VALUE OF FLUORESCENCE POLARISATION ASSAY IN COMPARISON TO TRADITIONAL TECHNIQUES IN DIAGNOSIS OF PORCINE BRUCELLOSIS

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Abstract

The aim of this study was the evaluation of fluorescence polarisation assay (FPA) in the diagnosis of porcine brucellosis in comparison with Rose Bengal test (RBT), serum agglutination test (SAT), complement fixation test (CFT), 2-mercaptoethanol test, and ELISA. Eight hundred seventeen sera from pigs, including 612 sera from healthy animals, seven sera from Brucella suis bv2 culture positive animals, and 198 sera classified as false positive, originated from confirmatory investigations, were used. All sera from healthy animals, negative in RBT, SAT, CFT, and ELISA were also negative in FPA. All sera positive in serological examination, originated from Brucella infected animals, were also positive in FPA. Among sera classified as false positive almost half of the samples tested (49.4%) reacted positively in FPA. The examinations confirmed the usefulness of FPA in diagnosis of porcine brucellosis, but the method, like the other tests, does not allow resolving the problem of discrimination cross-reacting from specific antibodies.

Key words: swine, brucellosis, serological diagnosis, methods.

Brucellosis remains a zoonosis of worldwide public health and economic importance (1). Ten species are recognised within the genus Brucella: B. abortus, B. melitensis, B. suis, B. ovis, B. canis, B. neotomae, B. cetaceae, B. pinniped, B. microti, and B. inopinata (5, 10, 15). The main role is played by 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. The species of B. suis consists of five biovars, but the infection in pigs is caused by B. suis biovars 1, 2, and 3 (14). Porcine brucellosis is of widespread occurrence; however, the prevalence is low, with the exception of South America and South-East Asia, where the prevalence is higher. In Europe, the disease is caused by B. suis biovar 2 (except for Croatia, where biovar 1 was observed), and the natural reservoir of this biovar, which is rarely pathogenic for humans, are wild boars and hares (1, 8, 14, 16). Common manifestations of brucellosis in pigs are abortions occurring at any time during gestation, temporary or permanent sterility, the birth of stillborn or weak piglets, orchitis, and swelling of one or both testicles of infected boars, and abscesses in subcutaneous tissues, kidneys, and muscles. Boars and sows can become lame or even paralysed because of severe arthritis. Porcine brucellosis is transmitted venereally. Infected boars infect sows during mating, and conversely, infected sows shed the bacterium in the discharges from their uterus, and boars can contract the disease during copulation. Sometimes, uninfected herds are contaminated by wild boars mating with domestic sows. The disease can also be transmitted by ingestion, by inhalation, via the conjunctiva, or cutaneously. As brucellosis in pigs does not always cause symptoms and abortions can be caused by a number of other conditions, proper diagnosis must be performed by laboratory testing including serological tests and the culture and identification of Brucella. The risk of introducing brucellosis into a swine herd is related to wild animals and purchased infected pigs (24). Semen for artificial insemination should also be considered as a risk factor. As regards the intra-Community trade in pigs, under Council Directive 64/432/EEC of 26 June 1964 on health problems affecting intra-Community trade in cattle and swine (6), until 1997 pigs had to be certified as originating from brucellosis-free countries. Since porcine brucellosis was thought to have disappeared from UE countries and due to the technical development of pig husbandry, Directive 97/12/EEC of 17 March 1997 removed this requirement (7).

The laboratory diagnosis of brucellosis is mainly based on serological tests. The main methods employed for diagnosing brucellosis are the Rose Bengal test (RBT) and ELISA. Additionally, according to the Instruction of Chief Veterinary Officer of Poland, serum agglutination test (SAT), 2-mercaptoethanol test...
(2-ME), and complement fixation test (CFT) can be used to explain doubtful results (19). If positive serological results are ascertained, pigs are slaughtered, and culture methods are used to isolate Brucella. 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 diagnosis resulting in false positive serological results (FPSR) (13).

In recent years, a new technique measuring antigen/antibody interaction - fluorescence polarisation assay (FPA) has been developed. The mechanism of the assay is based on random rotation of molecules in solution. Thus small molecules rotate faster than large molecules (complexes of antigen and antibodies) (17). If a molecule is labelled, the rotation can be determined by measuring polarised light intensity in vertical and horizontal planes (19). It is host species-independent method and can be performed also with whole blood or milk (9, 11, 20-22). According to OIE Terrestrial Manual 2009, the FPA is recommended as a prescribed test for bovine and porcine brucellosis for determining the health status of animals (19).

In a previous study the method was assessed in relation to bovine sera (27). The aim of this study was the application of FPA for the examination of porcine sera and a comparison of the results of FPA with the results of traditional serological techniques such as RBT, SAT, CFT, 2-ME, and ELISA.

Material and Methods

Serum samples. Eight hundred seventeen sera from swine, including 612 sera from healthy animals, seven sera from animals regarded as serologically positive for brucellosis (confirmed as Brucella suis biotype 2 positive in bacteriological examination), and 198 sera originated from confirmatory investigations (FPSR), finally classified as negative, were used. The sera were obtained from international or regional laboratories in 2000-2010 and sent to the National Reference Laboratory for Brucellosis in the National Veterinary Research Institute in Pulawy for further investigation or for research purpose. Prior to testing, the sera were preserved with sodium azide, stored in a refrigerator (2-8°C), and then allowed to reach room temperature for 30 min before the examination.

Serological tests. ELISA, RBT, SAT, CFT, and 2-ME were used for the serological examination. The diagnostic ELISA kit had been developed at the National Veterinary Research Institute in Pulawy, and it was described previously (25). Briefly, the lipopolysaccharide (LPS) obtained from B. abortus S19 was used as the antigen, anti-swine immunoglobulins conjugated with horseradish peroxidase were used as the conjugate, and ABTS with H2O2 as the substrate. Controls in the ELISA consisted of a strong positive swine serum (S++) with a high titre of Brucella antibodies, a weak positive serum (S+) with a low titre of Brucella antibodies, and a negative serum (N). The results of the ELISA were read when the absorbance values (OD-optical density) of the weak positive controls were above 0.250. At this level the cut-offs between positive and negative results were settled. The RBT, SAT, CFT and 2-ME were performed according to official instructions and protocols.

FPA. Fluorescence polarisation assay was conducted as described previously (27) with the major modification of millipolarisation (mP) negative value. In detail, the mean mP for three replicates of the negative control were previously appointed and calibrated to 70-95 mP, according to the manual obtained from the manufacturer. In this study, FPA reader (Diachemix, USA) was locally calibrated using control of strong positive, weak positive, and negative sera (19). The results were interpreted as negative, if millipolarisation units of the samples were less than 10 mP above the locally established mean negative control. Any results greater than 20 mP of the mean of the negative control were positive. The samples in range of 10-20 mP, which were recognised as suspected, were retested twice using 20 µl of the serum samples.

Bacteriological examination. To isolate brucellae, the samples from the lymph nodes, spleen, liver, and kidneys were cultured on Farrell’s medium. The samples (1 g) were homogenised with 10 ml of saline and 0.1 ml of the homogenate was transferred onto plates. The plates were incubated for 10 d at 37°C in an atmosphere with 5%-10% CO2 added. Typical Brucella colonies were stained by Gram’s method, then affiliation for the genus Brucella was confirmed by agglutination with anti-Brucella standard serum and positive results in tests for catalase and oxidase. Further antigenic characteristic was performed using monospecific anti-A and anti-M sera and conducting further tests: test for CO2 requirement, production of H2S and urease, growth on thionin and basic fuchsin, and lysis by phages (Tbilisi at its routine test dilution, RTD, and 104 × RTD).

Results

All sera originated from healthy animals were negative in ELISA, RBT, SAT, and CFT. In the FPA, all samples were also negative; the values below 90 mP were noted. Table 1 summarises results of serological examination of FPSR samples. Among 198 sera obtained from different laboratories for confirmatory investigations or research purpose, 27 were positive in only one of classical serological assays. Among SAT-positive sera (n=11), nine of them were also positive in FPA and among CFT-positive samples (n=15), 12 of them gave results greater than 20 mP of the mean negative control and were classified as FPA-positive. It was demonstrated that among samples, which were positive in two of assays used: RBT- and SAT-positive (n=4), or CFT- and SAT-positive (n=10), the positive results in FPA were observed in two and eight cases, respectively. Among minority of the sera, which were RBT-positive, SAT-positive, and CFT-positive (n=157) almost all were positive in ELISA (n=156). In this group 66 of the samples tested were FPA positive.
Table 1
Results of serological examination of FPSR porcine sera for Brucella antibodies in classical tests and FPA.

<table>
<thead>
<tr>
<th>Porcine sera according to the serological results</th>
<th>FPA+</th>
<th>FPA-</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAT+ (n=11)</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>RBT+ (n=1)</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>CFT+ (n=15)</td>
<td>12</td>
<td>3</td>
</tr>
<tr>
<td>RBT+ and SAT+ (n=4)</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>CFT+ and SAT+ (n=10)</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>RBT+, SAT+ and CFT+ (n=156)</td>
<td>66</td>
<td>90</td>
</tr>
<tr>
<td>ELISA- (n=1)</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

False positive serological results (n=198)

+: positive; -: negative

Table 2
The results of serological examination for Brucella antibodies in the animals infected with Brucella suis bv. 2 as determined in bacteriological examination.

<table>
<thead>
<tr>
<th>No. of animal</th>
<th>I examination</th>
<th>II examination</th>
<th>I examination</th>
<th>II examination</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RBT (IU/ml*)</td>
<td>SAT (ICU/ml**)</td>
<td>CFT (titer)</td>
<td>ELISA (titer)</td>
</tr>
<tr>
<td>1             + (574,5)</td>
<td>- (320)</td>
<td>1/320</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2             + (164)</td>
<td>+ (160)</td>
<td>4/80</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3             + (492,5)</td>
<td>+ (848)</td>
<td>2/320</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4             +</td>
<td>61,5</td>
<td>- (8,4)</td>
<td>2/40</td>
<td>+</td>
</tr>
<tr>
<td>5             -</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6             -</td>
<td>+ (61,5)</td>
<td>+ (106)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>7             +</td>
<td>+ (61,5)</td>
<td>+ (46,5)</td>
<td>1/10</td>
<td>+</td>
</tr>
</tbody>
</table>

*: international units per ml. **: international complement fixation test units per ml

Table 2 summarises the results of the serological examination of sera originated from animals, which were positive in serological and bacteriological investigation and from which Brucella suis bv. 2 was isolated. Seven samples reacted positively in all tests: RBT, SAT, CFT, 2-ME, and ELISA. The comparison of antibody titres of in SAT, CFT, and 2-ME shows that in most samples the level of antibodies detected in the second examination, which was performed after 30 d, was markedly higher. From the tissue samples, Brucella microorganisms were isolated. All isolates had the same characteristics: agglutination with anti-Brucella standard serum and monospecific anti-A serum, positive results of oxidase, catalase, and urease tests, no CO₂ requirement for growth, no H₂S production, growth on thionin, no growth on basic fuchsin, and lysis by TB phages at a higher concentration (10⁷ × RTD). These characteristics are typical of B. suis bv. 2

Discussion

Diagnosis of brucellosis in animals can only be made on the basis of laboratory testing – the demonstration of causal organism and the detection of significant levels of specific antibodies. Unequivocal diagnosis of Brucella infections can be made only by the isolation and identification of microorganisms. The most valuable samples from animals include aborted foetuses, foetal membranes, vaginal secretions, blood, milk, semen, tissues, and arthritis and hygroma fluids (1). In addition to bacteriological examination, the new molecular techniques based on DNA amplification of targeted genes can be used (26). All these techniques are expensive, laborious, and as brucellae are classified in risk group III, adequate accommodation, equipment, and hygienic facilities are necessary for safety of examinations. Thus taking into account the scale of investigations, the costs and the ability to be performed
by the laboratories, serological examinations play a dominant role in diagnosis of brucellosis (2-4, 12, 19).

In pigs, no serological method is fully reliable in diagnosing brucellosis in individual animals. One of the most important problems one should always bear in mind is the possibility of cross reactions between Brucella sp. and a number of other microorganisms, primarily Yersinia enterocolitica O:9, which are difficult to distinguish from specific anti-Brucella reactions. Similarity of the OPS, the immunodominant region of the LPS molecule, is responsible for most of the observed cross reactions (5, 13, 22). Thus, quick and accurate diagnosis of porcine brucellosis is very important for an effective outcome of eradication and monitoring programmes and precise establishing of epidemiological status of herds of pigs and individual animals. Among new methods, the FPA is increasingly used as a simple technique for measuring antigen/antibody interaction, which may be performed in a laboratory setting or in the field. The method has been validated for a large number of species, including humans, cattle, and bison, and is a prescribed OIE test applied for the examination of cattle and pigs during international turnover (19). The various studies concerning bovine sera showed the high sensitivity and specificity of the method (20-22). Unfortunately, there is a lack of information about the practical aspects and validation of FPA in diagnosis of porcine brucellosis (18, 19).

In our study, a full correlation between the results of examination of negative samples tested with RBT, SAT, CFT, ELISA and FPA was observed. Similar correlation was observed when samples from Brucella positive animals were tested in bacteriological culture. That means that FPA enables to classify correctly both groups of animals. Different situation was observed when sera with positive serological reactions, but classified as false positive, were examined. The earlier study recommended the way to distinguish Brucella infected pigs from those in which FPSR were observed (23). The investigators should take into account such parameters as: the percentage of positive results and titres of antibodies in respective tests, the absorbance values of positive samples in the ELISA, the presence of antibodies, which are not inactivated by 2-mercaptoethanol, and the permanent character of Brucella antibodies (long-lasting serological reply). The results of this study show that FPA is also susceptible to the presence of cross-reacting antibodies in porcine serum, though in a lesser scope than other methods – RBT, CFT, SAT, and ELISA. The results of our previous studies concerning evaluation of FPA in diagnosis of bovine brucellosis revealed that only eight out of 123 samples reacting in RBT, SAT, CFT, or ELISA (probable false positive reactors) were positive in FPA. In this group of samples all sera positive only in RBT and SAT were negative in FPA (27). In pigs, the percentage of positive reactions in FPA, regarded as FPSR, is much higher.

Taking into consideration the obtained results, FPA can be regarded as an additional method in serological diagnosis of porcine brucellosis, but does not constitute a tool for resolving all problems, particularly relevant to the presence of cross-reacting antibodies. Results obtained in FPA still do not allow making an unambiguous diagnosis regarding cross-reactions. Further evaluating studies are needed.

References


