Abstract

The aim of this study was the application of fluorescence polarisation assay (FPA) for the examination of bovine sera and comparison of the results of the assay with the results of Rose Bengal test (RBT), serum agglutination test (SAT), complement fixation test (CFT), and ELISA. Six hundred thirty-five sera from cattle, including 300 sera from healthy animals, 32 sera from animals regarded as serologically positive for brucellosis and culled, and 303 sera originated from confirmatory investigations were used. All sera originating from healthy animals, negative in ELISA, RBT, SAT, and CFT were also negative in FPA. Among 303 sera from confirmatory investigations, 269 were positive in both RBT and SAT, 21 were positive in SAT and remaining 13 were RBT-positive only. Only two sera, one positive in both tests (RBT, SAT) and one SAT-positive, were also positive in FPA. Among 32 sera originated from animals regarded as serologically positive, which reacted in RBT, SAT, and CFT, 14 gave positive results in ELISA, whereas 18 were negative. Among these ELISA-positive sera, 13 were also positive in FPA. All samples positive in SAT, RBT, and CFT, and negative in ELISA, were also negative in FPA.

Key words: Brucella, bovine brucellosis, RBT, SAT, CFT, FPA.

Brucellae are Gram-negative, facultative, intracellular bacteria that are pathogenic for humans and a variety of domestic 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 (1, 4, 15). Their classification is mainly based on differences in their 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 (26-28). In humans, the infection generally results from transmission via the gastrointestinal route by the consumption of unpasteurised dairy products, air borne transmission by inhaling dust contaminated by aborted tissues, and transmission caused by laboratory-associated exposure to aerosols (1, 4, 13-15). In regard of large scale of investigations, the laboratory diagnosis of brucellosis is mainly based on serological tests (1, 15, 16). 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 (8, 24). Traditional and well-documented techniques for serological diagnosis include the Rose Bengal test (RBT), serum agglutination test (SAT), complement fixation test (CFT) and, more recently, ELISA being put into more regular use (16, 17, 25, 28). The fluorescence polarisation assay (FPA) has a shorter history of use and has yet to become established within the routine testing procedures of most National Reference Laboratories for Brucellosis (15, 20, 21, 23). Fluorescence polarisation measures the excitation by plane polarised light of a fluorescent molecule. Measurement of returned photons in the planes parallel and perpendicular to the excitation plane allows for the assessment of the rotation of fluorescent molecule (fluorophore). While all other factors remain constant, the rate of rotation of this molecule is inversely proportional to its size (22). Thus, the rotation of a fluorophore conjugated to, in this case, Brucella O-chain, will slow down if bound by anti-Brucella antibodies. The FPA is rapid and requires no solid phase bound reagent or removal of excess reagents. It is host species-independent and can be conducted also on whole blood or milk (2, 5, 18, 27).

The aim of this study was the application of FPA for the examination of bovine sera and a comparison of the results of FPA with the results of traditional serological techniques such as RBT, SAT, CFT, and ELISA.
Material and Methods

Sera samples. Six hundred thirty-five sera from cattle, including 300 sera from healthy animals, 32 sera from animals regarded as serologically positive for brucellosis and culled, and 303 sera originated from confirmatory investigations, finally classified as negative, were used. The sera were obtained from regional laboratories in 2008-2010 and sent to the National Reference Laboratory for Brucellosis in the National Veterinary Research Institute in Pulawy for further investigation. 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 was performed using commercial Brucellosis serum ELISA (Pourquier, France), whereas RBT, SAT, and CFT were done according to official instructions and protocols (10-13, 15)

FPA. Fluorescence polarisation assay was conducted in borosilicate disposable glass (VWR, USA). Initially 1 ml of a dilution buffer was placed into four 10x75 mm borosilicate tubes, then 10 µl volumes of: negative control were added to three of them, and positive control to the remaining one. Both control samples were obtained from Diachemix, USA. Samples were mixed, incubated at room temperature for 5 min, and an initial reading, referred to as the blank intensity reading, was taken using FPA Reader Sentry 100 (Diachemix, USA). Subsequently, 10 µl of a conjugate was added to each sample, and after mixing and incubation at room temperature for 3 min., a second reading was taken, referred to as sample reading. The reader automatically subtracted the initial reading and calculated a value for every sample in millipolarisation units (mP). The mean mP for three replicates of the negative control should be noted between 70-95 mP and for the positive control over 120 mP, according to the manual obtained from the manufacturer. After these initial steps, the bovine sera samples were tested. The results were interpreted as negative, if millipolarisation units of the samples were less than 10 mP above the mean negative control. Any results greater than 20 mP of the mean of the negative control were positive. The samples in the range of 10-20 mP were recognised as suspected and then were retested in duplicate, using 20 µl of the sera samples.

Results

Table 1 summarises results of serological examination. All sera originated from healthy animals were negative in ELISA, RBT, SAT, and CFT. In the FPA, all samples remained negative. The values below 90 mP were noticed. Among 303 sera obtained from regional laboratories for confirmatory investigations, 269 were positive in RBT and SAT, 21 were positive in SAT, and remaining 13 were RBT-positive. Only two sera, one positive in RBT and SAT and one SAT-positive, were also positive in FPA with values of mP=153.4 and mP=136.1, respectively. Among 32 sera originated from animals regarded as serologically positive for brucellosis, reacting in RBT, SAT, and CFT, 14 gave the positive results in ELISA, whereas 18 were negative. Among ELISA-positive sera, 13 were also positive in FPA with mP value ranging from 129.4 to 178.1. One remaining sample, which was FPA-negative resulted in mP value of 89.4. All samples, positive in SAT, RBT, and CFT, but negative in ELISA, were also negative in FPA.

Discussion

Quick and accurate diagnosis of brucellosis is very important for a positive outcome of eradication and monitoring programmes. The isolation of Brucella sp. from blood, tissues, and milk cultures is the only mean of definitively proven infection. In addition to bacteriological examination, molecular methods based on DNA amplification of targeted genes are used in many laboratories (3, 6, 7, 9, 19). The disadvantages of these techniques are that they are expensive, time consuming, require specialised laboratories, and could not be used in many regions of the world (7, 19). Due to a scale of investigations, lower costs and the ability to be performed by most laboratories, serology still has a dominant role to play in diagnosis of the disease (13, 20, 21). The most economical and most widely used laboratory tests in diagnosis of the disease are the agglutination tests, SAT, and RBT (1, 13, 15, 16) but the interpretation of their results is largely subjective.

Table 1

<table>
<thead>
<tr>
<th>Bovine sera according to the serological results</th>
<th>FPA-positive</th>
<th>FPA-negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>All serological assays negative (n=300)</td>
<td>0</td>
<td>300</td>
</tr>
<tr>
<td>RBT-positive and SAT-positive (n=269)</td>
<td>1</td>
<td>268</td>
</tr>
<tr>
<td>SAT-positive only (n=21)</td>
<td>1</td>
<td>20</td>
</tr>
<tr>
<td>RBT-positive only (n=13)</td>
<td>0</td>
<td>13</td>
</tr>
<tr>
<td>RBT-positive, SAT-positive and CFT-positive (n=32)</td>
<td>ELISA-positive (n=14)</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>ELISA-negative (n=18)</td>
<td>0</td>
</tr>
</tbody>
</table>
Many other tests have been widely studied such as the CFT (warm and cold) and binding assays (ELISA) (5, 13, 17, 26, 28). The advantages of these tests are the high sensitivity and specificity and the ability to detect the non-agglutinating antibodies, which is a great importance in the chronic and localised forms of the disease, but some of these are complex, laborious, and expensive. Cross reactions between Brucella sp. and a number of other microorganisms has been well documented in the past and reviewed in a considerable detail by Corbel (1). Similarity of the OPS, the immunodominant region of the LPS molecule is responsible for most of the observed cross reactions (8, 24). To improve serological diagnosis at least two tests could be used in parallel to avoid false positive, or false negative results (1, 15, 16). FPA is an increasingly used method in diagnosis of animal brucellosis and has many advantages: is very quick (ca. 5 min per sample), does not require specialised staff, may be performed under field conditions, also with battery supply, and because data are obtained electronically, it is an objective test. The FPA has been validated for a large number of species, including humans, cattle, swine, and bison, and it is an alternative OIE test for diagnosis of bovine and swine brucellosis (5, 14, 15, 17, 18, 22, 23). The various studies of Nielsen et al. (22, 23, 25) showed that among the serological assays, the FPA has a higher sensitivity (99.0%-99.3%) and specificity (96.9%-100%), in comparison to ELISA (sensitivity - 92.5%-100%, specificity - 90.6%-100%), and CFT with sensitivity (23.0%-97.1%) or specificity (30.6%-100%), respectively. On the other hand, the study of Konstantinidis et al. (13) showed that the magnitude of sensitivity is: ELISA> FPA> RBT.

In our study we observed a full correlation between results of examination of negative samples tested with RBT, SAT, CFT, ELISA, and FPA. In regard to sera from confirmatory investigations, only two suspected samples of 303 tested (one positive in SAT and RBT and one positive in SAT only) gave also positive results in FPA, but these samples were CFT and ELISA-negative. Two different situations were observed when the positive samples from animals finally classified as serologically positive (RBT, SAT, and CFT-positive) were tested in FPA. In our study we found that all 18 samples positive in RBT, SAT, and CFT, but negative in ELISA, were also negative in FPA. On the other hand, among 14 samples positive in four serological assays (RBT, SAT, CFT, ELISA), only one sample showed the low value of mP in FPA and was classified as negative. Observing discrepancies between the results of FPA and other serological methods, regarding the sera from animals classified as positive for brucellosis it should be stressed that Brucella infection was not confirmed in these animals in any case by culture. So the origin of Brucella antibodies in the sera was not unequivocally established.

Taking into consideration the diagnostic value and other advantages of the FPA, such as the speed of obtaining the results, the objectivity of the interpretation of the results n, as well as the cost, this method should be included in routine serological examinations for brucellosis. However, further studies are needed to assess the reproducibility of FPA.

References