Sensitivity of Actual Laboratory Diagnostic Methods Used for Surveillance of Swine brucellosis in Croatia

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ABSTRACT

During three years (2009-2011) 240 organ and tissue samples from swine serologically positive on brucellosis were bacteriologically tested for brucellosis. The samples were obtained from country farms from seven counties and two wild boars organs were obtained from hunting ground. Brucella was isolated from 52 (21.5%) samples. Infection with Brucella suis bv. 2 was confirmed in 12 (29.3%) swine herds, and including the positive tests of wild boars, infection was confirmed in 5 counties. According to consecutive negative bacteriological testing in flocks and epidemiological investigation we also confirmed false positive serological reactions (FPSRs) in 29 seropositive herds (70.7%). Bacteriological investigation in future should be improved by using new selective media, but more imporant, better sampling management and prompt sample delivery should be achieved to provide higher method sensitivity. Detection of brucellosis in wild boars over years confirms permanent source of disease for free range swine in all parts of Croatia.

INTRODUCTION

Swine brucellosis is caused by *B. suis* biovars (bv.) 1, 2 and 3, though swine can, but rarely, be infected with *B. melitensis* and *B. abortus*. *B. suis* can also cause infection in cattle (Cook and Noble, 1984, Garin-Bastuji and Delcueillerie, 2001, Forbes and Tessaro, 2003), dogs (Barr et al., 1986), horses (Cvetnić et al., 2005) and humans (Hall, 1990). Besides in domestic animals, the disease was found in wild boars all around the world (OIE 2011). The disease is present in Croatia and it was detected in domestic and wild pigs in almost all counties in which

swine breeding production is developed (Cvetnić et al., 2003, 2004, 2009, Špičić et al., 2010). There are five biovars of *Brucella suis* species and the brucellosis in swine is caused by bv. 1, 2 and 3. Geografically, *B. suis* bv. 1 and 3 are spread all around world. *B. suis* bv. 1 is the leading cause of swine brucellosis in South America, Asia and Oceania, and *B. suis* bv. 3 in Southeast Asia and China (Cvetnić, 2002, Cvetnić et al., 2004, OIE, 2011). B. suis bv. 2 was found only in Europe, from Scandinavia to Balkan (Alton, 1990). In Croatia the infection with *B. suis* bv. 2 and bv. 3 was detected in domestic and wild pigs (Cvetnić et al., 2003, 2004, 2009, Špičić et al., 2010) and infection with *B. suis* bv. 3 was detected in horses (Cvetnić et al., 2005). Ideal serological test or combination several tests regarding apsolute specificity and sensitivity still not found (Paulo et al., 2000, OIE, 2011, McGiven et al., 2012, Muñoz et al., 2012, Praud et al., 2012). Also is not solve problem of satisfactory sensitivity for bacteriological testing of material. Recent attempts is based on introduction new media (Ferreira et al., 2011, de Miguel et al., 2011) or improving sheme of matherial preparing by mechanical homogenisation followed by host cell lysis prior to cultivation (Abril et al., 2011).

In this study we show the results of pig samples bacteriological testing for brucellosis in seropositive animals. Also, we show the correlation between positive serological and positive bacteriological tests. This study is also a review of current serological and bacteriological methods and their characteristics in context of various epizootiological situations in small (half-intensive) swine breeding herds in Croatia. We identified the spread and the importance of *B. suis* bv. 2 infecton in pig farming in Croatia in the period between 2009 and 2011 and suggest to importance of wild boars as reservoirs.

MATERIALS AND METHODS

Annual control of swine brucellosis in investigated period is carried out over serological testing of boars, once a year, before they are used for natural breeding and production of semen for artificial insemination. In addition, all cases of abortion in swine must be reported and samples of blood and aborted fetuses taken to be tested for brucellosis. All new pigs that will be used for breeding must be tested for brucellosis before entering the herd. The diagnosis of brucellosis was done by serological test Rose Bengal test (RBT), and by complement fixation test (CFT) and enzyme-linked immunosorbent assay (ELISA), serological tests which were used as confirmatory tests. The pigs that beside RBT were positive on at least one more serological test were removed from the herd and slaughtered and samples were taken for bacteriological testing for brucellosis.

Bacteriological testing

The material for bacteriological testing was taken during the slaughter of pigs that were positive on at least two serological methods. For the bacteriological testing, reproductive organs (testes, uterus), lymph nodes (supramamar, inguinal, mandibular, mesenterial), liver and spleen were used. During three years (2009-2011) 240 pig samples were bacteriologically tested for brucellosis in the Laboratory for bacterial zoonoses and molecular diagnosis of bacterial diseases in Croatian Veterinary Institute, Zagreb. Pig samples were obtained from country farms from seven counties and two wild boars' organs were obtained from Šumarija Đakovo hunting ground (Osijek-Baranja County) (Table 1).

A few grams of tissue (testes, uterus or lymph nodes) was processed and 1-2 mL of homogenate was inoculated on selective medium for *Brucella* (Brucella medium base, Oxoid CM0169, Oxoid Ltd, Basingstoke, Great Britain) with addition of 25 mL of equine serum (Oxoid SR0035C), 12 500 IJ bacitracin (Merck Calbiochen, cat. no. 1951) and 3000 IJ polymyxin B sulphate (Merck Calbiochem, cat.no. 5291) on 0,5 L agar, and on Farrell's selective medium (Alton 1988). Inoculated media were incubated at 37°C some with and some without 10% CO_2. The growth of the colonies was checked daily during next eight days. Colo-

nies were identified based on their morfology (round, small, translucent, convex, with smooth margins) and their ability to grow in presence of CO2, production of H2S, growth on the medium with addition of 20 µg/mL of thionin and fuchsin (Alton, 1988). Final identification of the species and the biovar was done by molecular methods (García Yoldi et al., 2006, INgene Bruce-ladder Suis kit, Ingenasa, Spain).

Molecular identification

Genomic DNA isolation

Twenty five Brucella isolates and three Brucella standard cultures were resolved in 100 µl of distilled water (UltraPure™ DNase/ RNase-Free Distilled Water, Invitrogen, Great Britain), incubated at 95ºC for 20 minutes with shaking at 400 rpm (Thermomixer comfort, Eppendorf) and then centrifuged at 14 000 g for 1 minute. Supernatant was used as DNA template in PCR reactions.

Identification of *Brucella* species

For the identification of *Brucella* species Bruce-ladder PCR (García-Yoldi et al., 2006) was used. With this multiplex PCR assay, most terrestrial *Brucella* species (*B. neotomae*; *B. abortus* biovars 1, 2, 3, 4, 5, 6, 7, 9; B. *melitensis* biovars 1, 2, 3; B. *suis* biovars 1, 2, 3, 4, 5), *Brucella* strains from marine mammals (*B. pinnipedialis* and *B. ceti*) and *Brucella* vaccine strains *B. abortus* S19, *B. abortus* RB51 and *B. melitensis* Rev.1 can be identified and differentiated in a single step. PCR reaction mix with volume 20 µL consisted of 10 µL of QIAGEN Multiplex PCR Master Mix (Qiagen,Germany), 2.5 µL RNase- Free Water (Qiagen, Germany), 0.4 µM BMEI0998f and BMEI0997r primers (Invitrogen, Great Britain), 0.1 µM of each of other primers (García-Yoldi et al., 2006), and 2 µL DNA. Amplification was done in Veriti Thermal Cycler (Applied Biosystems, USA). After initial denaturation (95°C/15 min), PCR program consisted of 35 cycles of denaturation (95°C/30 sec), annealing (64°C/45 sec) and elongation (72°C/3 min), with final elongation step (72°C/10 min).

Molecular identification was used for the differentiation of the *Brucella suis* biovars (bv.) 1-5 (INgene Bruce-ladder Suis kit, Ingenasa, Spain). Assay was carried out with 25 µL Reagent A, 25 µL Reagent B and 1 µL DNA. Amplification was done in Veriti Thermal Cycler (Applied Biosystems, USA), with initial denaturation at 95°C for 7 minutes, followed by 30 cycles of denaturation (95°C/35 sec), annealing (63°C/45 sec) and elongation (72°C/1 min), with final elongation step (72°C/6 min). Expected

Figure 1*. B. suis isolates in Croatia from 2001-2011. Counties: ZG – Zagreb; BB – Bjelovar-Bilogora; SM - Sisak-Moslavina; PS - Požega-Slavonia; BP – Brod-Posavina; OB – Osijek Baranja; VS – Vukovar-Srijem*

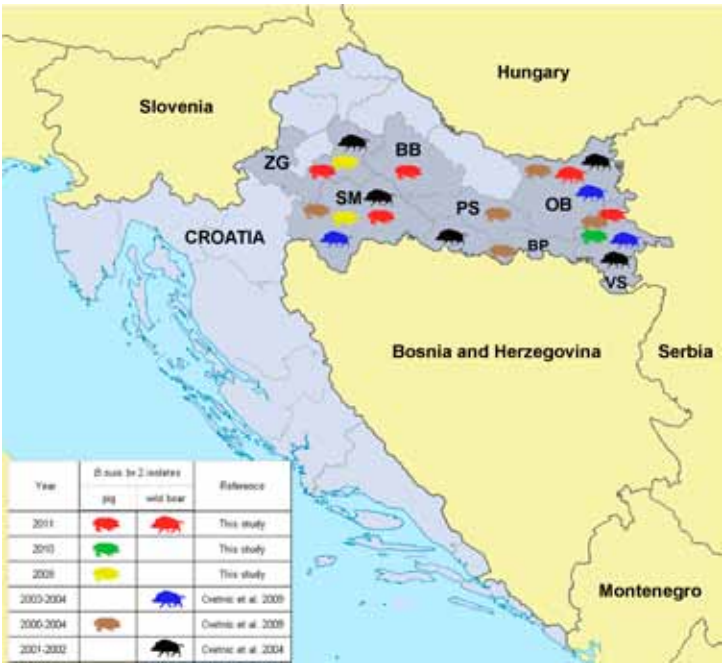

Table 1 *The results of serological and bacteriological swine brucellosis testing in period from 2009 to 2011*

County	Serologically positive herds	Bacteriological testing						Sensitivity of bacteriological tests: samples/isolates (%)/ positive herds	Year
		sows		boars		piglets			
		total	positive/ (%)	total	positive/ (%)	total	positive/ (%)		
Bjelovar-Bilogora	4	15	0	0	0	0	0	15/0 (0)/0	2009
	1	6	0	0	0	0	0	6/0 (0)/0	2010
	1	12	11/(91,6)	0	0	0	0	12/11 (91,6)/1	2011
Brod-Posavina	2	12	0	0	0	1	0	13/0 (0)/0	2009
Osijek-Baranja	12	20	0	1	0	0	0	21/0 (0)/0	2010
	1	0	0	2*	1*/(50)	0	0	2/1 (50)/1*	2011
Sisak-Moslavina	2	20	15/(75)	2	1/(50)	0	0	22/16 (72,7)/2	2009
	1	37	1/(2,7)	0	0	0	0	37/1 (2,7)/1	2011
Virovitica-Podravina	1	0	0	1	0	0	0	1/0 (0)/0	2009
Vukovar-Srijem	7	16	0	0	0	0	0	16/0 (0)/0	2009
	4	36	9/(25)	0	0	0	0	36/9 (25)/4	2010
	2	5	0	2	1/(50)	0	0	7/1 (14,3)/2	2011
Zagreb	2	17	1/(5,9)	4	1/(25)	3	0	24/2 (8,3)/1	2009
	1	26	7/(26,9)	1	1/(100)	3	3/(100)	30/11 (36,6)/1	2011
Total	**41**	**222**	**44/(19,8)**	**13**	**5/(38,5)**	**7**	**3/(42,8)**	**242/52 (21,5)/12**	

*** - wild boar organs**

amplicon sizes for *B. suis* bv. 1 were 197 and 425 bp; for bv. 2 278 and 548 bp; for bv. 3 197 and 302 bp; for bv. 4 197 and 611 bp; and for bv. 5 197, 278, and 611 bp. PCR products from all molecular tests were analyzed using the system for capilary electrophoresis QIAxcel (Qiagen, USA).

RESULTS

In the period from 2009 to 2011 240 boar, sow and piglet samples and 2 wild boar testes samples were tested. In 2009 swine brucellosis was confirmed in 2 herds in Sisak-Moslavina County and in one herd in Zagreb County. *Brucella suis* was isolated from 18 (19.8%) of 91 samples in 2009. During 2010 infection was confirmed in 4 herds in Vukovar-Srijem County with 9 (14.3%) isolates from 63 samples. In the last year of the study, 86 swine organ samples and 2 wild boar organ samples from Šumarija Đakovo hunting ground were tested. Brucella was isolated from 25 (28.4%) samples. Infection with *Brucella suis* was confirmed in 4 pig breeding herds, and including the positive tests of wild boars, infection was confirmed in 5 counties. Efficiency of isolation was 19.8% for sow, 38.5% for boar and 42.8% for piglet samples (Table 1, Figure 1). By molecular tests all 52 isolates were identified as *Brucella suis* bv. 2.

DISCUSSION

There are five biovars belonging to *Brucella suis* species, and bv. 1, 2 and 3 cause brucellosis in swine. Geografically, B. suis bv. 1 and bv. 3 are spread all around the world. *B. suis* bv. 2 was found only in Europe, from Scandinavia to Balkan (Alton, 1990, OIE, 2011). In Croatia the infection with *B. suis* bv. 2 and bv. 3 was detected in domestic and wild pigs (Cvetnić et al., 2004, 2009, Špičić et al., 2010), and infection with *B. suis* bv. 3 in horses (Cvetnić et al., 2005). In 2011 only *B. suis* bv. 2 was isolated from domestic and wild pig samples. Cvetnić et al. (2004, 2009) described identification of infection in swine breeding herds in Sisak-Moslavina, Osijek-Baranja, Zagreb and Vukovar-Srijem County. During 2011 we confirmed the infection in Bjelovar-Bilogora County. All cases of infection occured in herds with half-extensive swine keeping where domestic animals can come in contact with wild pigs. Although it is not according to law, it is common that one boar is used for natural breeding in more than one farm and that is the way how the infection can be spread.

The confirmation of infection in one wild boar's testes which were submitted to the Laboratory most probably because of orchitis, indicates brucellosis persistence, but also emphasises the need for extensive study on determining the prevalence of brucellosis in wild pigs in Croatia. Wild boars are present in almost all parts of Croatia with confirmed brucelosis (Cvetnić et al., 2004, 2009.). Risco et al. (2013) suggest that the size of wild boar population have great influence in probability of infection with *B. suis* in south-western Spain. Wild boars are the source of brucellosis for domestic pigs in Europe (Godfroid and Kasbohrer, 2002, Leuenberger et al., 2007) and in Croatia and in semi extensive management it is almost impossible to stop spreding of disease.

Bacteriological methods for *B. suis* bv. 1 and bv. 3 identification are as sensitive as serological methods (Ferris et al., 1995), while *B. suis* bv. 2 is very sensitive and isolation of B. suis bv. 2 from the samples is sometimes very difficult (OIE, 2011).

The efficiency of our bacteriological tests was 21.5% on average. The highest sensitivity was 28.4% in 2011, and the lowest was 14.3% in 2010. Similar sensitivity for this method (21%) reported Ferris et al. (1995). They tested 221 samples from 39 naturally infected herds. In our study, the highest sensitivity of bacteriological testing was shown for the boar isolates (testes, mostly epididymis) 38.5%, while for sow isolates sensitivity was 19.8%. Sensitivity of bacteriological testing of piglets isolates 42.8% must be considered with caution because only 7 samples were tested. The possible cause of difference in sensitivity can be inadequate collecting of samples on farms, longlasting and inappropriate transport of material to laboratory (more than 3 days) or

inhibitory effects of Farrell's medium on *B. suis* bv. 2 growth (de Miguel et al., 2011). Furthermore, the *Yersinia enterocolitica* O:9 LPS is able to induce serological cross-reactions indistinguishable from brucellosis (Al Dahouk et al., 2005).

Using two different selective media, we didn't detect significant difference in sensitivity. In order to increase sensitivity of bacteriological testing Ferreira et al. (2011) and de Miguel et al. (2011) suggest using new culture media. In addition, it is not known which serological method is the most sensitive and specific (Paulo et al., 2000, OIE, 2011, Muñoz et al., 2012, Praud et al., 2012). Thereby the strategy of treating the disease in swine is still not defined. Recent investigation of McGiven et al. (2012) shows that the protein based iELISAs demonstrated sufficient diagnostic sensitivity to resolve the false positive serological reactions (FPSRs) and suggests that use of rough lipopolysaccharide iELISA showed no cross reaction with the FPSRs. Unfortunately this test is not commercially available. The sensitivity of bacteriological tests is still the object of researches (de Miguel et al., 2011, Ferreira et al., 2011) and bacteria can be isolated even from serologically negative swine from infected herds (Ferris et al., 1995). In this study, in 2011 *B. suis* was isolated from samples from piglets less than 3 months old. We examined the samples from 41 herds, and *B. suis* was isolated from 12 (29.3%) herds. Regarding the results of epidemiological investigation in herds before and after negative bacteriological investigation, we can conclude that serological findings belong to FPSRs. In this study we found FPSRs in 29 seropositive herds (70.7%). High number of FPSRs indicates the presence of problems in the routine swine brucellosis diagnostics which is based on serological testing.

Regarding the obtained results of the study on spread of *B. suis* in Croatia, it is necessary to preserve this level of controlling brucellosis in swine. Farmers should be educated how to prevent infection with *B. suis*, mainly by preventing mating with wild boars, using controled boars and artificial insemination. In addition, veterinarians should be educated about sampling and delivery of material for laboratory testing. This would increase sensitivity of bacterial tests and therefore FPSRs could be more accurately distinguished from swine brucellosis.

AUTHORS' CONTRIBUTIONS

SS made concept and design the investigation, write the article; MZ-T and AV carried out the immunoassays and participate in presentation of results and discussion; IR and SD carried out the molecular studies and drafted the manuscript. ZC and MZT performed the bacteriological investigation and statistical analysis. All authors read and approved the final manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare to have no conflict of interest regarding this study.

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