations qualified as positive and collected in years 2002–2011 were cultured for isolation of *Brucella*. The specimens consisted of head, mammary and genital lymph nodes, spleen, liver, uterus and udder (females) or testes (males).

Bacteriological examinations. Serum dextrose agar (SDA – home made medium) was used for culture of specimens. The plates were incubated for 10 days at 37 °C in an atmosphere with 5–10% CO₂ added. In parallel, the specimens were cultured in similar conditions in an enriched liquid medium (serum dextrose broth supplemented with antibiotic mixture) for up to 6 weeks, with weekly subcultures on to a solid

Address for correspondence: Wojciech Iwaniak, Department of Microbiology, National Veterinary Research Institute, 24-100 Pulawy, Poland
e-mail: iwaniakw@piwet.pulawy.pl

Received: 31 July 2012; accepted: 7 February 2013



selective medium (Farrell's home made medium). Colonies typical for *Brucella* were checked with anti-*Brucella* standard serum, examined in catalase and oxidase tests and stained by Gram's method. Further characteristics were determined by using monospecific anti-A and anti-M sera (AHVLA, Weybridge, UK) and further tests for CO₂ requirement, production of H₂S (Hydrogen Sulfide Test Strip, Fluka) and urease, growth in the presence of thionin and basic fuchsin, and lysis by phages (Tbilisi at its routine test dilution – RTD and 10⁴ × RTD) [10]. Additionally, molecular methods – a multiplex PCR assay (Bruce – ladder) and a multi-locus analysis of variable number tandem repeats (MLVA) were applied to confirm the identification of isolated *Brucella* strains [11, 12].

DNA extraction. One individual colony of each *Brucella* isolate was suspended in 50 µl of sterile, DNase, RNase-free deionized water (ICN Biomedicals). The suspensions were heated at 99°C for 5 min, chilled on ice, and then centrifuged at 13,000 × g for 1 min to pellet the cellular debris. The supernatant (5 µl) was subsequently used as a source of the DNA template. Concentration of the DNA preparations was measured spectrophotometrically at 260 and 280 nm (GeneQuant 1300, GE Healthcare).

PCR assays. Each DNA amplification was performed in a 50 ml reaction mixture consisting of the DNA template, 1X PCR buffer (Fermentas), 200 mM of dNTPs, 3mM MgSO₄ (for Bruce-ladder) or 5 mM MgCl₂ (for VNTR's), 2U of Pfu DNA Polymerase (Bruce-ladder) or 2 U of Taq DNA polymerase (VNTR's), nucleotide primers, and water. Sequences, characteristics, and concentration of the primers used in the presented study were chosen following Lopez – Goni et al. and Le Fleche et al. [12]. Bruce-ladder and MLVA (Panel 1) were run in a thermocycler (T3, Biometra) under the following conditions: initial DNA denaturation at 95°C for 5 min, followed by 25 cycles at 95°C for 30 s, 64°C for 45 s and 72°C for 3 min (Bruce-ladder), or 30 cycles at 95°C for 30 s, 60°C for 30 s and 70°C for 1 min (MLVA). The final extension step was conducted at 72°C (Bruce-Ladder), or 70°C for 10 min (MLVA). The PCR product of Bruce-ladder was visualized by means of a standard gel electrophoresis in a 2% agarose gel (Type I, Low EEO, Sigma). For the MLVA, 3% High Resolution agarose (Fermentas) was used. After having been stained with ethidium bromide (50 mg/ml) for 0.5 min and washed in distilled water, the gels were photographed under UV light with the GelDoc 2000 documentation system (Bio-Rad). The sizes of the PCR amplicons were compared to the 100 bp DNA marker (Fermentas).

RESULTS

Of 176 examined animals, 5 cases exhibited *Brucella* organisms. *Brucella* colonies became visible on solid medium once the cultures had been incubating for 3 days. In all these cases, bacteria were recovered from the mammary glands, genital lymph nodes, spleen and liver samples. The growth was obtained from direct plating. Three of the culture-positive cows originated from the same herd, the other two were single reactors in their herds. All cows were RBT, SAT, Coombs and ELISA positive, whereas 2 negative results were obtained in the CFT (Tab. 1). What is crucial about the results

Table 1. Results of serological examination of *Brucella* culture positive cattle.

Animal	RBT	SAT (Titer, iu/ml ¹)	2-ME ² (Titer)	CFT (Titer, icftu/ml ³)	Coombs ⁴ (Titer)	ELISA
1	positive	Positive 4/80 164	negative	positive 2/10 40	positive 4/640	positive
2	positive	positive 4/20 41	negative	positive 3/5 23.3	positive 2/320	positive
3	positive	positive 3/20 36	negative	negative	positive 3/160	positive
4	positive	positive 3/20 36	negative	negative	positive 3/160	positive
5	positive	positive 2/160 246	positive 2/160	positive 2/80 320	positive 2/2560	positive

1 – iu/ml: international units per ml, all sera containing at least 30 iu/ml classified as positive;
2 – all sera with titers at the same level as SAT classified as positive;
3 – icftu/ml: international complement fixation test units per ml, all sera containing at least 20 icftu/ml are classified positive;
4 – all sera with titers at least 3 dilutions higher than SAT are classified as positive.

of the serological testing is that 170 sera were positive at least in RBT, SAT and CFT, whereas 6 sera were RBT and SAT positive, but negative in CFT. However, these sera were additionally positive in one or more of the following tests – ELISA, 2-ME, and Coombs, i.e. finally classified as positive.

The bacteriological examinations revealed that all isolates had the same characteristics: agglutination with anti-*Brucella* standard serum and monospecific anti-A serum, positive results in oxidase, catalase and urease (very fast rate – within a few minutes) tests, no CO₂ requirement for growth, no H₂S production, growth on thionin dye medium, no growth on basic fuchsin dye medium and lysis by TB phages at a concentration 10⁴ × RTD. These characteristics are typical for *B. suis* biovar 2.

The Bruce-ladder PCR assay confirmed that all the examined *Brucella* are *B. suis*. Seven DNA fragments were amplified: 1,682, 1,071, 794, 587, 450, 272 and 152 bp in size. MLVA (Panel 1) analysis revealed the same VNTR profile of the isolates from cows as reference strain *B. suis* biovar 2 Thomsen.

Table 2. Results of examinations of isolated *Brucella* strains in MLVA.

Strains	Nos. of repeated sequences in examined VNTRs (Panel 1)							
	VNTR6	VNTR8	VNTR11	VNTR12	VNTR42	VNTR43	VNTR45	VNTR55
<i>B. suis</i> biovar 2 Thomsen	2	4	8	15	6	1	5	2
Isolates from cows 1-5	2	4	8	15	6	1	5	2

DISCUSSION

The results of the presented study confirm that *B. abortus* is absent from the population of Polish cattle. Despite the lack of infected herds, a certain number of seropositive animals is observed each year. The cause of this phenomenon lies

in the brucellosis serological tests, which are 'imperfect' [13]. The restricted specificity of serological diagnosis yielding false positive serological results (FPSR) is primarily caused by cross-reactions demonstrated between smooth *Brucella* species and other microbes, such as *Escherichia coli* O:157, *Salmonella* serotypes of Kaufman-White group N, *Stenotrophomonas maltophilia* and *Yersinia enterocolitica* O:9 [10, 14], showing the antigenic relationships. The lower the prevalence rate of *Brucella* infections, the higher the percentage of false-positive results, and thus continued surveillance becomes harder to justify. The consequence is an excessive amount of slaughtered animals and higher costs related to the implementation of the Council Directive 64/432/EEC. The occurrence of such reactions in surveillance and diagnostics of brucellosis amounts to a serious problem in many countries [15, 16, 17, 18, 19, 20, 21].

Most commonly, FPSR are caused by infections with *Yersinia enterocolitica* O:9, as the bacterium possesses almost identical smooth lipopolysaccharide (S-LPS) to that present in *B. abortus* [20, 22, 23]. We have recently launched examinations of faeces samples from cows slaughtered due to positive serological reactions for brucellosis for the presence of *Y. enterocolitica* O:9, and in 7 out of 17 samples the bacteria was found to be present [24].

It has been proved that cattle can be infected by *B. suis* – causative agent of brucellosis in pigs, wild boars, caribou, reindeer, hares. The infection appears to be a noncontagious disease with limited induced pathology and no induction of abortion [25, 26, 27]. However, cattle infected with *B. suis* test seropositive on brucellosis surveillance tests, and antibody response cannot be readily differentiated from that caused by an infection with *B. abortus* [25, 27, 28]. There are several reports of isolation of *B. suis* biovar 1 from cattle, as recorded in Australia [25], North America [27, 29] and South America [30, 31, 32]. On the other hand, in Europe, *B. suis* biovar 2 has been isolated from cattle in Denmark [33]. Our investigations confirm that the *B. suis* biovar 2 in European conditions, where wild boars and hares are its natural reservoir [4, 13, 34, 35, 36], can infect cattle and influences the diagnosis of bovine brucellosis. It is worth emphasizing that all *Brucella suis* biovar 2 positive animals originated from regions with large forests adjacent to open grassland, and had easy contact with wild animals (grazing on unfenced pastures). It has already been shown that the prevalence of anti-*Brucella* antibodies in wild boars was higher than 20% [4] in these regions. Our investigations revealed that 3 out of 5 positive animals came from the same herd, suggesting that the exposure to the bacteria was intense rather than sporadic. The observed antibody titers were not high, with 2 samples being negative by CFT, but positive in Coombs. The latter method, so rarely used in routine testing in bovine brucellosis, enables detection associated with the chronic phase of infection, so-called incomplete antibodies of IgG class, which may not be detected in SAT and CFT; for this reason we continue to use this method in our laboratory. It is worth mentioning that this test fulfilled an important role in the campaign for the eradication of brucellosis in Poland [37].

In Poland, a country officially free from bovine brucellosis, positive serological reactions are recorded each year, the great majority being of unknown origin. The results of the presented study indicate that *B. suis* biovar 2 can easily infect cattle, and undoubtedly plays a role in the epidemiology and control of bovine brucellosis.

Acknowledgements

The authors express their thanks to Zofia Zaręba, Hanna Czepińska and Monika Szymajda for their excellent technical assistance.

REFERENCES

1. Council Directive 64/432/EEC of 26 June 1964 on animal health problems affecting intra Community trade in bovine animals and swine, 1964.
2. Commission Decision of 5 August 2009 amending Decision 2003/467/EC as regards the declaration that certain Member States and regions thereof are officially free of bovine brucellosis (2009/600/EC), 2009.
3. Szulowski K, Pilaszek J, Iwaniak W. Application of meat juice in diagnosis of brucellosis in hares and wild boars by ELISA. *Bull Vet Inst Pulawy*. 2000; 44(1): 45–52.
4. Pilaszek J, Szulowski K, Iwaniak W. Epidemiological condition of animal brucellosis in Poland. *Medycyna Wet.* 2000; 56(6): 363–366 (in Polish).
5. Szulowski K, Iwaniak W, Pilaszek J, Murat J. Wild boars and hares as reservoirs of *Brucella suis* biovar 2 in Poland. *Proceeding of the Brucellosis 2008 International Research Conference (Including the 61st Brucellosis Research Conference)*; Sep 10 – 13 2008, Royal Holloway College, University of London, UK, p. 137.
6. OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, Office International des Epizooties, Paris, France, 2009.
7. Commission Regulation (EC) No 535/2002 of 21 March 2002 amending Annex C to Council Directive 64/432/EEC and amending Decision 2000/330/EC, 2002.
8. Wiśniowski J, Królak M, Drożdżyńska M. Standard technique of anti-globulin test in diagnosis of bovine brucellosis. *NVRI Puławy, Poland*, 1978 (in Polish).
9. Królak M, Stryszak M. Standard technique of 2-mercaptoethanol test in diagnosis of animal brucellosis. *NVRI Puławy, Poland*, 1979 (in Polish).
10. Alton GG, Jones LM, Angus RD, Verger JM. Techniques for the brucellosis laboratory. *Institut National de la Recherche Agronomique, Paris, France*. 1988.
11. Lopez-Goni I, Garcia-Yoldi D, Martin CM, Miguel MJ, Munoz PM, Blasco JM, Jacques I, Grayon M, Cloeckart A, Ferreira AC, Carosso R, Correa de Sa, MI, Walravens K, Albert D, Garin-Bastuji B. Evaluation of a multiplex PCR assay (Bruce-ladder) for molecular typing of all *Brucella* species including the vaccine strains. *J Clin Microbiol.* 2008; 46(10): 3484–3487.
12. Le Fleche P, Jacques I, Grayon M, Al Dahouk S, Bouchon P, Denoel F, Nockler K, Neubauer H, Guilloteau LA, Vergnaud G. Evaluating and selection of tandem repeat loci for *Brucella* MLVA typing assay. *BMC Microbiol.* 2006; 6(9): 1–14.
13. Godfroid J, Cloeckart A, Liautard JP, Kohler S, Fretin D, Walravens K, Garin-Bastuji B, Letesson JJ. From the discovery of the Malta fever agent to the discovery of a marine mammal reservoir, brucellosis has continuously been re-emerging zoonosis. *Vet Res.* 2005; 36(3): 313–326.
14. Sixth Report. Joint FAO/WHO Expert Committee on Brucellosis. *World Health Organization, Geneva, Switzerland*, 1986.
15. Johnson RP, Boag L, Anderson S, Holtzlander R, Rahn K, Clarke RC, Renwick SA, Alves D, Wilson JB, Spika J. *Brucella abortus* serology in cattle naturally infected with *Escherichia coli* O:157:H7. *Vet Rec.* 1994; 135(16): 382–383.
16. Weynants V, Tibor A, Denoel P, Saegerman C, Godfroid J, Thiange P, Letesson JJ. Infection of cattle with *Yersinia enterocolitica* O:9, a cause of false positive serological reactions in bovine brucellosis diagnostic tests. *Vet Microbiol.* 1996; 48(1–2): 101–112.
17. Gerbier G, Garin-Bastuji B, Pouillot R, Very P, Cau C, Berr V, Dufour B, Moutou F. False positive serological reactions in bovine brucellosis: evidence of the role of *Yersinia enterocolitica* serotype O:9 in a field trial. *Vet Res.* 1997; 28(4): 375–383.
18. Pouillot R, Lescoat P, Garin-Bastuji B, Repiquet D, Terrier P, Gerbier G, Béné J, Sanaa M. Risk factors for false-positive serological reactions for bovine brucellosis in Saône-et-Loire (France). *Prev Vet Med.* 1998; 35(3): 165–179.
19. Godfroid J, Käsböhrer A. Brucellosis in the European Union and Norway at the turn of the twenty-first century. *Vet Microbiol.* 2002; 90(1–4): 135–145.

20. Muñoz PM, Marín CM, Monreal D, González D, Garin-Bastuji B, Díaz R, Mainar-Jaime RC, Moriyón I, Blasco JM. Efficacy of Several Serological Tests and Antigens for Diagnosis of Bovine Brucellosis in the Presence of False-Positive Serological Results Due to *Yersinia enterocolitica* O:9. Clin Diagn Lab Immunol. 2005; 12(1): 141–151.
21. Weiner M, Zlotnicka J, Iwaniak W, Szulowski K. Development of a multiplex PCR for identification of *Brucella* sp. and cross-reacting *Yersinia enterocolitica* O:9. Bull Vet Inst Pulawy. 2011; 55(4): 603–607.
22. Caroff M, Bundle DR, Perry MB. Structure of the phenol-phase soluble cellular lipopolysaccharide of *Yersinia enterocolitica* serotype O:9. Eur J Biochem. 1984; 139(1): 195–200.
23. Kittelberger R, Hilbink F, Hansen MF, Penrose M, De Lisle GW, Letesson JJ, Garin-Bastuji B, Searson J, Fossati CA, Cloeckaert A, Schurig G. Serological crossreactivity between *Brucella abortus* and *Yersinia enterocolitica* O:9. I Immunoblot analysis of the antibody response to *Brucella* protein antigens in bovine brucellosis. Vet Microbiol. 1995; 47(3–4): 257–270.
24. Szulowski K, Iwaniak W, Weiner M, Zlotnicka J, Szymajda M, Zaręba Z, Czępińska H. Diagnosis and epidemiological condition of bovine brucellosis in Poland. Medycyna Wet. 2012; 68(2): 110–113 (in Polish).
25. Cook DR, Noble JW. Isolation of *Brucella suis* from cattle. Aus Vet J. 1984; 61(8): 263–264.
26. Corbel MJ. Brucellosis: an overview. Emerg Infect Dis. 1997; 3(2): 213–221.
27. Ewalt DR, Payeur JP, Rhyon JC, Geer PL. *Brucella suis* biovar 1 in naturally infected cattle: a bacteriological, serological and histological study. J Vet Diagn Invest. 1997; 9(4): 417–420.
28. Olsen SC, Hennager SG. Immune response and Protection against Experimental *Brucella suis* Biovar 1 Challenge in Nonvaccinated or *B. abortus* Strain RB51-Vaccinated Cattle. Clin Vaccine Immunol. 2010; 17(12): 1891–1895.
29. Luna-Martinez JE, Mejia-Teran C. Brucellosis in Mexico: current status and trends. Vet Microbiol. 2002; 90(1–4): 19–30.
30. Garcia Carillo C. Animal and human brucellosis in the Americas. OIE, Paris, 1990.
31. Samartino LE. Brucellosis in Argentina. Vet Microbiol. 2002; 90(1–4): 71–80.
32. Lucero NE, Ayala SM, Escobar GI, Jacob NR. *Brucella* isolated in humans and animals in Latin America from 1968 to 2006. Epidemiol Infect. 2008; 136(4): 496–503.
33. Andersen FM, Pedersen KB. Brucellosis: a case of natural infection of a cow with *Brucella suis* biotype 2. Dan Vet. 1995; 78(8): 408.
34. Cvetnic Z, Mitak M, Ocepek O, Lojkic M, Terzic S, Jemersic L, Humski A, Habrun B, Sostaric B, Brstilo M, Krt B, Garin-Bastuji B. Wild boars (*Sus scrofa*) as reservoirs of *Brucella suis* biovar 2 in Croatia. Acta Vet Hung 2003; 51(4): 465–473.
35. Melzer F, Lohse R, Nieper H, Liebert M, Sachse K. A serological study on brucellosis in wild boars in Germany. Eur J Wildl Res. 2007; 53(2): 153–157.
36. Gyurancz M, Erdélyi K, Makrai L, Fodor L, Szépe B, Ráczné Mészáros A, Dán A, Dencsó L E, Fassang E, Szeredi L. Brucellosis of the European Brown Hare (*Lepus europaeus*). J Comp Path. 2011; 145(1): 1–5.
37. Królak M, Owadiuk Z. Immunogenic and diagnostic values of vaccine Duphavac in eradication of bovine brucellosis. Życie Wet. 1994; 10: 375–379 (in Polish).

