IDENTIFICATION OF Brucella DNA IN LYMPH TISSUE FROM DEER (Cervus elaphus) AND WILD BOARS (Sus scrofa) BY THE USE OF BCSP31 PCR AND AMOS-PCR

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Abstract

At first, a genus-specific PCR assay (BCSP31) to identify all known Brucella, generating an amplicon of 223 bp, was used. Then, positive samples were analysed with AMOS-PCR, which enables us to identify selected biovars of B. abortus, B. melitensis, B. ovis, and B. suis. The BCSP31 PCR analysis revealed that 27 (11.5%) of 235 samples originating from wild boars were positive and they all were negative in AMOS-PCR. Twenty-five were identified as B. suis biovar 2 through bacteriological examination. On the other hand, none of the 183 samples from deer was positive in PCR or bacteriological examination. The obtained results can provide a better knowledge of the potential reservoirs of infection and also should lead to great progress in molecular epidemiology.

Key words: deer, wild boar, lymph nodes, Brucella, PCR, identification.

Brucellae are Gram-negative, facultative, intracellular bacteria that are pathogenic for humans and a variety of livestock animals. Eight species are recognised within the genus Brucella: B. abortus, B. melitensis, B. suis, B. ovis, B. canis, B. neotomae, B. cetaceae, and B. pinnipediae (3, 13). Their classification is mainly based on differences in pathogenicity and host preferences. The main pathogenic species distributed worldwide are B. abortus, which is responsible for bovine brucellosis, B. melitensis, the main aetiological agent of ovine and caprine brucellosis, and B. suis, which is responsible for swine brucellosis (3, 6, 14). In humans, the infection generally results from transmission via the gastrointestinal route by the consumption of unpasteurised dairy products, airborne transmission by inhaling dust contaminated by aborted tissues, and transmission caused by laboratory-associated exposure to aerosols. On the other hand, there is a lack of information about the prevalence of Brucella sp. among wildlife animals, such as deer or wild boars, obtained on the basis of molecular or bacteriological examinations.

In respect of large scale of investigations, the laboratory diagnosis of brucellosis is mainly based on serological tests. However, the similarity of the O-antigenic side chain of Brucella LPS with other microbes, particularly Yersinia enterocolitica O:9, has restricted the specificity of serological diagnostics. On the other hand, the culture of Brucella is time-consuming, expensive, low sensitive, sometimes difficult in interpretation of results and requires biohazard conditions for handling with highly-contagious material. Due to the disadvantages of serological and bacteriological methods, the new methods based on molecular biology (PCR) have been introduced into routine diagnostics (4, 5, 8, 19, 21). Among them, genus specific BCSP31 PCR, which enables us to identify all known Brucella sp. and AMOS-PCR allowing to distinguish B. abortus (biovars 1, 2, and 4), B. melitensis (biovars 1, 2, and 3), B. ovis, and B. suis (biovar 1) are used.

The aim of this study was to determine the presence of molecular markers specific for Brucella in lymph-node samples originating from deer and wild boars from Poland.

Material and Methods

Bacterial strains. The Brucella reference strains B. abortus bv. 1 (strain 544), B. melitensis bv. 1 (strain 16M), B. ovis (strain 63/290), B. suis bv. 1 (strain 1330) and bv. 2 (Thomsen), B. canis (RM6/66), and also Yersinia enterocolitica O:9 strain were used in the investigations. At first, they were analysed by PCR for the presence of gene-encoding BCSP31 (universal protein of Brucella), and subsequently by a species-
distinguished protocol based on the differences of IS711 gene fragment (AMOS-PCR).

**DNA extraction.** Lymph tissue samples (1 g) originating from deer (n=183) and wild boars (n=235) were added to 9 ml of saline and homogenised in a stomacher. The suspension was heated at 99°C for 5 min (Thermomixer Comfort, Eppendorf), next chilled on ice, and centrifuged at 2,500 x g for 2 min. The supernatant was used for the DNA extraction according to commercial protocol (DNeasy Blood & Tissue Kit, Qiagen) and used as a source of DNA in PCR assays. On the other hand, one individual colony of each *Brucella* reference strains was suspended in 50 µl of sterile, DNase, RNase-free deionised water (ICN Biomedicals). The suspensions were heated at 99°C for 5 min, chilled on ice, and then centrifuged at 13,000 x g for 1 min to pellet the cellular debris. The supernatant (5 µl) was subsequently used as a source of DNA template.

**PCR assay.** In the first step, a universal PCR assay to identify all known *Brucella*, generating an amplicon of 223 bp, was used. Each DNA amplification was performed in a 50 µl reaction mixture consisting of DNA template, 1X Taq buffer, 1 U of Taq DNA polymerase, 200 µM of dNTPs, MgCl₂ (3 mM for BCSP31 and 5 mM for AMOS-PCR), nucleotide primers, and water. Sequences, characteristics, and concentration of the primers used in this study are shown in Table 1.

Only samples which gave positive results in the first test were then analysed with the second protocol, AMOS-PCR, which enabled us to identify selected species: *B. abortus*, *B. melitensis*, *B. ovis*, and *B. suis*, generating the amplicons of 498, 731, 976, and 285 bp, respectively. PCR and AMOS-PCRs were run in a thermocycler (T3, Biometra) under the following conditions: initial DNA denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 1 min, 60.5°C for 1 min, and 72°C for 1 min (BCSP31 PCR) or 35 cycles of 95°C for 1 min, 55.5°C for 2 min, and 72°C for 2 min (AMOS-PCR). The final extension step was done at 72°C for 10 min. After staining with ethidium bromide (50 µg/mL) for 0.5 min and washing in distilled water, the gels were photographed under UV light using the GelDoc 2000 documentation system (Bio-Rad). The sizes of the PCR amplicons were compared to the 100 bp DNA marker (Fermentas).

**PCR sequencing.** A 223-bp product of BCSP31 PCR was cleaned up using ExoSAP-IT (GE Healthcare), reamplified with DYEnamic™ ET Dye Terminator Kit (MegaBACE™) (GE Healthcare) and analysed using an automated sequencer Megabace 1000 DNA Analysis System (GE Healthcare). The obtained results were compared to GeneBank Database.

**Bacteriological analysis.** Parallel to the PCR assays, bacteriological examination was performed (17). Briefly, *Brucellae* were grown on Farrel’s medium at 37°C for 10 d in an atmosphere containing 5%-10% CO₂ and without CO₂ added. The bacteria from suspected colonies were stained by the Gram method and typed with routine typing tests: agglutination with anti-Brucella standard serum and monospecific anti-A and anti-M sera, requirement of added CO₂ for growth, oxidase, catalase, urease tests, production of H₂S, growth in the presence of thionin and basic fuchsin, and susceptibility to phages Tibilisi (Tb at its routine test dilution RTD and 10² x RTD) and R/C (RTD).

**Results**

The PCR analysis of the DNA extracted from the *Brucella* reference strains used in the investigations revealed the presence of the BCSP31 gene, generating the amplicon of 223 bp size (Fig. 1, upper gel). In parallel, the examination of the DNA from lymph tissue showed that 27 of 235 samples (11.5%) originated from wild boars were positive, as determined by the presence of the 223 bp-amplified product. The remaining 208 samples, as well as 183 samples from deer, did not generate the PCR amplicon of 223 bp size.

The 223-bp product of BCSP31 was analysed with Megabace 1000, compared to the GeneBank database, and recognised as *Brucella* sp. The lymph tissue samples, which were BCSP31-positive, were further analysed with AMOS-PCR and all results were negative, whereas the reference strains *B. abortus*, *B. melitensis*, *B. ovis*, and *B. suis* bv.1 showed the presence of predicted amplicons of 498, 731, 976, and 285 bp, respectively. Contemporaneously, the reference strain *B. suis* bv. 2 did not generate the product in AMOS-PCR (Fig. 1, lower gel).

![Fig. 1. PCR amplicons obtained in BCSP31 PCR (upper gel) and AMOS-PCR (lower gel). Lane 1 - *B. abortus*; lane 2 - *B. melitensis*; lane 3 - *B. ovis*; lane 4 - *B. suis* bv.1; lane 5 - *B. suis* bv.2; lane 6 - lymph tissue sample – direct DNA isolation; lane 7 - *B. suis* isolate received from lymph tissue sample; lane 8 - *B. canis*; lane 9 - *Yersinia enterocolitica* O:9; lane 10 - H₂O; lane M - 100 bp DNA marker.](image-url)
Table 1
Sequences and characteristics of the primers used in the study

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5’→3’)</th>
<th>Target gene</th>
<th>Amplicon (bp)</th>
<th>Concentration (µM)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>B4</td>
<td>TGG CTC GGT TGC CAA TAT CAA</td>
<td>BCSP31</td>
<td>223</td>
<td>0.05</td>
<td>(1)</td>
</tr>
<tr>
<td>B5</td>
<td>CGC GCT TGC CTT TCA GGT GTG</td>
<td></td>
<td></td>
<td>0.05 (1)</td>
<td></td>
</tr>
<tr>
<td>BA*</td>
<td>GAC GAA CGG AAT TTT TCC AAT CCC</td>
<td></td>
<td>498</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>BM*</td>
<td>AAA TCG CGT CCT TGC TGG TCT GA</td>
<td>IS711</td>
<td>731</td>
<td>0.1</td>
<td>(4, 5)</td>
</tr>
<tr>
<td>BO*</td>
<td>CCG GTT CTG GCA CCA TCG TCG</td>
<td>IS711</td>
<td>976</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>BS*</td>
<td>GCG CCG TTT TCT GAA GGT GGT TCA GG</td>
<td>IS711</td>
<td>285</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>IS711†</td>
<td>TGC CGA TCA CTT AAG GGC CTT CAT</td>
<td>IS711</td>
<td></td>
<td>0.2</td>
<td></td>
</tr>
</tbody>
</table>

*The amplicons were obtained using the forward primers BA (B. abortus) or BM (B. melitensis) or BO (B. ovis) or BS (B. suis) with common reverse primer IS711.

Table 2
Results of the bacteriological examination of wild boar samples

<table>
<thead>
<tr>
<th>Characteristics of isolates</th>
<th>Agglutination in sera</th>
<th>Growth on dyes</th>
<th>Lysis by phages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>Culture (+/-)</td>
<td>CO₂ requirement</td>
<td>Oxidase</td>
</tr>
<tr>
<td>All Brucella isolates from lymph tissue (n=25)</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Bacteriological examination showed that among 27 PCR positive samples, Brucellae were isolated in 25 cases. The isolates were Gram-negative, agglutinated with anti-Brucella standard serum and monospecific anti-A serum, gave positive results in oxidase, catalase, and urease tests, did not produce H₂S, did not require CO₂ for growth, grew in the presence of thionin and were susceptible to TB phages at 10⁴ x RTD (Table 2).

Discussion

Fast and accurate diagnosis of brucellosis is very important for a positive outcome of eradication programmes. PCR is a promising additional tool supplementing the problematic culture of Brucella and serological techniques (2, 9, 18). It is well known that Salmonella, Escherichia coli O:157, and particularly Yersinia enterocolitica O:9, frequently cause cross-reactions, due to the similarities of polysaccharide antigens with the Brucella (6, 11). This can lead to mistakes in the serological diagnostics of brucellosis. On the other hand, as mentioned, culture has also some disadvantages. Since 1987, numerous PCR-based assays for the identification of Brucella were developed (10, 16, 19, 20). In our study we used the assay described by Bailey et al. (1), based on the gene encoding BCSP31, the first published Brucella loci to be cloned and sequenced. This gene encodes an antigenic, periplasmic protein of unknown function. As was published by Bricker et al. (4, 5), it is conserved in all species and biovars of Brucella with the exception of B. ovis. We discovered that B. ovis reference strain 63/290, used in our laboratory, generated a typical amplicon of 223 bp, similarly as the other Brucellae.

In our study, in the direct examination of lymph tissues samples, we demonstrated that 27 of 235 of them (11.5%) originating from wild boars were positive in BCSP31 PCR, as determined by the presence of the 223 bp amplified product. We observed that these samples, confirmed by DNA sequencing, were negative in AMOS-PCR test. When we simultaneously bacteriologically tested the samples, we identified the isolates as B. suis bv. 2. Our results are in accordance with Bricker et al. (4, 5) who showed that among B. suis in AMOS-PCR assay, only bv. 1 and 3 are positive and bv. 2 is negative.

The high rate of positive results in the examination of samples from wild boars confirm the
results of other authors that these wild animals should be regarded as a natural reservoir of *B. suis* in Europe. Godfroid *et al.* (12) found *B. suis* in wild boars in Belgium, where it was isolated from 13 (9.2%) out of 141 analysed samples. In France, Garin-Bastuji *et al.* (11) also isolated *B. suis* in about 10% of the analysed material (spleen). They showed also, that in different regions of France, positive reactions to brucellosis were found in wild boars in the range 20%-35%. Hubalek *et al.* (15) reported that in the Czech Republic the frequency of positive reactions to brucellosis was 15%. In Poland, Szulowski *et al.* (21) showed the presence of *Brucella* antibodies in 12.3% of sera from wild boars.

The results showed small disagreements between the culture and molecular methods. In two samples we found bacterial DNA of *Brucella* sp. but we could not get isolates from the samples tested. It is due more to the higher sensitivity of PCR than culture. On the other hand, the positive results in PCR can be evoked by the presence of bacterial fragments (DNA) only, without any live bacteria in lymph tissue (nodes) (7, 18, 22).

The results of our investigations showed the high-prevalence of *B. suis* bv. 2 infection in wild boars in Poland and provided a better knowledge of the potential source and reservoir of infection for domestic animals, particularly pigs. The investigations should also lead to great progress in molecular epidemiology.

**References**