Brucellosis in wild boar (*Sus scrofa*) in the Republic of Croatia

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ABSTRACT: During the years 2001 and 2002 on seven localities in Croatia a survey on the prevalence of brucellosis in wild boar was carried out. The survey included 271 (52.7%) female and 243 (47.3%) male animals between 7 months and 4 years of age and weighing from 14 to 135 kg. On that occasion 514 blood samples of wild boar were serologically analysed. For serological analysis indirect enzyme immunoassay (iELISA), Rose Bengal test (RBT), complement fixation test (CFT) and slow agglutination test (SAT) were used. In all of the wild boar from all of the localities investigated positive reactions to brucellosis were established. Most of the positive reactions were established by iELISA (13.6%), then by RBT (11.5%), CFT (10.5%) and SAT (8.9%). Tissue samples of 106 animals: testes samples from 67 animals, uterus tissue from 38 animals and 5 fetuses of piglets from 1 mother were analysed bacteriologically. *Brucella suis* biovar 2 was isolated from 18 (17.0%) animals that originated from all of the localities investigated. Isolates were identified by PCR using BRU-UP and BRU-LOW primers specific for genus *Brucella* and primers specific for IS*711*. Based on our results it could be concluded that in Croatia wild boar are natural vector and/or reservoirs of *B. suis* biovar 2. This permanent risk factor is hazardious for domestic and wild animals in the Republic of Croatia.

Keywords: zoonoses; risk assessment; pig industry; wild boar; *Brucella suis* biovar 2

Brucellosis in pigs is a chronic disease manifesting most often by infertility and abortion in sows and by orchitis in boars. The causative agent is *Brucella suis* biovar 1, 2 and 3. Brucellosis in pig was first described by Hutyra in 1909 in Hungary and by Traum in 1914 in the U.S.A. (as quoted by Alton, 1990; Cvetnic, 2002). Brucellosis is a venereal disease and the most important routes of spreading are genital and digestive systems. Infected pigs excrete brucellas in urine, sperm, vaginal discharge, milk, and also by placenta, lochial secretion, aborted fetuses and the content of subcutaneous brucellous abscesses (MacMillan, 1999; Cvetnic, 2002). Brucellosis occurs in most countries in the world in which pigs, domestic as well as wild boar (*Sus scrofa*), reside (Drew *et al*., 1992; Van Der Leek *et al*., 1993; Kautzsch *et al*., 1995). Geographycally, brucellosis in pigs caused by *B. suis* biovar 1 prevails in Latin America, Asia, Oceania, and *B. suis* biovar 3 in the U.S.A. and China. *B. suis* biovar 2 was demonstrated in wild boar in the countries of Central Europe. Kormendy and Nagy (1982) reported on the findings of *B. suis* biovar 2 in Hungary, Hubalek *et al*. (2002) in Czech Republic, Brglez and Batis (1981) in Slovenia. Godfroid *et al.* (1994) reported on the findings of *B. suis* biovar 2 in wild boar in Belgium, where it was isolated from 13 (9.2%) out of 141 analysed samples of wild boar. In France Garin-Bastuji *et al*. (2000) also isolated *B. suis* biovar 2 in about 10% of the analysed materials (spleen) of wild boar. In Central and Western Europe the most often causal agent of brucellosis in pigs is *B. suis* biovar 2 whose natural carriers are hares (*Lepus capensis*) (Brglez and Batis, 1981; Szulowski

et al., 1999) and wild boar (Quinn *et al*., 1994; Garin-Bastuji *et al*., 2000; Hubalek *et al*., 2002). Brucellosis was diagnosed in different countries and continents by serological examination too. Garin-Bastuji *et al*. (2000) reported that in different regions of France positive serological reactions to brucellosis were found in wild boar in the range from 20% to 35%. Hubalek *et al*. (2002) reported that in the Czech Republic the frequency of positive reactions to brucellosis in wild boar was 15%. Dedek *et al*. (1986) reported serologically positive findings in 7.9% of wild boar in Germany. Becker *et al*. (1978) found positive reactions to brucellosis in 50 (52.6%) out of 95 analysed blood samples of wild boar in Florida. Van der Leek *et al*. (1993) described the findings of serologically positive reactions in wild boar in 6 out of 18 localities investigated. On particular locations in Florida positive reactions ranged between 5.5% and 33.3% of wild boar. Drew *et al*. (1992) reported on the findings of positive reactions in 23 (3.8%) out of 611 analysed blood samples of wild boar in California.

The aim of our investigation was to establish the prevalence of brucellosis in wild boar on different localities in the Republic of Croatia using serological, bacteriological and molecular methods of diagnostics.

MATERIAL AND METHODS

Serology examinations

Serum samples. During 2001 and 2002 on seven different localities (Velika Gorica, Sisak, Novska, Nova Gradiska, Djakovo, Vinkovci and Baranja) in the Republic of Croatia the blood samples from 514 wild boar were collected. From each animal one blood sample was taken from thoracic cavity, heart and pericardium.

Serological tests. The tests used for the analyses of blood sera included Rose Bengal test (RBT), slow agglutination test (SAT), complement fixation test (CFT) and indirect enzyme immunoassay (iELISA). The antigen for SAT was obtained from the culture of *B. abortus* strain 99 and produced in Croatian Veterinary Institute of Zagreb, for RBT a commercial antigen (VLA-Weybridge, UK) was used and for CFT an antigen (Bioveta, Ivanovice na Hane, Czech Republic).

RBT. Serum (25–30 µl) is mixed with an equal volume of antigen on a plastic plate to produce a zone approximately 2 cm in diametar. The mixture agitated gently for 4 minutes at ambiental temperature. Read for agglutination immediately after 4-minute period is completed. Any visible reaction is considered to be positive (Alton *et al*., 1988).

SAT. The suspected reactions reveled by SAT method were those in which it was established that 1 ml of sera contained 50 to 100 IJ of agglutinin, and the positive reaction was the one with the level of agglutinin higher then 100 IJ (Alton *et al*., 1988).

CFT. The findings of 20 IJ of complement fixation antibodies in 1 ml of serum was considered as positive reaction (RVK) (Alton *et al*., 1988).

iELISA. For indirect ELISA test we used the same antige as for CFT produced in Croatian Veterinary Institute in Zagreb, which was diluted 1 : 500 in a carbonate-bicarbonate buffer pH 9.6 and inoculated to microtitration plates (NUNC-polysorp, Germany). After the incubation of 24 hrs at +4°C and rinsing (PBS-T), the application of the tested sera of wild boar followed, using negative sera (from a known negative farm) and positive sera (the sera of immunized pig) as a control. After incubation and washing out, a peroxidase conjugat was added (Protein G-Sigma, Germany). After reincubation, washing out and adding the substrate (TMB-Sigma, Germany) and H_2SO_4, on the spectrophotometer at the wavelength of 450/630 nm, the absorbance was measured. The sera with the absorbance higher than the mean absorbance of the negative sera plus 2 SD (standard deviation) were considered positive.

Bacteriology examination

Tissue samples. From above mentioned 514 animals 106 animals were randomly selected, from which tissue samples were collected for bacteriological examinations: testes samples (from each male animal tissue samples from both testes were examined separately) 67 wild boars; uteri samples from 24 animals in different phases of gravidity (from one mother 5 dead fetuses at the time of collection aborted were taken for examination); non-gravid uteri sampels from 15 animals. The tissue samples were taken strictly from the sexually matured animals and from separately from each of aborted fetus. Animals originated from all investigated localities.

Bacteriological analysis. The tissue samples were homogenized in a stomacher, and then inoculated

on two plates of blood agars and two plates of Farell medium. Two nutritive media (one blood agar and one Farell medium) were incubated at 37°C, and two same media were incubated with in 5 to 10% CO_2 atmosphere. Growth and morphology of the colonies were monitored in daily intervals for seven days.

Standard strains. For the PCR identification standard strains of *B. abortus* strain 99, *B. suis* biovar 1 (strain 1330) and *B. suis* biovar 2 were used.

Isolates identification

Growth characteristics. Isolates were identified on the basis of the morphology of the colonies (S or R), their growth in CO_2, production of H_2S, growth on the media with the addition of 20 µg/ml of thionine and basic fuschin in the serum dextrose medium.

Identification by agglutination. Agglutination with monospecific sera for A and M antigens was carried out (Corbel *et al*., 1983; Alton *et al*., 1988).

PCR. Each of 18 isolates was suspended in 50 µl of redestilated water (Sigma, Germany). The suspension was heated in a thermo block at 100°C for 15 min and then centrifuged at 12 000 rpm for 2 minutes. The supernatant (5 µl and 2 µl) was collected and used for DNA amplification by PCR, as previously described by Bricker and Halling (1994) and Serpe *et al*. (1999). The PCR was carried out in two steps.

In the first step the target was a DNA fragment within the coding region of the *Brucella* genome which is responsibile for the synthesis of BCSP-31 protein. The BCSP 31 is a membrane antigen characteristic for *Brucella* genus (Mayfield, 1988). The primers we used were BRU-UP (GGG CAA GGA AGA TTT) and BRU-LOW (CGG CAA GGG TCG GTG TTT) (Qiagen Operon, Germany) that allowed the amplification of a fragment of approximately 443 bp (Serpe *et al*., 1999).

In the second step we used two specific primers for the genetic element IS*711* in the *Brucella* chromosome. The primers were originally created by Bricker and Halling (1994); the IS*711* specific primer (TGC-CGA-TCA-CTT-AAG-GGC-CTT-CAT) and the *B. suis* specific primer (GCG-CGG-TTT-TCT-GAA-GGT-TCC-GG). The reaction mixture for amplification of the DNA of IS*711* fragment for one sample consisted of 46 µ of the solution Hot Start Master Mix (Qiagen, Germany) kit in the concentration recommended by the manufacturer, 1 µl of each primer (100 µM/µl) (Invitrogen, Scotland) and 2 µl of the supernatant with DNA previously isolated from the investigated *Brucella* cultures. The samples were cycled (1 min at 94°C, 1 min at 57°C and 1 min at 72°C) 35 times in a termocycler (Corbett Research PC- 960C, Australia). The PCR products were separated by electrophoresis in 2% agarose gel, and strained by ethidium bromide. The results were documented by a video documentation system, which included a UV transluminator and camera (Bio-Capt, Vilbert Lourmat, France).

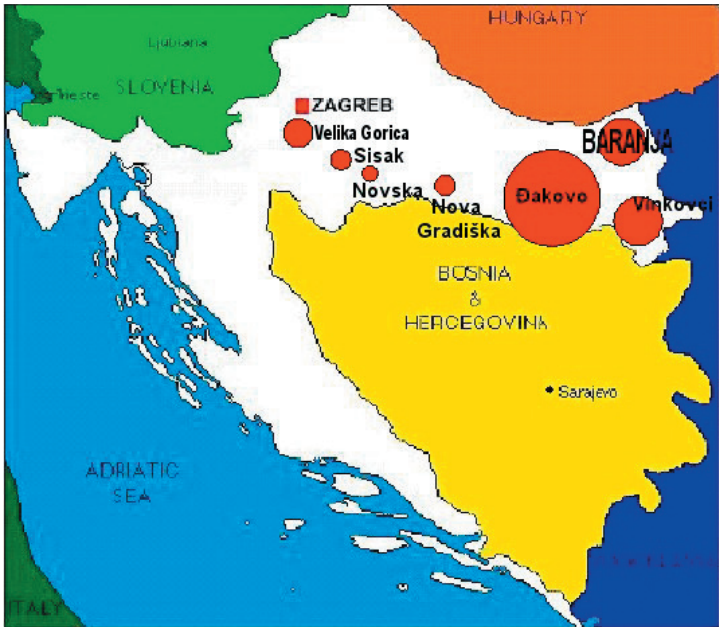

Figure 1. Presentation of the localities investigated considering the presence of brucellosis in wild boar in the Republic of Croatia

RESULTS

Animals characteristic

During 2001 and 2002 the blood sera of 514 wild boar from seven localities in Croatia were analysed: 271 (52.7%) were female and 243 (47.3%) male animals (Figure 1). The animals were of 7 months to 4 years of age and their weight ranged from 14 to 135 kg.

Serological examinations

The higher serological positivity was established by iELISA (13.6%), then by RBT (11.5%), followed by CFT (10.5%) and SAT (8.9%) examinations. Positive serological reactions were found in all localities investigated. Most sera analysed originated from the locality of Djakovo with positive reactions in 12.4% to 17% of blood sera of wild boar, depending on the method. In the locality Novska serological positivy ranged in different methods from 16.6% to 25%, in locality Sisak from 7.4% to 22.2%, in locality Velika Gorica from 8.5% to 11.9%, in locality Baranja from 6.3 % to 11.6%, in locality Nova Gradiška from 6.9% to 10.3% and in locality Vinkovci from 6.1% to 8.1% (Table 1).

Bacteriological examination

B. suis biovar 2 isolates were received from 18 samples from 18 (17.0%) from 106 animals examined. Smooth, radiant, honey-colour colonies were identified, not growing in the presence of CO_2, not producing H_2S, not growing on media with basic fuschin, but growing in the normal atmosphere at 37°C, on media with thionin, agglutinating with A monospecific antiserum, while being negative with M and R monospecific antiserum.

PCR analysis

The bacteriological identification of 18 *B. suis* biovar 2 isolates was confirmed by PCR analysis in two consecutive PCR analyses.

In the first PCR analysis a part of gene coding protein BCSP-31, a membrane antigen characteristic for *Brucellae* spp. genus, was multiplied. The expected result of multiplying (443 bp) was obtained in all examined type strains: *B. abortus* E99, *B. suis* biovar 1 (strain 1 330) and *B. suis* biovar 2 and in all investigated isolates. They were grooped according to the origin and designated as VG1 (Velika Gorica), S1 (Sisak), N1 (Novska), NG1 (Nova Gradiška), Dj1,6 (Djakovo), V1,4 (Vinkovci), B1-3 (Baranja). From

Table 1. Results of examined sera using different serological methods

Locality	Number of sera	RBT		SAT		CFT		iELISA	
		Positive	Dubiou	Positive	Dubiou	Positive	Dubiou	Positive	Dubiou
Velika Gorica	59	6	2	5	6	5	2	7	2
Sisak	27	2	1	2	6	4	0	0	0
Novska	12	3	1	2	1	2	0	2	1
Nova Gradiska	29	2	2	1	4	1	2	3	2
Djakovo	194	30	5	24	18	28	3	33	5
Vinkovci	98	7	3	6	13	6	1	8	1
Baranja	95	9	0	6	6	8	0	11	3
Total	514	59	14	46	54	54	8	70	14
%	100	11.5	2.7	8.9	10.5	10.5	1.6	13.6	2.7

iELISA = indirect enzyme-linked immunosorbent assay
RBT = Rose Bengal test
CFT = complement fixation test
SAT = slow agglutination test

Table 2. Isolation of *Brucella suis* biovar 2 from tissue samples from wild boar

Name of the locality	Number of examined animals				Total number of animals		
	Testes[1]	Uterus			Examined	Positive	%
		Non-gravid	Gravid	With fetuses[2]			
Velika Gorica	6	2	1	0	9	1	11.1
Sisak	4	2	1	1	8	1	12.5
Novska	0	1	1	0	2	1	50.0
Nova Gradiska	4	4	2	0	10	1	10.0
Djakovo	22	3	8	0	33	6	18.2
Vinkovci	13	1	7	0	21	5	23.8
Baranja	18	2	3	0	23	3	13.0
Total	67	15	23	1	106	18	17.0

[1]from each male animal testes were examined separately in two cultures
[2]from one mother gravid uterus with 5 aborted fetuses were examined

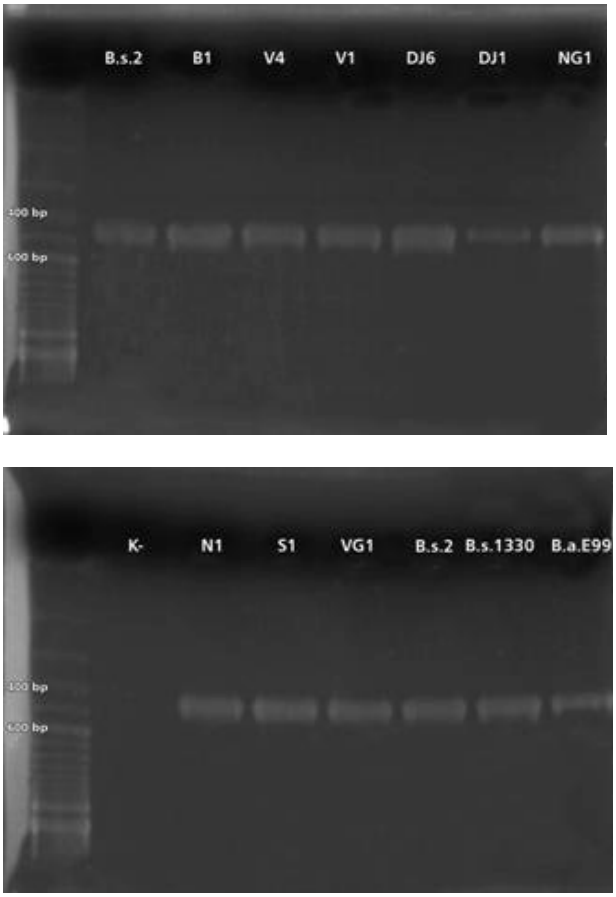

Figure 2. Presentation of the results of PCR for the investigated and standard strains of *Brucellae* spp. for protein BCSP-31 with the application of primer BRU-UP and BRU-LOW

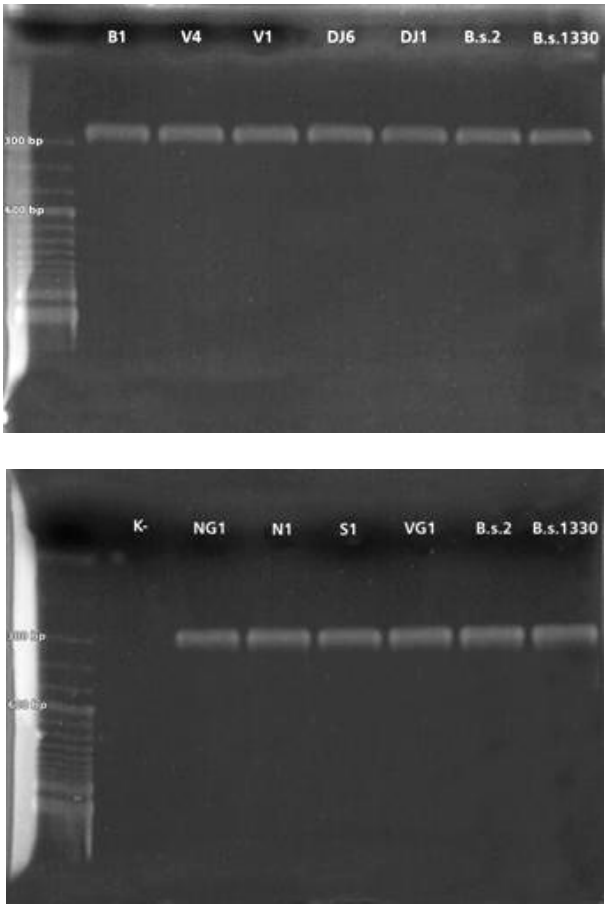

Figure 3. Presentation of the results of PCR for standard and investigated strains using specific primers IS*711* and *B. suis*

each locality one isolate was selected including type strains for results shown in Figure 2.

In the second PCR analysis the multiplication of the DNA of IS*711* fragment was carried out using two primers (IS*711* specific primer and *B. suis* specific primer). In all 18 isolates investigated as well as in type strains mentioned above the established result of multiplying was approximately about 285 bp. From each locality one isolate was selected including type strains for results shown in Figure 3.

DISCUSSION

The aim of this investigation was to demonstrate the level of prevalence of brucellosis in wild boar in the Republic of Croatia and for that reason seven localities with the largest number of wild boar were investigated. During two years of investigation in the hunting seasons in seven regions the blood sera of 514 wild boar were analysed although blood samples were collected from 627 wild boar. The reduction of examined sera samples happened due to the haemolitic changes occurred after the hunting the animal. The highest seroprevalence was found by iELISA (13.6%) followed by RBT (11.5%), CFT (10.5%) and SAT (8.9%). Positive serological reactions were established on all the localities. Most analysed blood sera were from the locality of Dakovo, and most positive reactions were established in more areas (Novska, Sisak, Djakovo, Velika Gorica, Baranja, Nova Gradiska, and Vinkovci). These results are not surprising due to the fact, that in other European and oversees countries antibodies against causal agent of brucellosis were detected as well (Becker *et al*., 1978; Dedek *et al*., 1986; Drew *et al*., 1992; Van der Leek *et al*., 1993; Garin-Bastuji *et al*., 2000; Hubalek *et al*., 2002).

It was demonstrated that the infection in pig caused by *B. suis* is transmitted to susceptible pig by direct or indirect contact with infected pig (Garin-Bastuji *et al*., 2000). Since wild boar live in joint herds and due to the character of the disease itself the only symptom of which sometimes being abortion, the aborted piglets and placenta are quite often eaten by other wild boar or other wild animals (Alton, 1990). Besides the venereal route, which is the most important route of spreading brucellosis in pig, the way of life of wild boar indicates that digestive system is also very often the route of entrance of brucellas into the host organism. In two regions of Republic of Croatia the existence of brucellosis in wild boar was demonstrated, being the source and reservoir of *B. suis* biovar 2 for domestic pig. They had contacts with domestic pig kept in those regions on pastures, thus brucellosis was transmitted either directly (sexual contact) or indirectly to domestic pig (Cvetnic *et al*., 2003).

Serological analyses of blood samples are the fastest and most frequently used diagnostic means by which the presence of brucellosis in a herd can be demonstrated. On the basis of serological results it is not always possible to diagnose brucellosis in an individual animal however, most serological tests are good for demonstrating the disease in a herd. Due to various stages of the disease, in an infected herd of pig there will almost always be some infected animals in which no antibodies to brucellosis will be found (Deyoe, 1967).

In Croatia RBT is used as a screening test for brucellosis, and CFT and iELISA for verification. In our investigations RBT, SAT, CFT and iELISA were used. It is known that bacteria from *Salmonella* genus, *E. Coli* O:157, and particularly *Yersinia enterocolitica* O:9 frequent cause cross-reactions, due to the similarities of polisaharide antigens with brucellas (Corbel, 1985; Garin-Bastuji *et al*., 1999, 2000). This can lead to heavy mistakes in serological diagnostics of brucellosis in pig (Corbel, 1985). The slow agglutination test, although highly sensitive, is not specific enough, and for that reason not reliable enough for diagnosing brucellosis in pig. The reduction of the pH of brucella antigen will result in preventing agglutination with non-specific agglutinins, and for that reason RBT is as sensitive as any other serological test for diagnosing brucellosis in pig, but also more specific than SAT. CFT is also often used in serological diagnosing brucellosis in pig, and particularly as a serological test for confirming the results of other tests. The disadvanage is its relatively low sensitivity, but it is highly specific because it does not reveal IgM in earlier stages of the disease but the disease in the chronic phase (Alton, 1990).

In our investigations according to the literature data the most sensitive serological test was the iELISA. Godfroid *et al*. (1994) reported 39.72% positive reactions found by iELISA test, 19.48% by CFT, 14.67% by RBT and only 0.71% by SAT. MacMillan (1999) reported that regardless the test used for making the diagnosis to brucellosis in pig it was hard to demonstrate brucellosis in more than 80% to 90% individually infected pig. For that reason the

control of brucellosis in pig by serological testing and slaughtering always fails, because new positive serological reactors show up over and over again (Wrathall *et al*., 1991).

The most accurate, and probably the most sensitive method of diagnosing brucellosis in pig is isolation and identification of brucellas. Alton (1990) reported that it was demonstrated that bacteriological diagnostics was as positive as a serological one. A significant number of testes and uteri were bacteriologically analysed, certifying the findings of brucellosis on particular localities with certainty. Brucellas were isolated also from the organs of pig in which neither serologically positive reactions nor pathological changes were established. Averagely 10.1% of the analysed samples of uteri or testes of wild boar were found positive. All isolated brucella species were identified as *B. suis* biovar 2. This was supported also by the previous findings in two locality Lonjsko Polje i Djakovo (Cvetnic *et al*., 2003), and now it was established also on six other localities in Croatia.

Identification of each isolate was also confirmed by PCR. In the first analysis it was demonstrated by PCR that the isolates belonged to *Brucella* spp. genus. The results of the second PCR analysis confirmed their belonging to *B. suis* species. However, by PCR it is not possible to differentiate *B. suis* biovar 1 and biovar 2, thus this was carried out by traditional bacteriological procedure (Bricker and Halling, 1994).

By these investigations it was demonstrated that brucellosis is present in wild boar on all the investigated seven localities in Croatia. The brucellas isolated were identified as *B. suis* biovar 2, as that was also the case in other countries of Central and Western Europe. Wild boar could be the reservoirs of *B. suis* biovar 2 in Croatia as of *Mycobacterium bovis* (Machackova *et al*., 2003) and in this way should be wild boar populations assessed and carcasses handled.

The risk for diseases transmission from wild boar to game and domestic animals (esp. for domestic pigs) is due to the high density of population relatively high.

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Received: 03–06–14
Accepted after corrections: 04–02–06

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