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Comparison of fluorescence polarization assay with Rose Bengal (RB) test and complement fixation tests for the diagnosis of buffalo (*Bubalus bubalis*) brucellosis in a high-prevalence area

S. Montagnaro[1], U. Pagnini[1], T. Diana[1], L. Bruno[1], L. Baldi[2], G. Iovane[2]

[1] Department of Pathology and animal Health – School of Veterinary Medicine University of Naples "Federico II", Naples, Italy

[2] Istituto Zooprofilattico sperimentale del Mezzogiorno- Portici - Naples, Italy

Corresponding author: S. Montagnaro. Department of Pathology and animal Health - School of Veterinary Medicine - University of Naples "Federico II" - Via Delpino n°1 - 80137 - Naples. Email: serena.montagnaro@unina.it

ABSTRACT: The fluorescence polarisation assay (FPA) was evaluated for the serological diagnosis of bovine brucellosis in buffalo (*Bubalus Bubalis*) in southern Italy. This assay uses O-polysaccharide prepared from *Brucella Abortus* lipopolysaccharide conjugated with fluorescein isothiocyanate as a tracer. It has many methodological advantages over older, more established tests and can be performed in short time. To measure the fluorescence polarization, a Tecan GENios Pro (Prionics) fluorescence-polarization analyzer was used with the procedure described by Nielsen *et al.* 1996. A cut-off value of 117 millipolarization (mP) units was used for testing 912 buffalo sera from Campania Region (526 positive sera and 386 negative sera according to the complement fixation test; CFT). All samples were tested with the Rose Bengal plate (RB). Sensitivity and specificity (Sn) for RB were 84.0% and 87.8% and for FPA were 92.6% and 88.9 percent. The FPA gave a kappa coefficient of agreement with respect to CFT of 0.755, while RB (relative to the CFT) gave coefficients of 0,715. Finally, ROC analysis suggested a cut-off value which did not agree with the one recommended in the test procedure for cow.

Key words: Brucellosis; *Bubalus bubalis*; FPA.

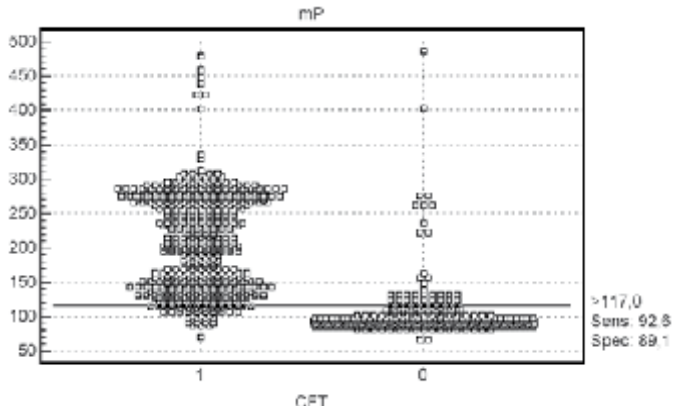
INTRODUCTION - Brucellosis in bovine and buffaloes is responsible for important economic losses in bovine and buffalo farming. It is also a widespread zoonosis constituting a serious public health problem wherever the infectious agent is endemic, like in Mediterranean and Middle-East countries. Diagnosis of brucellosis is complicated by variable incubation time and the absence of clinical signs other than abortion. Serological techniques based on the detection of antibodies to the LPS antigen of smooth Brucella strains are the mainstay of diagnosis and mass testing programmes. The Rose Bengal Test (RBT) and complement fixation test (CFT) are the most accepted tests worldwide for this purpose. FPA is

based on the rotational differences between a small fluorochrome labelled antigen molecule in solution and the antigen molecule complexed with its antibody. Thus, the rotation of a fluorescent molecule (fluorophore) conjugated to Brucella O-chain will slow if bound by anti- Brucella LPS antibodies. The FPA is rapid and requires no solid phase bound reagent or removal of excess reagents. It is host species-independent and can also be conducted on whole blood (Nielsen, K. *et al.*, 2001a)and milk (Nielsen, K. *et al.*, 2001b). The aim of the present study was to evaluate FPA for the diagnosis of *B. abortus* infection in buffalo, determinate the cut-off offering the highest performance index and compare its performance with that of the other tests used for this purpose.

MATERIAL AND METHODS - For this study, buffalo serum samples were obtained from the Istituto Zooprofilattico Sperimentale del Mezzogiorno (IZSM). Official or accredited veterinarians performed buffalo bleeding as a part of the eradication program in Campania Region, Italy. A total of 912 samples, 386 tested as CFT negatives and 526 CFT positives were supplied by the IZSM for the present study. RB and CFT were performed at IZSM using antigens and procedures according to the Italian and European Official Norm (D.M. n.651 27/08/1994; D.M. n.429 12/08/1997; OIE Manual, 2000). The antigens were prepared by Istituto Zooprofilattico Sperimentale di Teramo using *B. abortus* strain 99 (Weybridge). A negative diagnosis for a sample is the result of a RB negative test or RB positive combined with a negative CFT result, whereas a positive diagnosis consists of positive RB and CFT results. The FPA was performed using a *Brucella* FPA Antibody Test Kit (Prionics, Italia S.r.l), using a 96-well microtitre plate format. The first step of the test required the addition of 180 µl of buffer to 20 µl of test serum within each test well. Control samples were added to each plate, a minimum of 3 wells for the negative control and also 1 high positive control wells. Buffer and test sample were mixed by repeated pipette action, and the test plates were incubated and read on a GENios Pro microplate reader (Prionics Italia S.r.l.) (reader set to gain of 53 and number of flashes per well at 25) to obtain a background reading, in relative fluorescent units (RFUs), for each sample. Subsequently, 10 µl of antigen (*Brucella* O-polysaccharide conjugated isothiocyanate fluorophore) was added to each well and mixed by pipette action followed by a further 2-min incubation as above. The plate was read again as before to obtain the raw parallel and perpendicular data for each sample. The analyzer automatically subtracts the background reading and calculates a value for the sample in millipolarization units (mP). The sensitivity (Sn) and specificity (Sp) of the FPA and RBT relative to the CFT (plus 95% confidence limits), the kappa measures of agreement between tests and the receiver operator characteristic (ROC) analysis curve were calculated with the aid of the MedCalc program. Results were expressed in a ROC curve analysis graph that plots the true positive rate related to the false positive rate at different cut-offs points. In addition, MedCalc provides a DOT graph displaying the results of individual tests according to the value of the classification variable and a horizontal line that represents the cut-off value (maximum value for both the Sn and Sp). This value may be changed for different purposes; in a screening test, a cut-off value with a higher Sn must be selected, and in positive cases, a second, more specific test should be performed (Schoonjans, F. *et al.*, 2005).

RESULTS AND CONCLUSIONS - To Compare FPA and RBT with CFT, 912 buffalo sera samples, 526 CFT positive and 386 CFT negative samples were evaluated by RBT, FPA test and ROC curve analysis. Sn of FPA was 92.6% (C.I. 95%, 90,0% - 94,7%), Sp was 89.1% (C.I. 95%, 85,6% - 92,0%) and accuracy was 95.5% (C.I. 95%, 94,0% – 96.8%) at a cut-off of 117 mP. The DOT diagram for FPA compared with the CFT is showed in Figure 1, where every square represents a single FPA result; CFT negative samples are showed as CFT = 0, CFT positive samples are showed as CFT = 1, and the horizontal line located at 117 mP represents the cut-off line for Sn (92.6%) and Sp (89.1%) calculated by ROC analysis.

Figure 1. DOT diagram of FPA relative to CFT results. Each square represents a single sample result. The horizontal line at 117 mP is the cut-off value determined by the ROC curve analysis. Points above or below this line are considered as positives or negatives by FPA, respectively. Increasing this level will make the test more specific, but less sensitive, and vice versa. 1, CFT positive; 0, CFT negative.



As shown in the figure, the cut-off of 117 mP provided the highest combined Sn and Sp values. Sn, Sp and kappa index for the various tests are reported in table 1. The Sn, Sp and performance index were lower in RBT than FPA. Finally, kappa values between FPA and CFT and between RBT and CFT were 0.755 and 0.715 respectively (Table 1).

Diagnostic procedures for brucellosis should be specific, sensitive, and detect all stages of the infection. The RB test (Nielsen, K. *et al.*, 1996) is considered suitable for screening individual animals, however, false negative reactions occurs. Antibody resulting from *B. abortus* S19 vaccination and some cross reacting antibodies are detected by this test and it

Test	% Sn	95% C.I.	% Sp	95% C.I.	Pi	AUC	Agreement whit CFT	
							Kappa	95% C.I.
FPA	92.6	90.0 – 94.7	89.1	85.6 – 92.0	181.7	0.955	0,755	0,711 to 0,799
RBT	84	81.2 – 87.5	87.8	84,1 - 90,9	171.8	0.862	0,715	0,669 to 0,76

is necessary to use other test(s) to confirm reactor animals as infected (Morgan WJ *et al.*, 1969; Nielsen, K. *et al.*, 2002). The CFT is recognised as a highly sensitive and specific test when correctly performed, yet its comparative underperformance in this study questions these properties. The CFT also has many practical drawbacks, not least of all is its relative technical complexity. The test is also subject to anticomplementary reactions and will not work on haemolysed samples. For acceptance of a new test for the presumptive diagnosis of brucellosis, it would be required to perform as well as or better than the in-use test or possess an advantageous attribute. The cut-off of FPA was determined using Medcalc and CFT like golden standard. Sensitivity (Sn) and specificity for RBT were 84.0% and 87.8% and for FPA were 92.6% and 89.1%, respectively. This may suggest that FPA is more sensitive than RB. Taken together, these results suggested that FPA might replace RB as a screening test for its better performance compared with CFT, its adjustable cut-off useful in different epidemiological situations, and for its reliability, ease of performance and potential application in field and high-throughput laboratories. However, more studies are required for its approval as a diagnostic tool for buffalo brucellosis, by testing a larger number of serum samples from brucellosis-free areas, and bacteriologically positives animals.

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