Praca oryginalna *Original paper*

False positive serological reactions to brucellosis in pigs: a growing problem in international trade

KRZYSZTOF SZULOWSKI, WOJCIECH IWANIAK, MARCIN WEINER, JOLANTA ZŁOTNICKA, MONIKA SZYMAJDA, ZOFIA ZARĘBA, HANNA CZĘPIŃSKA

Zakład Mikrobiologii Państwowego Instytutu Weterynaryjnego – Państwowego Instytutu Badawczego, Al. Partyzantów 57, 24-100 Puławy

Szulowski K., Iwaniak W., Weiner M., Złotnicka J., Szymajda M., Zaręba Z., Czępińska H.

False positive serological reactions to brucellosis in pigs: a growing problem in international trade

Summary

The diagnosis of brucellosis in pigs is based almost entirely on serological assays. None of the tests has been shown to be reliable in routine diagnosis in individual pigs. The biggest problem are false positive serological reactions (FPSRs) caused primarily by Yersinia enterocolitica O:9. The OPS component of the sLPS of Brucella is almost identical with that of Y. enterocolitica O:9. Thus no routinely used serological tests based on this antigen can distinguish between antibodies raised to these two infections. This paper presents the results of the examinations of 6 batches of pigs (total of 452 serum samples) traded between countries and causing major diagnostic problems. Positive reactions in RBT, SAT, CFT and I-ELISA were observed in all these batches of animals. Additionally 2 out of 21 samples from one of the batches were positive in 2-Me. FPSRs in the diagnosis of pigs for brucellosis seem to be a growing problem in international trade. The absence of provisions explicitly regulating the problem of FPSRs may have serious consequences, such as the slaughter of animals or even international repercussions. Clear guidelines for dealing with such cases should therefore be formulated.

Keywords: pigs, brucellosis, FPSRs, Yersinia enterocolitica O:9.

Porcine brucellosis is a zoonotic disease of widespread occurrence and global significance. However, the prevalence is low with the exception of South America and South-East Asia, where it is higher (9). Within the European Union (EU), the epidemiological situation is varied, with some countries free of the disease, others reporting sporadic outbreaks, and yet others reporting this disease as an emergent problem (10). Available epidemiological evidence shows that *B. suis* biovar 2 is the most common agent in Europe, and wildlife (wild boars and hares) constitutes a source of infection for pigs (3, 13, 14). There is a lack of systematic epidemiological data on porcine brucellosis in the member states of the EU, as there is currently no requirement for monitoring and surveillance of *B. suis* in domestic pigs and wild animals. However, in many disease-free countries statutory diagnostic testing is required, for example concerning boars in insemination stations, and is often a prerequisite for the movement of live animals. Testing is based almost entirely on serological assays, though the unequivocal diagnosis of *B. suis* infection can be made only by the isolation and identification of *Brucella*. Methods and tests used for the diagnosis of porcine brucellosis are very similar or identical to those applied for the diagnosis of brucellosis in cattle. To date none of the serological tests has been shown to be reliable in routine diagnosis in individual pigs. The Rose Bengal test (RBT), the complement fixation test (CFT), indirect and competitive enzyme-linked immunosorbent assays (I-ELISA and C-ELISA), and the fluorescence polarisation assay (FPA) are the prescribed tests for international trade purposes (9). In Poland the methods employed for diagnosing porcine brucellosis are RBT, I-ELISA, and additionally, to explain doubtful results, CFT, the serum agglutination test (SAT) and the 2-mercaptoethanol test (2-Me).

All available serological diagnostic tests are based on the detection of antibodies to the smooth lipopolysaccharide (S-LPS) of *Brucella* strains. The O-polysaccharide (OPS) component of the S-LPS of *Brucella* is almost identical with that of *Yersinia enterocolitica* O:9. No routinely used serological tests based on this antigen can therefore distinguish between antibodies raised to these two infections. *Y. enterocolitica* O:9 infection in pigs is apparently common in some areas of the EU, thus constituting a major complication for the diagnosis of porcine brucellosis.

The aim of the studies reported here was to show, referring to major examples, the importance of false positive serological reactions (FPSRs) for brucellosis in pigs traded between countries.

Material and methods

Sera. Six batches of sera from pigs traded between countries and causing major diagnostic problems in serological diagnosis for brucellosis were examined by the National Reference Laboratory for Brucellosis (NRLB) of the Department of Microbiology of the National Veterinary Research Institute (NVRI) in Puławy. A total of 452 sera were tested. Five batches were examined twice with a one-month interval, the sixth batch only once.

Methods. RBT, I-ELISA, SAT, CFT, 2-Me were used for the serological examination. The RBT, SAT, CFT and 2-Me were performed according to official instructions and protocols (4-6, 8, 18). The diagnostic kit used in I-ELISA had been developed in the NVRI in Puławy (14).

Results and discussion

Table 1 summarizes the results of the examinations of six batches of animals traded between countries and causing diagnostic problems due to false positive serological reactions. In all batches positive reactions in RBT, SAT, CFT and I-ELISA were observed. Exceptionally positive results in 2-Me were observed in one batch of animals in which the presence of anti-*Brucella*

Tab. 1. Results of examination for the presence of anti-*Brucella* antibodies in swine sera from 6 batches of animals causing diagnostic problems due to false positive serological reactions

No. of animals	No. of animals in particular tests									
	First testing					Second testing				
	RBT	SAT	2-Me	CFT	I-ELISA	RBT	SAT	2-Me	CFT	I-ELISA
44*	23	16	0	9	11	14	8	0	7	8
21	9	9	2	9	9	7	6	1	4	6
240	1	16	0	5	37	Not tested				
32	4	8	0	5	10	0	9	0	2	9
95	11	14	0	13	18	10	7	0	10	17
20	3	6	0	3	9	4	3	0	1	2

Explanation: * – *Yersinia enterocolitica* O:9 was isolated from 6 animals from this batch (15)

Tab. 2. Detailed results of examinations for the presence of anti-*Brucella* antibodies in a batch of pigs presenting typical false positive serological reactions

Sample number	First testing					Second testing				
	RBT	SAT*	2-Me	CFT**	I-ELISA	RBT	SAT	2-Me	CFT	I-ELISA
1	+	31.0	–	20.0	+	–	20.5	–	13.3	–
2	–	61.5	–	26.5	–	–	61.5	–	10.0	+
3	–	–	–	26.5	+	–	–	–	10.0	–
4	+	31.0	–	11.6	+	–	20.5	–	–	–
5	–	31.0	–	23.3	+	–	41.0	–	20.0	+
6	–	31.0	–	26.5	+	–	20.5	–	13.3	+
7	–	31.0	–	26.5	+	–	31.0	–	–	+
8	+	61.5	+/–	13.3	+	–	13.3	–	11.6	–
9	–	61.5	–	–	–	–	36.0	–	–	–
10	–	25.5	–	13.3	–	–	20.5	–	10.0	+
11	–	15.5	–	11.6	–	–	20.5	–	10.0	+
12	+	15.5	–	–	–	–	–	–	–	–
13	–	15.5	–	–	–	–	31.0	–	13.3	+
14	–	13.0	–	20.0	+	–	31.0	–	–	–
15	–	15.5	–	10.0	–	–	–	–	10.0	–
16	–	51.5	–	–	–	–	15.5	–	–	–
17	–	–	–	–	–	–	15.5	–	13.3	–
18	–	31.0	–	8.4	–	–	25.5	–	–	–
19-32	–	–	–	–	–	–	–	–	–	–

Explanations: * – results expressed in international units per ml (iu/ml) – sera with a level of anti-*Brucella* antibodies higher than 30 iu/ml are considered as positive; ** – results expressed in international complement fixation test units per ml (icftu/ml) – sera with a level of anti-*Brucella* antibodies higher than 20 icftu/ml are considered as positive

Tab. 3. Detailed results of the examination of pigs from the batch causing major diagnostic problems due to false positive serological reactions

Sample number	First testing					Second testing				
	RBT	SAT (iu/ml)	2-Me	CFT (icftu/ml)	I-ELISA	RBT	SAT (iu/ml)	2-Me	CFT (icftu/ml)	I-ELISA
1	+	123.0	+/–	53.0	+	+	72.0	+/–	26.5	+
2	+	164.0	+	134.0	+	+	123.0	+/–	53.0	+
3	+	82.0	+/–	20.0	+	+	41.5	–	–	–
4	+	246.0	–	20.0	+	–	25.5	–	–	–
5	+	492.5	–	53.0	+	+	72.0	–	13.3	+
6	+	82.0	+	106.0	+	+	102.5	+/–	26.5	+
7	+	246.0	+/–	53.0	+	+	51.5	–	20.0	+
8	+	123.0	+/–	26.5	+	–	–	–	–	–
9	+	123.0	–	26.5	+	–	–	–	–	–
10-21	–	–	–	–	–	–	–	–	–	–

antibodies was not transient, and they were also observed one month later. Detailed results of examinations for the presence of anti-*Brucella* antibodies in the batch of pigs presenting typical false positive serological reactions are shown in table 2. The results are characterized by a low percentage of positive results, low titers of antibodies observed in CFT and SAT, the absence of positive results in 2-Me, discrepancies between different tests, declining levels of antibodies observed in the second examination, and low levels of antibodies in other samples. The results of tests on another batch of 21 animals proved more difficult to interpret (Tab. 3). The results obtained in the first examination were very similar to those observed in pigs infected with *B. suis* (15), particularly in the case of samples 2 and 6, which were positive in all tests. There was a relatively high percentage of positive results in all tests, high titers in CFT and SAT, and even 2 positive results in 2-Me. Only the second examination, performed one month later, made it possible to define the samples as false positive: the level of reactions declined, and some of the samples became entirely negative. The epidemiological status of the herd of origin was also taken into account.

FPSRs to brucellosis in pigs are recorded in different countries. Some of these reactions may be due to nonspecific antibodies contained in porcine sera, thought to be of the IgM isotype, which further reduce the specificity of conventional tests, especially SAT (10). But the biggest problem is related to infections with *Y. enterocolitica* O:9, often found in pigs in different countries (7, 11, 15, 17, 19). As testing is dependent almost entirely on serological assays, it may result in a significant number of FPSRs. Not much is gained by the application of an additional method, the fluorescence polarization assay (FPA), recently adapted to the serology of porcine and bovine brucellosis (16). The NRLB is familiar with the problem, which does not affect the native Polish pig population, but concerns animals imported into Poland or transferred between other countries. In an earlier paper we recommended a serology-based method for distinguishing *Brucella*-infected pigs from those in which FPSRs are observed (12). The investigators should take into account such parameters as the percentages of positive results and titers of antibodies in each test, the absorbance values of positive samples in ELISA, the presence of antibodies that are not inactivated by 2-mercaptoethanol, and the permanent character of anti-*Brucella* antibodies (long-lasting serological response).

The results obtained in our studies were typical of FPSRs, meeting the above characteristics. The batch of samples presented in table 3 was the most difficult to interpret. Exceptionally, the results of the first examination appeared to be typical of brucellosis, but the results of the second examination, compared with the earlier results, were decisive.

However, the interpretation of the results as FPSRs does not solve the problem. According to the current EU legislation concerning brucellosis, pigs destined for international trade, as well as boars taken to approved semen collection centers, should be negative in serological examinations (1, 2). These regulations do not differentiate between specific positive results of serological investigations, evoked by *B. suis* infection, and false positive results due to *Y. enterocolitica* O:9. Thus, in practice, pigs are often slaughtered even when the results are interpreted by experts as false positives because regulations do not indicate what to do in such cases. Such results may even cause international repercussions. For instance, because of positive serological reactions to brucellosis, presenting features typical of FPSRs, that were observed in pigs imported into Russia from the EU in December 2011, Russian Federation decided to kill and destroy 1600 animals, and to impose restrictions on the import of pigs from two countries (http://www.fsvps.ru/fsvps/print/news/

4572.html). Given the negative economic impact of that decision, the Health and Consumers Directorate General of the European Commission intervened in the matter.

FPSRs to brucellosis are a growing problem in international trade, leading to extra costs related to the performance of additional tests, the extension of the quarantine, as well as the slaughter and destruction of animals. The experts are familiar with the problem in their own countries, but there is no procedure for dealing with FPSRs on the EU level. In our opinion, pigs originating from herds with FPSRs to brucellosis should generally be avoided in international trade. But this is not always possible, and pigs may be exported from herds where the problem has not been well recognized. If FPSRs are found, two different options should be considered in order to avoid slaughtering the animals. One is to ignore FPSRs and introduce the pigs into breeding herds. This solution, involving the presence of *Y. enterocolitica* O:9 infection in the herds, may produce long-lasting diagnostic, administrative and epidemiological problems in future. The other option is to exclude the affected animals from breeding and allocate them for fattening. Clear guidelines for dealing with such cases need to be created.

References

1. Council Directive 64/432/EEC of 26 June 1964 on animal health problems affecting intra-Community trade in bovine animals and swine (OJ No. 121, 29.7.1964, p. 1977/64).
2. Council Directive 90/429/EEC of 26 June 1990 laying down the animal health requirements applicable to intra-Community trade in and imports of semen of domestic animals of the porcine species (OJ L 224, 18.8.1990, p. 62-73).
3. *Gyuranecz M., Erdélyi K., Makrai L., Fodor L., Szépe B., Ráczné Mészáros A., Dán A., Dencső L., Fassang E., Szeredi L.*: Brucellosis of the European Brown Hare (Lepus europaeus). J. Comp. Path. 2011, 145, 1-5.
4. Instruction no 26/2003 of the Chief Veterinary Officer, Warsaw 2003.
5. Instruction no 27/2003 of the Chief Veterinary Officer, Warsaw 2003.
6. Instruction no 28/2003 of the Chief Veterinary Officer, Warsaw 2003.
7. *Jungersen G., Sörensen V., Giese S. B., Stack J. A., Riber U.*: Differentiation between serological responses to Brucella suis and Yersinia enterocolitica serotype O:9 after natural or experimental infection in pigs. Epidemiol. Infect. 2006, 134, 347-357.
8. *Królak M., Stryszak M.*: Standard technique of 2-mercaptoethanol test in diagnosis of animal brucellosis. Nat. Vet. Res. Inst. Pulawy, Poland 1979.
9. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, OIE. Paris, France 2009.
10. Porcine brucellosis (Brucella suis). Scientific opinion of the Panel on Animal Health and Welfare. EFSA Journal 1144, 1-112.
11. *Singh D. K., Warayan K. G.*: Serological study of swine yersiniosis in a farm. Indian J. Anim. Sci. 1991, 5, 506-508.
12. *Szulowski K., Iwaniak W., Pilaszek J.*: Porcine brucellosis in Poland: problems accompanying serological surveys conducted in 1995-2000. Bull. Vet. Inst. Pulawy 2001, 45, 153-161.
13. *Szulowski K., Iwaniak W., Pilaszek J., Truszczyński M., Chrobocińska M.:* The ELISA for the examination of hare sera for anti-Brucella antibodies. Comp. Immun. Microbiol. Infect. Dis. 1999, 22, 33-40.
14. *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, 45-52.
15. *Weiner M., Szulowski K., Iwaniak W.*: Porcine brucellosis – evidence of the role of Yersinia enterocolitica O:9 in occurrence of false positive serological reactions. Pol. J. Vet. Sci. in press.
16. *Weiner M., Szulowski K., Iwaniak W.*: The value of Fluorescence Polarisation Assay in verification of problematic sera from cattle and pigs for brucellosis. Pol. J. Vet. Sci. 2012, 15, 801-802.
17. *Weiner M., Złotnicka 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, 603-607.
18. *Wiśniowski J., Królak M., Drożdżyńska M.*: Standard technique of anti-globulin test in diagnosis of bovine brucellosis. Nat. Vet. Res. Inst. Pulawy, Poland 1978.
19. *Wrathall A. E., Broughton E. S., Gill K. P., Goldsmith G. P.*: Serological reactions to Brucella species in British pigs. Vet. Rec. 1993, 132, 449-454.

Corresponding author: dr hab. Krzysztof Szulowski prof. nadzw., Al. Partyzantów 57, 24-100 Puławy; e-mail: kszjanow@piwet.pulawy.pl