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Brucellosis in Turopolje pig breeding in the 2008-2011 period: an overview of laboratory diagnostics and eradication system

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ABSTRACT

In the period 2008-2011, a program of diagnostics and control of swine brucellosis was conducted in the remaining two farms of the Turopolje pig breed, including a total of 729 samples of porcine blood. Serological diagnostics were carried out by means of the Rose Bengal test (RBT), complement fixation test (CFT) and enzyme-linked immunosorbent assay (ELISA). Agreement between serological methods was determined by the Kappa test. Almost perfect agreement was found between the results obtained by the CFT and RBT methods (0.764-0.936), while the agreement between the results obtained by RBT and CFT in comparison with the ELISA test results was substantial (0.547-0.767; 0.647-0.843). In addition, in 2009 and 2011, samples from 50 pigs were subjected to bacteriological tests, and a total of 15 isolates were obtained. Bacteriology was conducted with various time delays from the determination of positive serological reactions, and the method efficacy was determined considering the duration of the infection in pigs. The average efficacy of the isolation of *Brucella* (*B.*) *suis* ranged between 10-43.3 % (P = 0.01) and was significantly higher in acute brucellosis. Isolation was successful for all age categories, regardless of their sexual maturity. Molecular identification confirmed *B. suis* bv. 2 for the first time in this breed. The extensive breeding of Turopolje pigs and daily contact with live reservoirs of the disease agents are the reasons why the disease cannot be completely eradicated according to the test-and-remove principle. A condition for the successful control and eradication of brucellosis in a Turopolje pig herd is to ensure the conditions for individual and permanent supervision of each breeding pig.

Key words: swine brucellosis, *Brucella suis*, Turopolje pig, Croatia

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Introduction

Brucellosis is a zoonotic infectious disease of global importance. Swine brucellosis is caused by *B. suis* bv. 1, 2 and 3, although rarely, pigs are also susceptible to infection with *B. melitensis* and *B. abortus*. *B. suis* may also infect cows (COOK and NOBLE, 1984; GARIN-BASTUJI and DELCUEILLERIE, 2001; FORBES and TESSARO, 2003; GARIN-BASTUJI et al., 2006; SZULOWSKI et al., 2013), dogs (BARR et al., 1986), horses (CVETNIC et al., 2005) and humans (TEYSSOU et al., 1989; HALL, 1990; PATON et al., 2001). In addition to domestic swine, the disease affects wild boar worldwide (OIE, 2011). In Croatia, swine brucellosis has been detected in domestic pigs and wild boars in almost all counties with developed pig farming (CVETNIĆ et al., 2003; CVETNIĆ et al., 2004; CVETNIĆ et al., 2009; ŠPIČIĆ et al. 2010; ŠPIČIĆ et al., 2013). Of the five biovars of *Brucella suis*, bv. 1, 2 and 3 are responsible for the disease. *B. suis* bv. 1 and bv. 3 are widespread all around the world. *B. suis* bv. 1 predominantly causes brucellosis in pigs in South America, Asia and the Pacific, while *B. suis* bv. 3 is more prevalent in Southeast Asia and China (CVETNIĆ, 2002; OIE, 2011). *B. suis* bv. 2 has been found only in Europe, from Scandinavia to the Balkans (ALTON, 1990). In Croatia, infections with *B. suis* have been detected in domestic pigs and wild boars (bv. 2 and bv. 3) and in horses (bv. 3), (CVETNIĆ et al., 2003; 2004; 2005; 2009).

The Turopolje pig is an autochthonous Croatian primitive swine breed, dating back to the early Middle Ages and originating from the region of Turopolje in the Sava river basin, between the cities of Zagreb and Sisak (Fig.1). Its greatest commercial significance was in late 19th and early 20th century, when it was largely exported to the countries of the former Austrian-Hungarian Monarchy. After World War 2, the breed lost its commercial significance and currently there are only 2 major extensive breeding farms which protect the breed from extinction. These pigs have always been bred outdoors, in the oak woods of the Turopolje region. Their basic nutrition consists of pastures and acorn; in addition corn is given to the extent required to preserve contact with humans. In the 2008-2011 period, we performed serological, bacteriological and molecular tests of samples taken from Turopolje pigs from two major breeding farms in the region of Turopolje, and determined the prevalence of brucellosis in this breed. Due to the importance of the breed, eradication of the disease, based on depopulation of a positive herd, was impossible; instead, the test-and-remove principle was applied. The agent of swine brucellosis in this Croatian pig breed was detected for the first time. With a review of the test-and-remove approach for disease eradication, we also discuss possible sources and forms of infection transmission under outdoor pig breeding conditions.

Fig. 1. Brucellosis-infected Turopolje pigs in the slaughterhouse depot and the geographical position of the Turopolje region

Materials and methods

Serology. Serological diagnostics of brucellosis was based on the Rose Bengal test (RBT), the complement fixation test (CFT) and enzyme-linked immunosorbent assay (ELISA). During a 4-year period (2008-2011), we investigated a total of 729 blood samples of Turopolje pigs reared for breeding, which were the property of the Noble Municipality of Turopolje. The pigs originated from the two largest extensive breeding farms in the region of Turopolje, namely *Turopoljski lug* (TU) and *Lukavečki gater* (LG) (Table 1). Since 2012, Turopolje pigs reared for breeding have no longer been subject to compulsory controls, except when transferred to new farms. Therefore, no brucellosis data are available for these farms. All porcine blood samples were subjected to RBT (InstitutPourquier, Montpelier, France), ELISA (Ingesim Brucella Porcina, Ingenasa, Madrid, Spain) and CFT (Institut Pourquier, Montpelier, France). The tests were conducted in compliance with the OIE manual (2011). A result of ≥20 IU complement fixation antibodies by CFT was regarded as positive, and the valorisation of ELISA results was performed in compliance with the manufacturer's recommendations (Ingenasa, Madrid, Spain).

Bacteriology. The samples for bacteriological investigation were taken upon the slaughter of pigs which had been found positive in at least one serological test. Due to the traditional outdoor rearing method, most pigs could not be transported to the slaughterhouse, some of them died, and some were isolated from the farms without being sampled for bacteriology (veterinary inspectorate data). Samples taken from pigs included reproductive organs (testis, uterus), lymph nodes (supramammary, inguinal, mandibular,

mesenteric), liver and spleen. From one boar, only the testes were collected. In 2009, a total of 20 pigs with positive serological reactions were subjected to bacteriological investigation. As it was difficult to conduct bacteriology immediately, the samples were submitted for bacteriology 10 months after the serology was performed. In 2011, the bacteriological investigation was carried out within 30 days after the serology testing. Bacteriology was conducted on samples from 30 pigs in total. Several grams of the tissue (testis, uterus or lymph nodes) were inoculated onto Brucella agar (Brucella medium base, Oxoid CM0169, Oxoid Ltd, Basingstoke, United Kingdom) with the addition of 25 mL of horse serum (Oxoid SR0035C), 12 500 IU of bacitracin (cat. no.1951, Merck Millipore, Calbiochen, Darmstadt, Germany) and 3000 IU of polymyxin B sulphate per 0.5L of agar (cat. no. 5291, Merck Millipore Calbiochem, Darmstadt, Germany), and onto selective Farrell's agar (ALTON, 1988). The inoculated agars were incubated at 37 °C, both in ambient air and in 10 % CO_2. The growth of colonies was checked on a daily basis over the following 8 days. The colonies were then identified on the basis of morphology (small, transparent, convex, smooth), growth capacity in the presence of 10 % CO_2, production of H_2S, and growth on agar with the addition of 20μg/mL thionine and fuchsin (ALTON, 1988). The final identification of type and biotype was done using molecular methods (Garcia-Yoldiet al., 2006) using INgene Bruce-ladder Suis kit, Ingenasa, Madrid, Spain.

Molecular testing. Extraction of genomic DNA. Brucella isolates (15) and strains of *Brucella* suis bv. 1, 2 and 3 from our strain collection (3) were resolved in 50 μL of PCR-grade water (Invitrogen, Paisley, Scotland), and heated for 15 minutes at 99 ºC with occasional shaking. The suspensions were then centrifuged for one minute at 14 000 g. Two μL of the supernatant were used for PCR.

Identification of Brucella. The Bruce-ladder test enables a single step identification of the following Brucella members: all terrestrial Brucella biovars (*B. neotomae, B. abortus* biovars 1,2, 3, 4, 5, 6, 7, 9, *B. melitensis* biovars 1, 2, 3 and *B. suis* biovars 1, 2, 3, 4, 5), strains from marine mammals (*B. pinnipedialis* and *B. ceti*) and vaccine strains (*B. abortus* S19, *B. abortus* RB51 and *B. melitensis* Rev.1). The test was conducted as described by GARCIA-YOLDI et al. (2006).

Molecular type determination of *B. suis* isolates: the INgene Bruce-ladder Suis kit (INgene Bruce-ladder Suis, Ingenasa, Madrid, Spain) was used for identification of *Brucella suis* biovars (bv.) 1-5. The expected sizes of the amplicons for B. suis are as follows: for bv. 1, 197 bp and 425 bp; for bv. 2, 278 bp and 548bp; for bv. 3, 197 bp and 302 bp; for bv. 4, 197 bp and 611 bp; and for bv. 5, 197 bp, 278 bp and 611 bp. PCR products were separated by electrophoresis in 2 % agarose gel, stained with ethidium bromide, and visualized by means of a UV transilluminator and BioCapt Document System camera (VilbertLourmat, Marne La Vallee, France).

Statistical analysis. The numerical data were statistically processed by Stata 13.1 software (Stata corp. USA). The incidence of the infection, determined by the three different serological methods and by isolation of the causative agent, was compared between the two farms for each observation year by means of the chi-square test. The agreement of the various tests was compared using the Kappa test. According to SOLANO-GALLEGO et al. (2014), the kappa agreement between the serological diagnostic techniques was determined as follows: no agreement ($k<0$), slight agreement ($0<k<0.2$), fair agreement ($0.2 < 0.4$), moderate agreement ($0.4 < k < 0.6$), substantial agreement ($0.6 < k< 0.8$) and almost perfect agreement ($k> 0.8$).

Results

In 2008, positive serological reactions to RBT in both farms were determined in 79 (21.6 %) pigs, by CFT in 77 (21.1 %) pigs and by ELISA in 179 (49.0 %) pigs. In the subsequent years, no positive serological reactions were detected on the LG farm. In the following year, 2009, RBT detected 4 (4.2 %) positive pigs, while CFT revealed 6 (6.3 %) and ELISA 25 (26.3 %) pigs with positive reactions. All seropositive pigs were from the same farm (TL). In 2010, not a single pig blood sample showed a seropositive reaction, and blood samples were tested from breeding pigs from both farms. In 2011, positive serological reactions were found again on the TL farm. RBT detected 26 (13.3 %) positive pigs, CFT 28 (14.3 %) and ELISA 35 (17.9 %) pigs (Table 1). The Kappa agreement of the serological tests used was most prominent when RBT and CFT were compared (Table 2). In 2009, bacteriological tests were conducted on organs from 19 sows and one boar. *Brucella* was isolated from the testes of the boar and from the inguinal lymph nodes of one sow. In 2011, the organs of 30 pigs were tested, including one boar, two barrows, 23 sows and 4 piglets aged approximately 4 months. *Brucella* was isolated from samples from the boar ($n = 1$), barrow ($n = 1$), sows ($n = 7$) and piglets ($n = 4$). Molecular tests identified all isolates ($n = 15$) as *Brucella suis* bv. 2 (Table 3).

Discussion

To date, *B. suis* has been detected in Croatia in both domestic pigs and wild boars with bv. 2 and bv. 3 (CVETNIĆ et al, 2003; 2004; 2009; ŠPIČIĆ et al. 2010) and with bv. 3 in horses (CVETNIĆ et al., 2005). While *B. suis* bv. 1 and bv. 3 are widespread all around the world, *B. suis* bv. 2 has been found only in Europe, from Scandinavia to the Balkans (ALTON, 1990; OIE, 2011). Interactions between wild boar and outdoor pigs are common and pose a risk for pathogen spill-over (CVETNIĆ, 2002; KÖPPEL et. al., 2007). The Turopolje breed is an example of an outdoor pig, with an extensive breeding phase in the forests, and an intensive indoor fattening phase. Serological methods are standardly used for surveillance studies of brucellosis in domestic animals (OIE, 2011). Regarding our serological investigation, in general, ELISA seems to be the most sensitive

Table 1. Serological tests of swine blood samples in the 2008-2011 period

Year	2008				2009				2010				2011			
Serological method	No. of tested blood samples		No. of positive blood samples		No. of tested blood samples		No. of positive blood samples		No. of tested blood samples		No. of positive blood samples		No. of tested blood samples		No. of positive blood samples	
Farm	TU	LG	TU	LG	TU	LG	TU	LG	TU	LG	TU	LG	TU	LG		
Rose bengal test (RBT)	173	192	36	43	75	20	4	0	39	34	0	0	132	64	26	0
Complement fixation test (CFT)	173	192	32	45	75	20	6	0	39	34	0	0	132	64	28	0
Enzyme-linked immunosorbent assay (ELISA)	173	192	74	105	75	20	25	0	39	34	0	0	132	64	35	0

TU - Turopoljski lug; LG - Lukavečki gater

Table 2. Agreement between serological methods in 2011

	Kappa	SE	95 % Confidence Interval
RVK:RBT	0.85	0.0438	0.764-0.936
RBT:ELISA	0.657	0.0562	0.547-0.767
RVK:ELISA	0.745	0.05	0.647-0.843

SE - standard error of Kappa

Table 3. Bacteriological tests of swine organs and tissues

Year of examination	Sample type	No. of samples	No. of isolates*	Organ/tissue from which *Brucella suis* was isolated	Successful isolation of *B. suis* from samples	P (Fisher's exact)
2009	Reproductive organs and lymph nodes	20	2	Inguinal lymph node (1); testes (1)	10.0 %	0.01
2011	Reproductive organs and lymph nodes	30	13	testes** (1); uterus** (1), ln. mix (13)	43.3 %	
	Total	50	15		30 %	

*- one isolate = one animal; **- *B. suis* was isolated from lymph nodes and testes/uterus in the same animal

test (49.0 %, 26.3 % and 17.9 %). Significantly lower sensitivity was found for both RBT and CFT (21.6 %, 4.2 %, 13.3 % and 21.1 %, 6.3 %, 14.3 %, respectively). Almost perfect agreement was found between the results obtained by CFT and RBT. Substantial agreement was found between the results obtained by RBT and CFT in comparison with the results obtained by the ELISA test.

Similar investigation results in wild boar were obtained in Belgium by GRÉGOIRE et al. (2012) and the seroprevalence determined by iELISA was 54.9 %; in Germany, it was 22.0 % (AL DAHOUK et al., 2005), in Switzerland 35.8 % (WU et al., 2011) and in Spain 25 % to 46 % in different regions studied and comparing different serological methods (MUÑOZ et al., 2010). ELISA proved to be more sensitive for serodiagnostics of porcine brucellosis than CFT (94 % versus 58-93 %) (NIELSEN et al., 1999). As for *B. suis* bv. 1 and 3, the bacteriological test is almost equally as sensitive as serological methods (FERRIS et al., 1995) but in the case of *B. suis* bv. 2, its cultivation from samples is sometimes very difficult (OIE, 2011). We determined a correlation between successful pathogen isolation from serology-positive samples and the delay of the bacteriology test on the serology-positive samples. The observed differences in the efficacy of isolation of the causative agent from the tissue samples between the two years compared are statistically significant, which is attributed to the time that elapsed between the serological test and the sampling of the tissue for the bacteriological test.

Porcine brucellosis is a venereal disease, also transmitted by contact with foetal membranes, post-parturient discharges and milk. The pathogen is also able to survive in soil and water for several weeks. The ingestion of contaminated food and water, as well as inhalation of brucellas may be other possible routes of transmission in animal husbandry (GODFROID et al., 2005). The successful isolation of *B. suis* from three piglets, aged about three to four months, with a limited antibody response in our case confirms that this age category can also be susceptible to brucellosis. As brucellosis has been detected in Croatia in wild boar (CVETNIC et al., 2003; 2004), including locations currently populated with Turopolje pigs, we presume that direct contact and cohabitation with wild boar were the source of infection. The results of this study (high seroprevalence and bacteriological confirmation of the disease) prove that the Turopolje breed has survived regardless of the constant presence of brucellosis on the farms. As opposed to wild boar, due to semi-extensive breeding, this breed represents a higher risk for spreading the disease to domestic pigs, other animal species and humans. Eradication of brucellosis on the farm, in such extensive breeding conditions by application of test-and-remove principle, was impossible in our case. To be able to definitely control brucellosis, a nucleus of brucellosis free pigs should be formed, without the possibility of contact with reservoirs, and with continuous control of the health status of all pigs reared for breeding.

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SAŽETAK

U razdoblju od 2008. do 2011. godine proveden je program dijagnostike i kontrole bruceloze u preostala dva uzgoja turopoljskih svinja na ukupno 729 uzoraka krvi. Serološka dijagnostika provedena je rose bengal testom (RBT), reakcijom vezanja komplementa (RVK) i imunoenzimskim testom (ELISA). Međusobna podudarnost metoda određena je kappa testom. Između rezultata dobivenih primjenom RVK i RBT utvrđena je vrlo visoka podudarnost (0,764-0,936). Nešto niža, ali još uvijek visoka podudarnost utvrđena je između rezultata dobivenih RBT i RVK u usporedbi s rezultatima dobivenim ELISA testom (0,547-0,767; 0,647-0,843). Također, tijekom 2009. i 2011. godine bakteriološki je pretražen materijal podrijetlom od 50 svinja te je ukupno izdvojeno 15 izolata. S različitim vremenskim odmakom od utvrđenih pozitivnih seroloških reakcija učinjena je i bakteriološka pretraga te određena uspješnost metode s obzirom na starost infekcije u uzgoju. Prosječna uspješnost izdvajanja vrste *Brucella* (*B.*) *suis* varirala je od 10 - 43,3 % (P = 0,01), te je bila značajno veća pri akutnoj brucelozi. Izdvajanje uzročnika bilo je uspješno u svih dobnih kategorija, bez obzira na spolnu zrelost svinja. Molekularnom identifikacijom prvi je put u ove pasmine potvrđena infekcija vrstom *B. suis* bv. 2. Ekstenzivni način uzgoja turopoljske svinje i svakodnevni kontakt sa živim rezervoarima uzročnika određuju da se bolest ne može u potpunosti iskorijeniti prema principu pretraži i ukloni. Preduvjet uspješne kontrole i eradikacije bruceloze u stadu turopoljskih svinja jest osiguravanje uvjeta individualnog i trajnog nadzora nad svakom rasplodnom svinjom.

Ključne riječi: bruceloza svinja, *Brucella suis*, turopoljska svinja, Hrvatska