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# WILD BOARS *(SUS SCROFA)* AS RESERVOIRS OF *BRUCELLA SUIS* BIOVAR 2 IN CROATIA

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This work presents the results of findings for brucellosis in wild boars and domestic swine in two regions of Croatia. In the region of Djakovo the blood samples of 211 wild boars were analysed and in 29.4% of the samples serologically positive reactions were established. In the same region the blood samples of 1080 domestic swine on pastures were also analysed and positive serological reactions were established in 12.3%. In the regions around Lonjsko Polje the blood samples of 53 wild boars were analysed and in 22.6% of them positive serological reactions were established. On several locations around Lonjsko Polje the blood samples of 901 domestic swine were serologically analysed and 13.5% of the swine were found to be seropositive. Bacteriological analyses of submitted materials from 24 wild boars resulted in isolation of *Brucella* from seven (29.2%) samples, and from 43 samples originating from domestic swine that had aborted and had been serologically positive, *Brucella* were isolated from 25 (58.1%) swine, as well as from 10 (62.5%) out of 16 aborted piglets. In all the isolates *Brucella suis* biovar 2 was identified. Wild boars are carriers and reservoirs of *Brucella suis* biovar 2 in Croatia.

**Key words:** Wild boars, reservoir, *Brucella suis* biovar 2, Croatia

Brucellosis is a chronic contagious disease of domestic and wild animals as well as humans (zoonosis). It manifests in necrotic and inflammatory changes in the attacked organs and causes abortion in pregnant animals. Most countries are free from brucellosis; however, sporadic outbreaks can occur in feral pigs and hares (Drew et al., 1992; Van Der Leek et al., 1993; Kautzsch et al., 1995). In central and western Europe the cause of brucellosis in swine is most often *Brucella suis* biovar 2 whose natural carriers are hares and wild boars (Körmendy and Nagy, 1982; Quinn et al., 1994; Godfroid et al., 1994; Kautzsch et al., 1995; Szulowski et al., 1999; Garin-Bastuji and Delcueillerie, 2001; Hubalek et al.,

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2002), while in North and South America and Asia *B. suis* biovars 1 and 3 occur more frequently (Van Der Giessen and Priadi, 1988; Cornell et al., 1989; Lord et al., 1997). Since brucellosis occurs also in wild boars, they can be a significant source of infection for domestic swine. It is important to know the reservoirs of the infection so that certain measures for preventing the disease in domestic swine could be applied. The aim of our work was to determine the distribution of brucellosis in wild boars, the possible sources and the distribution of brucellosis in domestic swine kept extensively on pastures in fields or forests and in which abortions and problems in reproduction had been established. The investigation included many herds in the regions where swine are traditionally kept on pastures, which regions in Croatia are located along the river Sava.

## Materials and methods

### *Serology*

In the period from 1996 to 2000 in two regions of Croatia blood samples of 264 wild boars (117 males and 147 females at the age from one to four years) were analysed and in 1997 and 2000, due to the occurrence of abortions in swine in some particular regions, blood samples of domestic swine kept extensively were tested. Blood samples of a total of 1981 swine (1907 sows and 74 boars at the age of one to five years) were serologically analysed. In domestic swine the blood samples for serological analyses were taken by venipuncture from the vena cava cranialis, vena jugularis or the ear vein, while in wild boars blood from the pericardium or thoracic cavity was taken immediately after killing them or if the boars were caught alive, in the way previously described. Since the investigations started in the period when indirect ELISA was not commercially available in Croatia, the blood sera were analysed by the rose bengal test (RBT) and confirmed by the complement fixation test (CFT). The antigens for complement fixation were derived from the culture of *Brucella abortus* strain S99 and produced in the Croatian Veterinary Institute (Zagreb), and the antigen for the rose bengal test was derived from the Central Veterinary Laboratory (Weybridge, UK). All RBT suspected and positive reactions were confirmed by CFT. The finding of 20 IU of complement fixing antibodies in 1 ml of the serum was considered a positive reaction (Alton et al., 1988).

### *Bacteriology*

During slaughtering, materials from seropositive animals were collected for bacteriological and histopathological analyses. The materials included lymph nodes (parotid, submandibular, retropharyngeal, portal, subiliac, mesothelial, supramammary), and pieces of the liver and reproductive organs (testicles and

uterus). From 43 serologically positive domestic swine kept extensively materials were collected (35 samples of uteri, testicles of eight boars and lymph nodes of all the swine) and 16 aborted piglets were also submitted for examination. Immediately after killing the wild boars, samples were taken from 24 animals (15 samples of uteri, testicles of nine boars, livers of eight swine and lymph nodes of all the animals). In addition, the organs of nine hares from different regions were also bacteriologically analysed for brucellosis.

For bacteriological analysis the submitted material was processed in the following way: first it was homogenised in a 'stomacher' and from that suspension directly cultured on four nutritive media (2 plates of blood agar and 2 plates of Farrell medium). Two media (1 plate of blood agar and 1 plate of Farrell medium) were incubated at 37 °C in normal atmosphere and other two in the presence of 5–10% $CO_2$. Growth and morphology of the colonies were monitored daily. Furthermore, the colonies were subcultured and the grown colonies were examined by microscopy. Identification was carried out by determining the morphology of the colonies (S or R), their growth in $CO_2$, production of $H_2S$, growth on media with the addition of 20 µg/ml of thionine and basic fuchsin to the serum dextrose medium, as well as agglutination with A and M monospecific antisera (Corbel et al., 1983; Alton et al., 1988).

*Histopathology*

Immediately after sampling, the tissues for histopathological analysis were fixed in 10% buffered formalin and, with one change of the fixative, they were fixed for 2 weeks. After that period, the samples dehydrated in increasing concentrations of alcohol by standard methods on the histokinette (MICROM HMP 110). After embedding in paraffin, histological sections were cut on the microtome (LEITZ 1512), stained with haematoxylin-eosin and studied by microscopy. During the whole course of fixation and the analysis of histological specimens the generally accepted methods were used (Luna, 1979).

*Polymerase chain reaction (PCR)*

A culture of *Brucella* sp. was suspended in 50 µl twice-distilled water (Sigma). The suspension was heated in a thermo block at 100 °C for 15 min and then centrifuged at 12,000 rpm for 2 min. The supernatant (5 µl) was collected and used for DNA amplification by PCR as previously described by Bricker and Halling (1994). 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 that is responsible for the BCSP-31 protein synthesis. The primers we used were BRU-UP (GGG CAA GGA AGA TTT) and BRU-LOW (CGG CAA GGG TCG GTG TTT) that allowed the amplification of a fragment of approximately 443 bp (Serpe et al., 1999). In the second step specific primers for *B. abortus*, *B. meli-*

*tensis*, *B. ovis* and *B. suis* biovar 1 (strain 1330) as well as *B. suis* biovar 2 were used for the identification of our isolates. The amplification was carried out under the conditions described by Bricker and Halling (1994). Products of the PCR were separated by electrophoresis in 2% agarose gel and stained with ethidium bromide. Results were documented by a video documentation system, which included a UV transluminator and camera (Gel Doc 1000, BioRad).

## Results

During the period between 1996 and 1998 from the region of Djakovo blood samples of 211 wild boars were serologically analysed. Positive reactions were established in 62 (29.4%) and suspected reactions in 28 (13.3%) wild boars. In addition, blood samples from 1080 domestic breeding swine from the same region were analysed. Positive reactions were established in 133 swine (12.3%) and suspected ones in 270 (25.4%) swine (Table 1).

**Table 1**

Number of positive and suspected blood samples of wild and domestic swine in the investigated regions

| Region | No. of samples analysed | Positive reactions | | Suspected reactions | |
|---|---|---|---|---|---|
| | | number | % | number | % |
| Wild boars | | | | | |
| Djakovo | 211 | 62 | 29.4 | 28 | 13.3 |
| Lonjsko Polje | 53 | 12 | 22.6 | 7 | 13.2 |
| Total / % | 264 | 74 | 28.0 | 35 | 13.3 |
| Domestic swine | | | | | |
| Djakovo | 1080 | 133 | 12.3 | 270 | 25.4 |
| Lonjsko Polje | 901 | 122 | 13.5 | 135 | 15.0 |
| Total / % | 1981 | 255 | 12.9 | 305 | 15.4 |

During the period from 1998 to 2000 the blood samples of 53 wild boars from the region of Lonjsko Polje were serologically analysed. Positive reactions were established in 12 (22.6%) and suspected ones in seven (13.2%) wild boars. In the places around Lonjsko Polje the blood samples of 901 breeding swine were serologically analysed. Positive reactions were established in 122 (13.5%) swine and suspected reactions in 135 (15%) swine (Table 2).

The samples of 24 wild boars were analysed bacteriologically and *Brucella* were isolated from seven (29.2%) samples. From 43 submitted samples

originating from serologically positive domestic swine brucellae were isolated from 25 (58.1%) samples. The fetuses of 16 piglets were also submitted for examination and brucellae were isolated from the stomach contents of 10 (62.5%) piglets. From the samples of nine hares no brucellae were isolated.

Smooth, bright, honey-coloured colonies were identified that did not grow in the presence of $CO_2$, did not produce $H_2S$, did not grow on media with basic fuchsin but developed well in normal atmosphere at 37 °C on media with thionine and gave agglutination with monospecific antiserum A while being negative with monospecific antiserum M. On the basis of these indicators *B. suis* biovar 2 was identified. The DNA from isolated colonies was detected by PCR when specific *B. suis* primers were used. The bacteriological finding of *B. suis* biovar 2 was also confirmed in AFSSA (OIE/FAO Reference Laboratory, Maisons Alfort, France) (Table 2).

**Table 2**

Number of bacteriologically analysed samples and identified isolates

| Origin of the material | No. of materials bacteriologically analysed | Isolates | | Identified isolates |
|---|---|---|---|---|
| | | number | % | |
| Wild boars | 24 | 7 | 29.2 | *B. suis* biovar 2 |
| Domestic swine (serologically positive) | 43 | 25 | 58.1 | *B. suis* biovar 2 |
| Aborted piglets | 16 | 10 | 62.5 | *B. suis* biovar 2 |
| Hares | 9 | 0 | 0 | – |

Pathological changes were most often found in the testicles of male animals in the form of inflammation of the testicles and/or epididymis. Necrotic and purulent foci of various sizes were present in the testicular parenchyma and epididymis. Histopathologically, a large mass of connective tissue could be observed in the affected epididymis, and the complete disappearance of epithelium, as well as purulent content consisting mostly of polymorphonuclear neutrophils and eosinophils with some detritus in the duct lumen were found. Changes were frequently seen in the uterine mucosa with characteristic miliary yellowish-white nodules which could also be purulent and caseous. Placentas and fetuses were also noticeably changed. The placenta was usually hyperaemic and oedematous and multiple areas of haemorrhage were seen on the fetuses.

## Discussion

Brucellosis is a contagious venereal disease of swine. The reproductive tract is the most important route of spreading the infection in this species. Breeding swine are most often infected by direct contact while mating with infected animals. Natural admission is the most common way of mating in swine kept under extensive conditions. Another important route of *Brucella* infection in swine is the oral route. Contaminated feed and water in the excreta of swine infected with brucellosis can be the source of infection. Keeping swine together on pastures or in pens gives them opportunity to eat aborted fetuses and placenta and contract infection in that way.

The existence of brucellosis in domestic swine and wild boars has been demonstrated on the territory of Croatia in the regions along the river Sava. In the regions mentioned breeding swine are often kept on pastures or in forests, so contacts with many other swine and also with wild boars are possible. In this paper we present several outbreaks that occurred in different regions of Croatia in swine kept on pastures.

The finding of serologically positive reactions to brucellosis in 29.4% of wild boars in the region of Djakovo and in 22.6% of them in Lonjsko Polje indicates a relatively high rate of presence of the disease in wild boars in these regions. These findings were confirmed by isolating brucellae from 29.2% of organ samples of wild boars. Serological and bacteriological findings in wild boars from both regions show good agreement. *Brucella suis* biovar 2 has been described in hares and feral pigs in other Central European countries: Slovenia (Brglez and Batis, 1981), Hungary (Körmendy and Nagy, 1982), Slovakia (Nižnansky et al., 1957; cit. Hubalek et al., 2002), Czech Republic (Hubalek et al., 2002) and Poland (Szulowski et al., 1999). Godfroid et al. (1994) have reported the occurrence of brucellosis in wild boars. They described the finding of *B. suis* biovar 2 in wild boars in Belgium, isolating the same bacteria from 13 (9.2%) out of 141 analysed samples of wild boars. Garin-Bastuji et al. (2000) reported that in different regions of France positive serological reactions to brucellosis in wild boars ranged from 20% to 35%. From spleen samples of wild boars *B. suis* biovar 2 was bacteriologically isolated and diagnosed. They also indicated that the possibility of the contacts of wild and domestic swine favoured the spreading of brucellosis in swine. Van Der Leek et al. (1993) described the findings of serologically positive reactions in wild boars in six out of 18 examined locations in Florida. Positive reactions in particular locations ranged from 5.5% to 33.3% in wild boars. Becker et al. (1978) found positive reactions to brucellosis in 50 (52.6%) out of 95 analysed blood samples of wild boars in Florida. Drew et al. (1992) described positive reactions in 23 out of 611 analysed blood samples of wild boars in California, which corresponds to a seropositivity

rate of 3.8%. MacMillan (1989) reported that, besides infected domestic swine, the most significant potential reservoir of *B. suis* biovar 2 were wild boars.

Brucellosis in domestic swine has also been demonstrated in the above-mentioned regions of Croatia. In the region of Djakovo the presence of positive serological reactions was demonstrated in 12.3% of swine and in Lonjsko Polje in 13.5% of swine. By bacteriological analysis of 43 samples from seropositive slaughtered swine, brucellae were isolated from 25 (58.1%) samples and from 10 (62.5%) out of 16 aborted piglets. Although there is no direct evidence that wild boars are the source of brucellosis for domestic swine, in our opinion the identification of the isolates (*B. suis* biovar 2) and the fact that domestic swine live in the same area where wild boars live, and thus indirect and even direct (reproductive) contact among them is possible, may provide sufficient basis for connecting the occurrences of this disease among the investigated groups.

Until recently, the rose bengal test was used as a screening test for the diagnosis of swine brucellosis and CFT as a method of verification of the positive results in Croatia. Positive reactions obtained by the RBT were established in blood serum samples in 109 feral pigs, and 74 were confirmed by CFT as well. Other reactions (16) were suspicious by CFT (< 20 IU) or were positive only by RBT (19). In blood serum samples from domestic pigs more suspicious (15.4%) than positive reactions (12.9%) for brucellosis were found. It is possible to diagnose brucellosis in a swine herd by the RBT and CFT for sure; however, it is difficult to estimate the individual status of pigs regarding brucellosis. The problem of unspecific reactions is more explicit in domestic swine.

For the detection of *Brucella* DNA a gene fragment responsible for the synthesis of protein BCSP-31 was amplified. This membrane protein is known as a *Brucella* gender (Mayfield et al., 1988). Furthermore, our isolates have been amplified by specific primers for *B. abortus*, *B. melitensis*, *B. ovis*, *B. suis* biovar 1 and *B. suis* biovar 2 (in a reference laboratory). The PCR products have shown to be of the same size as those of *B. suis* (1330) and *B. suis* biovar 2. According to these findings it could be assumed that the isolate was probably *B. suis* biovar 2, which was also confirmed by a traditional bacteriological identification.

European hares (*Lepus europaeus*) are natural hosts of *B. suis* biovar 2. Kautzsch et al. (1995) also indicated hares, but also wild boars, as natural reservoirs of *B. suis* biovar 2. Garin-Bastuji et al. (2000) also reported on finding brucellosis in hares in France. Kovačić and Karlović (1982) described serological analyses of blood samples of 58,929 hares in a years-long investigation on brucellosis carried out in several regions in Croatia when 1,000 hares were also bacteriologically analysed for brucellosis. Positive serological reactions were established in only 13 cases and the diagnosis was bacteriologically confirmed. The isolated agent was regularly identified as *B. suis*. On the basis of those findings temporally distributed to ten years, the authors reported that it could not be concluded that hares were significant reservoirs of brucellosis in nature in Croa-

tia. In our investigations from the organs of 9 hares from the above-mentioned regions brucellae were also not isolated.

By these investigations it was demonstrated that *B. suis* biovar 2 prevailed in domestic and wild swine on the territory of Croatia. Wild boars are the reservoirs of *B. suis* biovar 2 and in that way also the possible source of these bacteria in nature, while hares are probably not significant for spreading the disease in domestic swine. For this reason it is important to recognise the disease, and when abortions or problems in reproduction occur in domestic swine, this is the disease which should be suspected by veterinarians.

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