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Field evaluation of fluorescence polarization assay, and comparison with competitive ELISA for the detection of antibodies against *Brucella melitensis* in sheep in Sicily, Italy.

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Abstract

The relative sensitivity of the fluorescence polarization assay (FPA) was estimated by testing blood sera from 1515 sheep, belonging to flocks infected with *Brucella melitensis* in Sicily, which were previously shown as positive to at least two of the following tests: rose bengal test (RBT), complement fixation test (CFT), competitive ELISA (c-ELISA). FPA specificity was estimated by testing sera from 719 sheep from *B. melitensis*-free flocks from Sicily and Sardinia. Based upon results of a receiver-operating-characteristic (ROC) curve, optimal FPA cut-off value in millipolarization units (mP) was chosen at the mean of negative controls tested in the same assay, plus 6, which corresponded to 96.0% sensitivity (95% confidence interval: 93.9, 97.3) and 100% (99.5, 100) specificity. Such a cut off value was the one yielding the maximum sum of sensitivity and specificity (196.0), and it was adopted for subsequent analyses. Maximum FPA sensitivity (98.8%; 91.7, 99.8) was obtained on sera which were positive to CFT and c-ELISA, but negative to RBT. Median FPA values (first, and third quartiles) (109.7, 53.7, 169.3) were greatest for sera which were positive to RBT, CFT, and c-ELISA. Sensitivity of c-ELISA, which was estimated as the proportion of positive results on RBT and CFT positive sera, was 99.8% (99.4, 1.0), whereas specificity was 100.0% (98.5, 100.0). Although FPA resulted as an accurate test, c-ELISA was characterized by a superior sensitivity and it is, therefore, proposed to be used, in parallel with RBT, to increase the testing sensitivity in regions and flocks which can be considered as at a great risk of infection.

Keywords: Brucella melitensis, fluorescence polarization assay, ELISA, sensitivity, specificity, Sicily.

Introduction

Ovine and caprine brucellosis, caused by *Brucella melitensis*—a gram-negative, facultative, intracellular bacterium—is mostly associated with reproductive failure. The agents are disseminated through fetal and vaginal fluids, or via colostrums and milk (Garin-Bastuji et al., 1998). In humans, *B. melitensis* causes a disease of variable severity, and it is a persistent threat to animal and public health in certain European countries (EFSA and ECDC, 2014), where eradication plans are co-funded by the European Commission, as part of the EU Animal Health Strategy¹. In certain Mediterranean regions, eradication might be delayed by difficulties in the application of measures such as identification, isolation, and prompt removal of infected animals (SANCO, 2009). Under these circumstances, inaccurate diagnostic tests have a particularly negative impact on disease eradication programs. In fact, imperfect test sensitivity (Se), and consequent false negative results, may lead to the persistence of infected animals serving as sources of infection. On the other hand, imperfect specificity (Sp) and false positive tests might cause the loss of qualification of free status and the adoption of unnecessary disease control measures.

In Southern Italy, testing strategy includes the execution of rose bengal test (RBT) on all animals in a flock (flock screening test). In case of positive result in at least one animal, the entire flock is, subsequently, tested by complement fixation test (CFT) (Anon., 2006, 2012). Sensitivity of RBT at the individual level is considered as relatively low (Blasco et al., 1994, Nielsen et al., 2005; Minas et al., 2005, Garin-Bastuji et al., 2006), whereas the probability of at least one animal resulting as positive in an infected flock (flock-level sensitivity) increases with within-flock prevalence.

In Sicily (Messina province), within-flock prevalence of *B. melitensis* (as estimated by means of the above described testing strategy) was shown to be generally low. In 2012, less than 5 positive animals were detected in 74% of infected flock, and single reactors were detected in approximately 41% of positive flocks (Fiasconaro et al. unpublished). As a consequence, flock-level sensitivity of RBT might be reduced, and infected flocks might go undetected. Furthermore, single reactors in an otherwise negative flock might be

¹ http://ec.europa.eu/food/animal/diseases/index_en.htm, accessed 2 September, 2013.

indicators of the introduction of the infection, or the consequences of false positive test results. Therefore, increasing the sensitivity of the flock screening testing, as well as the specificity at the individual level are critical for eradication of ovine and caprine brucellosis. Increased sensitivity and specificity might be achieved by using laboratory tests for the detection of antibodies against *B. melitensis*, such as ELISA and fluorescence polarization assay (FPA), which are based upon the direct detection of binding between antibodies and antigens. Indeed, sensitivity of RBT, which is based upon the detection of secondary effects of bindings, might be reduced by the prozone effect (Nielsen and Duncan, 1990), which might lead to false negative RBT results in individuals characterized by high antibody concentrations, resulting in very small antigen-antibody complexes that do not form visible agglutination.

FPA is a rapid assay, not requiring repeated washing procedures. It is based on the use of a fluorescence polarization analyzer to measure the reduced rate of rotation of large molecules in solution, such as antigen-antibody complexes which may be present in blood sera from infected animals (Nielsen et al., 1996). FPA was shown as an accurate test in the detection of antibodies against *B. abortus* in cattle. It was, subsequently, used for the diagnosis of brucellosis in goats and sheep, where Se and Sp were estimated as > 95%. FPA was, therefore, judged as a promising diagnostic test, and further, field validation was suggested, especially in those areas where eradication of brucellosis in small ruminants is still to be achieved (Nielsen and Gall, 2001, Burriel et al., 2004; Minas et al., 2005, Minas et al., 2007).

The objective of this study was to estimate accuracy of FPA by using a sample of sera from sheep populations from Southern Italy, where *B. melitensis* eradication and surveillance are currently implemented, and to obtain indications on the usefulness of FPA to increase Se and Sp of the testing procedure within ovine and caprine eradication programs. Accuracy of FPA was, then, compared with that of c-ELISA, which is a currently available, rapid test, which was previously proposed for improving accuracy and reducing costs of antibody testing for small ruminant brucellosis in Italy (Portanti et al., 2006).

Material and methods

Selection of sera

Blood samples were collected from sheep over six months of age, during the Italian eradication program of ovine and caprine brucellosis, and submitted to the official laboratories at the Istituto Zooprofilattico Sperimentale (IZS) for testing. Group A sera were collected from 1515 sheep, belonging to 174 *B. melitensis*-infected flocks in Sicily, which yielded positive results for at least two of these tests: RBT, CFT, c-ELISA. Sera selected by this criterium may lead to an overestimated test Se. In fact, sera from infected animals, but with an undetectable antibody response, might yield negative results to all serological tests (including FPA) and would, therefore, have been excluded from our sample. An early, or a chronic stage of infection might explain such and undetectable antibody response in infected animals. Therefore, overestimation of Se in our study might be attributable to a *spectrum of disease bias* (Greiner and Gardner, 2000). Furthermore, a limited antibody response in infected sheep might lead to positive results to one test only. As a consequence, our sample would only include strongly reactive sheep, which would be characterized to a relatively high probability of positive FPA results.

The sample size of group A sera allowed to estimate Se of FPA with a maximum, exact binomial error equal to 1.2%, with a 95% confidence level, and after assuming an expected sensitivity of 95% (*binom.test* function in the R software, R core team, 2012). Group B included sera from 417 sheep from officially free flocks in Sicily, plus 248 sera from the officially free region of Sardinia. Such a sample size (n=719) allowed the estimation of specificity with a maximum exact binomial error equal to 1.9 %, with a 95% confidence level, and after assuming an expected specificity of 95%. To estimate c-ELISA Sp, an independent sample of sera was, subsequently, collected from sheep from the Southern Italian region of Apulia (n=244), from flocks which were never found infected by *B. abortus*.

Laboratory test

After clotting of blood, sera were separated by centrifugation at 930 rcf/g for 20 min, then stored at -75 °C, to allow the execution of serological tests at different times. RBT and CFT were carried out according to the methods described in the 5th edition of the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE, 2000). In RBT, standardized, killed *B. abortus* strain 99 (Weybridge) (S99) cells, stained with rose Bengal and buffered to pH 3.65 ± 0.05 were used as antigens. Aliquots of 30 µl of sera, and 30 µl of antigen were dispensed, by a Tecan Freedom Evo[®] robot, on a white glass plate, and mixed by a ASAL 711[®] orbital mixer for 4 min. The wells were examined by incident light to identify sera-agglutination reactions. Any specimen showing any degree of agglutination was considered to be positive.

The CFT test was carried out after sera complement was inactivated at 60 °C for 30 s. The antigen was provided by the IZS of Lombardy and Emilia Romagna, validated with National Standard Serum and OIESS (International Standard Serum). Guinea pig complement was obtained from ID.VET Innovative Diagnostics[®], Hemolytic Sera from Biomerieux[®], whereas Sheep Blood in Alsever's Solution was provided by IZS of Sicily. A serum sample was considered positive if at least 50% of fixation was evident at dilution 1:4 ($\geq 20\text{UI/ml}$).

The c-ELISA (Brucella-Ab c-ELISA) was manufactured by IZS of Abruzzo and Molise, for the detection of *Brucella* antibodies in cattle, sheep and goat sera, using a monoclonal antibody (MAb 4B5A), produced against *Brucella melitensis* biotype 2. The kit was used as describes in Portanti et al (2006).

The FPA test was performed by using a commercial kit (*Brucella abortus* Antibody test kit, manufactured by Meridian Life Science[®], Inc., for Diachemix[®]), following manufacturer's instructions, and a single-channel multi-mode microplate reader (Synergy HT Multi-Mode Microplate Reader[®]). A 485 nm wavelength (20nm bandpass) excitation filter and a 528 (20) emission filter, with a 510 nm cut off dichroic mirror, were used². Briefly, 20 µl of sera supernatant, as well as 20 µl of controls (three negatives, and one positive controls provided by the kit manufacturer) were placed into flat-bottom wells of microtiter plates (Greiner bio-one[®] Art. 655209). Subsequently, 180 µl of diluted reaction buffer (one part of Reaction Buffer Concentrate with

² http://www.biotek.com/assets/tech_resources/Diachemix_App.pdf

24 parts of deionized water) were added to the same wells. Plates were incubated for 3 minutes at room temperature. A background reading, in mP, was taken and, subsequently, 10 μ l of conjugate (O-polysaccharide extracted from *B. abortus* bacteria and labeled with fluorescein-isothiocyanide), were added into wells. The plates were then incubated for 2 minutes at room temperature and a second reading was taken. To reduce the effect of inter-assay variability on test results, FPA values were expressed as the difference between FPA reading (mP) for each tested serum, and the mean reading of the negative controls which were tested in the same plate.

Statistical analysis

Median and first (Q_1) and third (Q_3) quartiles of FPA values (mP) were calculated for sera belonging to groups A and B, by using the *summary* function in the R software (R Development Core Team, 2012). Sera belonging to group A were further classified into four subgroups based on combination of results of RBT, CFT, and ELISA: subgroups A1 included sera which were positive to all of the three tests; A2 included sera resulting RBT and CFT-positive, but c-ELISA-negative; A3 included RBT and c-ELISA-positive sera, but CFT-negative; A4 included RBT-negative sera, but c-ELISA and CFT positive.

A receiver-operating-characteristic (ROC) curve was built to identify combination of Se (estimated as the % of animals belonging to group A yielding positive results by FPA test) and Sp (% of group B animals yielding negative FPA results) at varying cut off values (Greiner et al., 2000, OIE, 2009). The area under the curve (AUC) was calculated as a measure of overall accuracy of the test. The packages *ROCR* and *verification*, in the R software, were used. The cut off value for subsequent analyses was selected as the one yielding the maximum sum of Se and Sp.

Percent of FPA positivity, as an estimate of test Se, and 95% confidence intervals were obtained for each subgroup: A1, A3, A4, by using a intercept-only, generalized estimating equation (GEE), which was implemented by the GENMOD procedure, in the SAS System 9.3 (SAS, 2011). Such an approach was used to take into account the potential non independence of FPA results which were obtained on sera from sheep belonging to the same flock (Greiner and Gardner, 2000, Molenberghs and Verbeke, 2005). In fact, the

probability of obtaining positive test results on infected sheep might vary due to common factors affecting animals belonging to the same flock, including age, stage of pregnancy, health status as well as the stage of infection (latent, chronic and incubation period) (Blasco et al., 1994, Minas et al., 2005). GEE was also used to estimate percent of c-ELISA positivity, as an estimate of test Se, and 95% confidence interval, on sera which were positive to RBT and CFT (subgroups A1-A2).

Results

The ROC curve obtained by testing sera from non infected, and infected sheep, by FPA, yielded an area under the curve of 0.99. At a cut off corresponding to the mean FPA value of negative controls plus 4, both Se and Sp of FPA were > 95% (Table 1). Considering that, by moving the cut off from 4 to 6, there was a 1.3% reduction in test sensitivity (from 97.3 to 96.0), and a corresponding 3.3% increase in Sp (from 96.7 to 100%), the mean of negative controls plus 6 was chosen as the optimal combination of Se and Sp and was adopted in subsequent analyses. In fact, such a cut off corresponded to the maximum sum of Se and Sp estimates (196.0).

Cut-off	Sensitivity (95% CI)	Specificity (95% CI)
3	98.5 (97.1, 99.2)	93.0 (90.9, 94.8)
4	97.3 (95.7, 98.3)	96.7 (95.1, 97.8)
5	96.4 (94.5, 97.7)	98.7 (97.6, 99.4)
6	96.0 (93.9, 97.3)	100.0 (99.5, 100)
7	95.7 (93.6, 97.1)	100.0 (99.5, 100)

Table 1. Estimates of sensitivity and specificity of the fluorescence polarization assay (FPA) at different cut off values, by using 1515 infected and 719 non infected sheep as the reference sample. Cut-off values were calculated as the difference between FPA millipolarization values of tested sera and the mean of 3 negative controls tested in the same assay. CI: confidence interval.

Median (Q1, Q3) FPA mP values for sera belonging to group A are shown in Table 2. Median FPA values were greater for sera belonging to subgroup A1 than for other groups of sera. Sera from non infected sheep (group B) resulted in homogeneously low FPA values, median (Q1, Q3) was 0.0 (-1.6, 1.3). Estimates of percent of positive FPA results on sera which were positive at different combinations of RBT, CFT, and c-ELISA are summarized in Table 2. Greatest percent of positive results, as an estimate of Se relative to each sub group, was recorded by using sera belonging to groups A4 and A1 as positive sample. Whereas relative Se of FPA was lower for sera belonging to category A3 (positive to RBT and ELISA, negative to CFT). One out of two sera of class A2 was positive but it is not possible to draw conclusions based on such a limited sample.

Subgroup (n)	RBT	CFT	c-ELISA	Median (Q ₁ , Q ₃) FPA mP values	Percent FPA positive (95% CI)
A1 (1297)	+	+	+	109.7 (53.7, 169.3)	97.2 (95.1, 98.4)
A2 (2)	+	+	-	29.8 (nd, nd)	50.0 (0.6, 94.1)
A3 (131)	+	-	+	27.7 (12.3, 61.8)	86.1 (78.7, 91.3)
A4 (85)	-	+	+	63.0 (33.7, 114.7)	98.8 (91.7, 99.8)
Overall (1515)				97.3 (45.7, 163.7)	96.0 (93.9, 97.3)

Table 2. Result of a fluorescence polarization assay (FPA) for the detection of antibodies against *B. melitensis*, on sera from infected sheep, classified based on results of other serological tests. RBT: rose Bengal test. CFT: complement fixation test. c-ELISA: competitive ELISA. Q₁: first quartile. Q₃: third quartile. mP: milli-polarization units. CI: confidence interval. nd: not determined.

Se of c-ELISA on sera of subgroups A1-A2, combined was 99.8% (99.4, 1.0), which was greater than Se of FPA on the same sera: 97.2 (95.0, 98.4). Sp of c-ELISA on sera from non infected sheep from Apulia was 100.0 (98.5, 100.0).

Discussion

Based upon results of this study, FPA was confirmed as an accurate method for the detection of antibodies against *B. melitensis* in sheep (Minas et al., 2005, 2007). By choosing a cut off value equal to the negative control mean plus 6 mP, Sp was estimated at 100%, whereas Se was lower (96%). Considering that Se might have been overestimated due to the our criteria for the selection of sera from infected animals, such an imperfect Se of FPA (and of official diagnostic tests, such as RBT and CFT) must, however, be taken into account in when considering this test for use in *B. melitensis* eradication plans. Variations were observed in FPA relative Se on different groups of sera, which were classified based on positivity to two out of three test (RBT, CFT, c-ELISA). Best Se (98.8%), which was obtained on RBT-negative sera (group A4). Although the occurrence of prozone effect was not investigated in our study, this might have accounted for RBT-negative results on sera which were, subsequently, classified as positive by FPA.

The very high accuracy of c-ELISA that we found in this study is in agreement with previous findings (Portanti et al., 2006). Indeed, although FPA was shown as an accurate and rapid test, c-ELISA appears to be the superior in terms of Se, which is a critical factor in the eradication of *B. melitensis* in Southern Italy. An increased Se would be particularly useful in traditional husbandry systems where frequent testing of animals is problematic and where infectious individuals might establish frequent direct, or indirect contact with susceptible individuals. Furthermore, the use of RBT and c-ELISA in all animals in a flock would be favored by the relatively rapidity of c-ELISA. Such an approach would favor Se of the testing strategy, and it could be adopted in high risk situations, where the negative consequences of false negative test results would outweigh the risk of false positives. When c-ELISA is not used in the screening process, the excellent Sp of this tests would also make it useful as a further confirmatory test in those cases where RBT and CFT yielded positive results on single, or few animals in low-prevalence flocks or regions. Integration of a risk assessment process with accurate diagnostic tests would be useful to complete the eradication of *B. melitensis* in Southern Italy, where remaining foci of infection have shown to be difficult to detect, by means of traditional testing approaches.

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