Comparison of complement fixation test, immunoblotting, indirect ELISA, and competitive ELISA for detecting antibodies to *Mycoplasma mycoides* subspecies *mycoides* small colony (SC) in naturally infected cattle from the 1995 outbreak in Botswana

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ABSTRACT

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Four serological tests were compared in order to evaluate their efficacies in detecting antibodies to *M. mycoides* subspecies *mycoides* SC in cattle sera sampled in 1995 from herds affected with contagious bovine pleuropneumonia (CBPP) in the north-western part of Botswana.

Tests that were compared included immunoblotting test (IBT), indirect enzyme-linked immunosorbent assay (i-ELISA), competitive enzyme-linked immunosorbent assay (c-ELISA) and complement fixation test (CFT). The percentages of seropositive samples in the iELISA (48%) and in the c-ELISA (48 %) were similar but were comparatively lower than those obtained by the IBT (57 %) and CFT (61 %). The percentages of positive sera in the IBT and CFT were also similar and overall the efficacy of these tests was better than that of the two ELISA tests. There was 95.5 % agreement between the IBT and CFT, 85 % agreement between the IBT and c-ELISA, 90.9 % agreement between the IBT and i-ELISA, 88.6 % agreement between the i-ELISA and CFT, 80 % agreement between the c-ELISA and CFT and 90 % agreement between the two ELISA tests.

It became clearly evident from this comparative study that no single serological test was capable of detecting all animals affected by CBPP under natural field conditions of infection.

Keywords: Botswana, CBPP, competitive ELISA, complement fixation test, immunoblotting, indirect ELISA

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INTRODUCTION

The complement fixation test (CFT), improved by Campbell & Turner (1953), is widely used for the serodiagnosis and/or serosurveillance of contagious bovine pleuropneumonia (CBPP), a contagious and fatal disease of cattle caused by *Mycoplasma mycoides* subspecies *mycoides* small colony (SC) type (Provost, Perreau, Breard, Le Goff, Martel & Cottew 1987). With a specificity of 99.5 % the CFT is capable of detecting nearly all sick animals with

acute disease, but the test can detect only a small pecentage of infected animals in the early stages of the disease (Amanfu, Masupu, Adom, Raborokgwe & Bashiruddin 1998a; Anon 2000). CFT also shows lack of sensitivity in chronically infected animals. Some false positives may also occur. CFT is difficult to standardise due to the use of labile biological reagents (complement, sheep red blood cells), hence comparisons between laboratories may be difficult in case of slight positives. Some cases cannot be analysed with CFT because of serum incompatibility.

In view of the drawbacks of the CFT, alternative serological tests with a reported higher sensitivity than this test have been developed and improved. Such tests have included the indirect ELISA (i-ELISA) (Onoviran & Taylor-Robinson 1979), the competitive ELISA (c-ELISA) (Le Goff & Thiacourt 1998) and immunoblotting (IBT) (Gonçalves, Regalla, Nicolet, Frey, Nicholas, Bashiruddin, De Santis & Goncalves 1998). Comparative studies between the CFT and ELISA tests carried out previously have shown that the ELISA tests are more sensitive than the CFT in detecting CBPP antibodies in sera with very low titres, but are less specific than the CFT (Amanfu, Sediadie, Masupu, Benkirane, Geiger & Thiaucourt 1998b; Thiaucourt 1998; Nicholas, Santini, Clark, Palmer, De Santis & Bashiruddin 1996).

In the present study, IBT, i-ELISA, c-ELISA and the CFT were compared to evaluate their relative effectiveness in detecting antibodies to *M. mycoides* subspecies *mycoides* SC in sera taken during the outbreaks in Botswana in 1995 from indigenous Tswana cattle naturally exposed to CBPP.

MATERIALS AND METHODS

Animals

Local Tswana breed of cattle (*Bos indicus*) were collected from foci of the 1995 CBPP outbreak in north-western Botswana (Amanfu *et al.* 1998a) and kept in a government quarantine station at Nokaneng for two months and then slaughtered for laboratory investigations. Thorough post mortem examination of the lungs and other visceral organs was carried out in some animals.

Serum samples

Blood samples (n = 44) were taken from animals before slaughter using Vacutainer® tubes without preservative and anticoagulant. The samples were allowed to clot at a cool ambient temperature and the sera thereafter separated and aliquoted into sterile sample tubes which were then labelled, packed on ice and dispatched to the National Veterinary Laboratory (NVL), Gaborone, the samples arriving within 48 h post-sampling. At NVL the serum samples were stored at –20 °C and then sent to the Veterinary Laboratories Agency (VLA), UK, where they were stored at –70 °C until used.

Complement fixation test

The CFT was the improved test (Campbell & Turner 1953) used for studies on CBPP in Australia. The tests were carried out in microplate format according to the procedure recommended by the Office International des Epizooties (OIE) (Anon 2000). All sera were heat-inactivated for 30 min at 56 °C before use. The inactivated sera were diluted in a two-fold series to cover a dilution range of 1/10-1/10240. The reagents were antigen (CIRAD-EMVT), complement, haemolytic serum (Biomerieux, France) and sheep red blood cells (RBCs). Positive and negative sera were included in each CFT plate to validate the results. End-points of the serum dilutions examined in the complement fixation test were the highest dilutions of sera which produced > 50 % haemolysis of sheep RBCs and reciprocals of such dilutions were the estimates of the respective serum titres. Samples with a titre of <1:10 were considered negative.

Immunoblotting test (IBT)

The IBT technique used was similar to that described in a previously published work (Regalla, Gonçalves, Ribeiro, Duarte, Nicholas, Bashiruddin, De Santis, Garrido Abellan & Penha-Gonçalves 2000). Briefly, proteins of *M. mycoides* subspecies *mycoides* SC strain M378, isolated from one of the early cases of CBPP in Botswana, were separated with SDS-PAGE and transfered electrophoretically onto a nitrocellulose membrane. The nitrocellulose membrane was soaked in 5 % skimmed milk powder (Marvel) solution (5 g Marvel, 1 g chicken egg albumin, 7.5 g glycine and 100 mℓ phosphate buffered saline (PBS) pH 7.2) for 2 h at room temperature (RT) to prevent non-specific binding. The membrane was then washed five changes of 10 mℓ PBS Tween (PBST) pH 7.2. Sera were diluted 1/50 in PBST, added to the membrane strips and then incubated at 37 °C for 2 h. Thereafter the membrane strips were washed in three changes of PBST and then soaked in 1/200 diluted anti-bovine horse-

radish peroxidase conjugated IgG (Dako) and then incubated at 37 °C for 1 h. The membrane strips were then soaked in five changes of PBS, soaked in 4-chloronaphthol substrate and incubated at RT until bands developed to the desired intensity. The reaction was then stopped by rinsing the membrane strips in distilled water. Strips in which there was a simultaneous presence of four antigenic bands of 110, 98, 69 and 48 kDa were considered positive and characteristic of CBPP.

Enzyme immunoassay

The i-ELISA test was a modification of the method of Onoviran and Taylor-Robinson (1979) and was conducted as described by Nicholas *et al.* (1996) and Ayling *et al.* (1999b) for the detection of antibodies against *M. mycoides* subspecies *mycoides* in cattle sera. Carbonate/bicarbonate buffer, pH 9.8, was used to reconstitute the antigen; the latter was dispensed into microtitre plate wells in aliquots of 100 µl. Plates were incubated at 37 °C for 2 h (to allow for adsorption of antigen onto the solid phase) after which they were washed in five changes of 0.1 M PBS and then 100 µℓ of test serum (diluted 1/500 in PBS containing 0.05 % Tween, 1 % Marvel) was added to each well. Plates were incubated for 1 h at 37 °C after which they were washed thoroughly with 0.1 M PBS, 100 µℓ of anti-bovine horseradish peroxidase conjugated IgG (diluted 1/4000) was added to all wells and the plates were then incubated for 30 min at 37 °C. Plates were then washed with 0.1 M PBS, blotted dry and thereafter 100 µℓ substrate was added to all wells. Colour was allowed to develop for 10–15 min at RT after which time the reaction was stopped by the addition of 50 µℓ of 1.0 M citric acid to each well. Optical density (OD_{450}) values were read at 450 nm using an automatic plate reader. Serum samples with OD_{450} values of ≤ 0.29 were considered negative and those with OD_{450} values > 0.29 were considered positive for antibodies to *M. mycoides* subspecies *mycoides* SC (Ayling *et al.* 1999b).

The c-ELISA was carried out using commercial CBPP-ELISA kit supplied by CIRAD-EMVT (France). The tests were performed in Nunc polysorb microtitre plates following procedures according to the manufacture·s instructions (Amanfu *et al.* 1998b). The percentage inhibition (PI) value for each serum sample was calculated by the following formula:

$$PI = (OD_{Mab} - OD_{Test})/(OD_{Mab} - OD_{Conjugate}) \times 100$$

Where OD_{Mab} is the mean of the OD_{450} values of 0 % control and $OD_{Conjugate}$ is the mean of the OD_{450} values of 100 % control. The cut off point for the test was set at PI value of 50 % (Amanfu *et al.* 1998).

Statistical analysis

Percentages and standard errors (SE) at 95 % confidence level were calculated according to Swinscow (1980).

RESULTS

Table 1 presents the number of serum samples found positive or negative by each of the four tests covered in the comparative study. The CFT demonstrated the highest percentage of sero-positive samples (61 %) followed by the IBT (57 %) while both ELISA tests showed similar percentages of positive sera, i-ELISA (48 %) and the c-ELISA (48 %), which were comparatively lower than those obtained either in the IBT or in the CFT. The CFT-positive sera ($n = 27$) had antibody titres that ranged from 320–5 120. The IBT positive sera showed distinct bands of expected sizes (110, 98, 69, 48 kDa) and were clearly separated from the negative sera which showed no distinct bands. The i-ELISA values for positives ranged from 0.30–0.62 and the negatives ranged from 0.05–0.29. For the c-ELISA, positives ranged from 50–71 % and the negative values were from 0–46 %. The values obtained by any particular test did not equate to the other tests, e.g. the serum sample with the highest CFT titre of 5 120 gave a value of 0.31 for i-ELISA and 46 % for c-ELISA. Two of the CFT-positive sera (N202 and N23a) were negative both in the IBT and in the two ELISA tests; each of the two sera had a complement fixing antibody titre of 320. Animal N23a had chronic CBPP on post mortem examination while N202 was not necropsied. The four serological tests were positive while post mortem examination results were negative for CBPP for animal number 31. In three animals, N12, N16, N69, the post mortem examination results were negative while the serological tests were positive for CBPP antibodies.

Tables 2–7 show the percentage agreement and disagreements between the various serological tests. Whereas two of 27 (7 %) CFT-positive sera reacted negatively in the IBT test, none of 17 (0 %) CFT-negative serum samples reacted positively in the IBT test, resulting in a percentage agreement of 95.5 % (Table 2). The percentage agreement was

TABLE 1 Comparison of complement fixation test (CFT), immunoblotting test (IBT), indirect ELISA (i-ELISA) and competitive ELISA (c-ELISA) for sera from CBPP affected cattle in Botswana

Animal no.	Post[a] mortem	Serological tests			
		CFT[b]	IBT[c]	i-ELISA[d]	c-ELISA[e]
N115	ND	640	+	0.27	45
N202	ND	320	–	0.21	24
N211	+	640	+	0.29	31
N23a	+	320	–	0.18	28
N28	+	640	+	0.62	56
N281	ND	320	+	0.58	59
N282	ND	640	+	0.40	62
N283	ND	640	+	0.60	60
N284	ND	320	+	0.52	61
N285	ND	640	+	0.18	58
N31	–	640	+	0.50	59
N38	+	5 120	+	0.31	46
N57	+	1 280	+	0.43	60
N60	+	1 280	+	0.51	52
N67	+	2 560	+	0.39	59
N72	+	1 280	+	0.25	35
N84	+	640	+	0.48	53
N87	+	2 560	+	0.37	50
N89	ND	320	+	0.59	42
NK2	ND	320	+	0.35	56
NK6	ND	1 280	+	0.47	66
NK61	ND	640	+	0.48	66
NK62	ND	640	+	0.33	68
NK63	ND	640	+	0.36	71
NK64	ND	1 280	+	0.30	68
NK65	ND	640	+	0.33	67
No label	ND	640	+	0.32	39
N1	–	–	–	0.05	29
N11	ND	–	–	0.16	19
N12	+	–	–	0.13	24
N15	ND	–	–	0.15	ND
N16	+	–	–	0.12	18
N17	+	–	–	0.12	ND
N18	ND	–	–	0.13	26
N19	–	–	–	0.10	0
N2	–	–	–	0.10	12
N23b	ND	–	–	0.13	14
N3	ND	–	–	0.11	1
N4	+	–	–	0.10	ND
N5	ND	–	–	0.10	19
N69	+	–	–	0.15	27
N7	+	–	–	0.08	ND
N8	ND	–	–	0.09	20
SAQ441	ND	–	–	0.08	17

[a] = Post mortem lesions included sequestration and liquefaction necrosis, enlarged mediastinal and bronchial lymph nodes, pleural effusions, pleuritis and marbling

[b] = Titres are expressed as reciprocals of the dilution where 50% haemolysis was observed. Samples with a titre of <1:10 were considered negative

[c] = A sample in which there was a simultaneous presence of bands of sizes 110, 98, 69 and 48 kDa was considered positive

[d] = OD450 values are stated and those greater than 0.29 were considered positive

[e] = Percentage competition is stated and values ≥50% were considered positive

ND = Not done

TABLE 2 Comparative efficacy of IBT and CFT in the detection of antibodies to *Mmm*SC in 44 sera collected from naturally infected cattle in Botswana

	IBT Total number of sera		Total agreement in (%) ± SE[a]
	Positive	Negative	
CFT			95.50 ± 3.12
Positive	25	2	
Negative	0	17	

[a] Standard error at 95 % confidence

TABLE 3 Comparative efficacy of IBT and c-ELISA in the detection of antibodies to *Mmm*SC in 40 sera collected from naturally infected cattle in Botswana

	IBT Total number of sera		Total agreement in (%) ± SE[a]
	Positive	Negative	
c-ELISA			85.00 ± 5.65
Positive	19	0	
Negative	6	15	

[a] Standard error at 95 % confidence

TABLE 4 Comparative efficacy of IBT and iELISA in the detection of antibodies to *Mmm*SC in 44 sera collected from naturally infected cattle in Botswana

	IBT Total number of sera		Total agreement in (%) ± SE[a]
	Positive	Negative	
i-ELISA			90.90 ± 4.34
Positive	21	0	
Negative	4	19	

[a] Standard error at 95 % confidence

TABLE 5 Comparative efficacy of iELISA and CFT in the detection of antibodies to *Mmm*SC in 44 sera collected from naturally infected cattle in Botswana

	i-ELISA Total number of sera		Total agreement in (%) ± SE[a]
	Positive	Negative	
CFT			88.64 ± 4.78
Positive	21	0	
Negative	5	18	

[a] Standard error at 95 % confidence

TABLE 6 Comparative efficacy of c-ELISA and CFT in the detection of antibodies to *Mmm*SC in 40 sera collected from naturally infected cattle in Botswana

	c-ELISA Total number of sera		Total agreement in (%) ± SE[a]
	Positive	Negative	
CFT			80.00 ± 6.03
Positive	19	0	
Negative	8	13	

[a] Standard error at 95 % confidence

TABLE 7 Comparative efficacy of c-ELISA and iELISA in the detection of antibodies to *Mmm*SC in 40 sera collected from naturally infected cattle in Botswana

	c-ELISA Total number of sera		Total agreement in (%) ± SE[a]
	Positive	Negative	
i-ELISA			90.00 ± 4.5
Positive	18	3	
Negative	1	18	

[a] Standard error at 95 % confidence

85 % between the IBT and the c-ELISA (Table 3), 90.9 % between the IBT and the i-ELISA (Table 4), 88.6 % between the i-ELISA and the CFT (Table 5), 80 % between the c-ELISA and the CFT (Table 6) and 90 % between the c-ELISA and the i-ELISA (Table 7). From these data it was evident that there were discrepancies between all pairs of tests compared, and the level of conflict varied from one pair of test to another.

DISCUSSION

The CFT remains the most commonly used and standard method for the detection of antibodies to *M. mycoides* subspecies *mycoides* SC in cattle sera (Anon. 2000). It is rapid, simple to perform and easy to interpret the results. Although the test is more specific than the ELISA tests, it lacks sensitivity for serum samples having very low antibody levels (Onoviran & Tylor-Robinson 1979; Le Goff & Thiaucourt 1998; Amanfu *et al.* 1998b).

ELISA tests detect late and persistent infections while the CFT detects early infections (Abdo, Nicolet, Miserez, Gonçalves, Regalla, Griot, Bensaide, Krampe & Frey 1998). In the present study, the CFT is seemingly more sensitive than the ELISA tests; however, this observation is contrary to the claims that were made in previous studies (Le Goff

& Thiaucourt 1998; Thiaucourt 1998). These differences may be due to the status of the herds sampled, that is, in the study reported here, the sampling might perhaps have involved more early cases, while the previous studies involved chronic cases from endemic areas (Le Goff & Thiaucourt 1998).

Sera from cattle infected with *M. mycoides* subspecies *mycoides* SC have antibodies that are capable of recognizing the immunodominant antigens thus giving a typical pattern. On the basis of this characteristic pattern, the IBT was developed to facilitate the detection of antibodies against *M. mycoides* subspecies *mycoides* SC in cattle sera (Regalla *et al.* 2000). The significance of the IBT test lies in the fact that animals infected with *M. mycoides* subspecies *mycoides* SC have antibodies against four or five specific bands of molecular mass: 110, 98, 95, 62 and 48 kDa in their sera, that persist over a period of infection, and that it may therefore be useful for the detection of chronically infected animals (Gonçalves, Regalla & Penha-Gonçalves 1994; Gonçalves *et al.* 1998; Regalla, Gonçalves, Ribeiro, Duarte & Penha-Gonçalves 1996). The apparent molecular mass of some bands may vary with differing gel systems and in this study the reported 62 kDa band appeared to be 69 kDa, while the 95 kDa band was not resolved.

Nicholas *et al.* (1996) compared the IBT test with the CFT, ELISA and post mortem examinations in two groups of cattle in Italy in which CBPP had been diagnosed and found the IBT to be the most sensitive and specific immunological test. In the present study the proportion of positive samples obtained by the IBT is similar to that obtained by the CFT (Table 1; $\Delta\% = 4.58$: SE = 10.47; $P > 0.05$) but these percentages are relatively higher than those obtained by the ELISA tests (Table 1). The total agreement between the IBT and the CFT is by and large better than that between the ELISA tests and the CFT (Tables 2–7). There is a discrepancy of only 4.5 % between the IBT and the CFT where about 7 % of the CFT-positive serum samples gave negative results in the IBT test (Table 2).

Studies in Switzerland showed that the immunoblot patterns produced by sera with false positive reactions in the CFT consisted of variable bands which differed from those of CBPP affected animals, in particular, the false positive sera did not react with the major immunologic polypeptides (Cheng, Frey, Krawinkler & Nicolet 1994). No immunoblot patterns were produced by the two CFT-positive serum samples (Animal N202 and N23a). These sera could be falsely positive or they could be true positive samples, which the IBT failed to detect. Proteins of low molecular mass (30–50 kDa) were demonstrated in the IBT with these two samples. Since animal N23a was positive on post mortem examination, the IBT and ELISA gave false negative results which CFT was able to detect. No post mortem examination was conducted on animal N202 and as such it is difficult to make a rational conclusion on the serological status of this animal as revealed by CFT, IBT and ELISAs. True CFT positives with negative IBT and ELISA results are possible, if animals are sampled at an early stage of the disease, when IgM (complement fixing) antibodies predominate (Abdo *et al.* 1998) and IgG antibodies which are revealed by IBT and ELISA conjugates are not yet formed (Le Goff & Thiaucourt, 1998). The animal from which the CFT positive but IBT and CFT-negative serum originated, was probably at an early stage of infection with only IgM antibodies in circulation.

In the three animals that were serologically negative by all tests, but positive on post mortem examination, it is possible that they were at the chronic stage of disease. In chronic cases where sequestration and encapsulation has occurred, antibody formation is not always stimulated and the animals might be missed with serological tests. It is also possible that the lung lesions were due to some other causes like pneumonic pasteurellosis, actinobacillosis, infectious bovine rhinotracheitis and foreign body pneumonia (Schneider, Van der Lugt & Hubschle 1994).

Currently, it would be reasonable to conclude that no single serological test is capable of detecting all CBPP affected animals in the field, and which are useful for diagnosis at the herd level only. In the absence of a "gold standard" test for the serological diagnosis of CBPP, some uncertainties remain unresolved as demonstrated in the present study. For this reason, suspicious CBPP cases identified by positive serology must be confirmed by further investigations that demonstrate the presence of antigen in the respiratory tissues of animals (Bashiruddin, Santini, De Santis, Visaggio, Di Francesco, D'angelo & Nicholas 1999a; Bashiruddin, De Santis, Vacciana & Santini 1999b). In CBPP-free countries like Botswana, CFT should be used in conjunction with other serological tests where possible, so that every stage of disease could be followed serologically should the disease enter the country.

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