Field validation of a competitive enzyme-linked immunosorbent assay (cELISA) for the detection of contagious bovine pleuropneumonia in Botswana

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Key words

Contagious bovine pleuropneumonia - *Mycoplasma mycoides* - ELISA - Specificity - Botswana.

Summary

The newly developed cELISA for the detection of contagious bovine pleuropneumonia (CBPP) was applied at the National Veterinary Laboratory, Gaborone, on a significant number of sera harvested during the epizootics in 1995 and 1998 after complete depopulation of cattle in the outbreak zone. Results confirmed the excellent specificity of the test with only one serum, out of 895, from non infected areas having a titer slightly above the cutoff value. Comparisons with other serological tests such as the complement-fixation test and the rapid slide-agglutination test on sera collected during the 1995 outbreaks also showed that all three tests have similar sensitivities. The main advantages of the cELISA reside in its specificity, reproducibility and the possibility to monitor a quality control program through the use of internationally developed software such as the ELISA Data Interchange (EDI).

■ INTRODUCTION

Contagious bovine pleuropneumonia (CBPP) is an infectious disease caused by *Mycoplasma mycoides mycoides* biotype SC (MmmSC) that is characterized by pleuritis and pneumonia. It is now one of the most important threat to cattle production in Africa. Until 1990, the disease seemed to have been under control in most countries through annual vaccination campaigns. However, it reappeared recently in countries that were considered free of the disease. Botswana had remained free of infection for over fifty years since the disease was eradicated in 1939 from the Chobe district of the country (9). In 1995, cattle were reported to be dying in large numbers at Xaudum village, about 70 km from Mohembo in Northwestern Botswana. Field and laboratory investigations supported a CBPP diagnosis (1). The government of Botswana adopted a stamping-out policy which involved the slaughter of 320,000 cattle from the outbreak zone in Ngamiland district of Northwestern Botswana. By the end of 1997 about 70,000 cattle had been restocked in the former outbreak zone. Although a double cordon fence was built on the Namibian border it is imperative that serosurveillance be carried out in order to monitor the movement of cattle across the border with Namibia, from where the disease originally entered Botswana.

Serosurveillance is one of the means that permits the monitoring of efficient control measures against CBPP. In many infected countries, CBPP cases are not properly reported because of the unwillingness of owners to declare the presence of the disease or of improper control of cattle movements. Therefore, the assessment of CBPP prevalence can be obtained through seromonitoring based on a sound sampling frame. The measurement of CBPP prevalence is the only way to define cost-effective control strategies or to check that the adopted strategies yield the expected results. In CBPP free countries, serosurveillance is an additional tool that allows to check on the status of cattle herds. Serological testing can also be used for rapid confirmation of new outbreaks in order to adopt efficient control strategies such as the slaughter of all animals during an outbreak.

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The serological test that is currently recommended by the Office international des épizooties (OIE) is the complement-fixation test (CFT) (2, 8). This test proved to be very useful in the past for confirmation of CBPP outbreaks and it was extensively used during the CBPP eradication campaign in Australia or, more recently, in Italy. It has however some drawbacks. It is difficult to standardize (3) because of the use of various biological reagents: antigen batch, sheep red blood cells, thermolabile complement, etc. It sometimes gives false positive reactions whose origin has not been clearly elucidated. In the case of a CBPP free country these false positive reactions are particularly worrisome as they should normally be followed by field investigations. Moreover, they sometimes lead to restrictions in cattle imports.

A test based on a competitive ELISA has recently been described for CBPP (4). It was designed to give a higher specificity through the use of a monoclonal antibody that is specific to MmmSC. It was found sensitive enough to detect an antibody rise in experimentally infected cattle as well as in natural outbreaks. This test was developed jointly with the International Atomic Energy Agency (IAEA) in order to be compatible with a uniform ELISA format used in national reference laboratories throughout Africa. It is now undergoing a wide field validation both in Europe and Africa within the framework of an EU DGVIII research grant devoted to CBPP involving ten countries: Cameroon, Ethiopia, France, Germany, Kenya, Italy, Namibia, Portugal, Uganda. The results presented here were obtained through project number TCP/BOT/6712(A) of the Food and Agriculture Organization (FAO).

■ MATERIALS AND METHODS

Sera

Sera (n = 320) were collected during the CBPP outbreaks in Northwestern Botswana (Ngamiland District) in 1996. Fifty-five cattle from infected herds were kept in a government quarantine station and serially bled weekly until they were finally all slaughtered according to the stamping-out policy that was implemented by the Animal Health and Production Department of Botswana. Sera were stored at -20°C.

Other sera (n = 895) were collected in 1998 from various parts of the country that had never experienced the disease. They represented a statistically significant proportion of the cattle population of Botswana that is free of CBPP.

Complement-fixation test

The complement-fixation test was carried out in a microplate format according to the recommended procedure of the OIE (8). The reagents used were as follows: antigen (Cirad-emvt, France), complement, hemolytic serum, sheep red blood cells (Biomérieux, France).

Slide-agglutination test

The slide-agglutination serum test (6, 7) was carried out following standard procedures, using a methyl violet colored antigen (Cirad-emvt). Antigen and sera were mixed in equal volumes of 25 µl, agitated during one minute precisely, a positive reaction being evidenced by a clear agglutination. A negative control serum was always run in parallel to ensure the absence of autoagglutination of the antigen.

cELISA

The test was carried out according to the protocol of Le Goff and Thiaucourt (4) with slight modifications to ensure compatibility with the ELISA data interchange (EDI) program of the IAEA. Briefly, Nunc polysorb plates were coated with a lysed antigen solution in PBS and incubated overnight at +4°C. One in ten diluted sera were incubated with the monoclonal antibody for exactly one hour. The reaction was then revealed by a peroxidase antimouse conjugate (Dako P260) and an ABTS substrate. The plates were read at a wavelength of 405 nm after 20 min incubation when the optical density of the control monoclonal exceeded 0.8 but was lower than 1.6. The plate reading and recording were monitored with EDI version 2.1 software of the IAEA connected to a Multiskan MS reader.

■ RESULTS

cELISA

Reproducibility

This parameter was assessed by comparing the results obtained with three control sera that were included in each plate (figure 1). All control titers remained stable throughout the study that involved two technicians and ten sets of plates. This could be further evaluated with the distribution of the control results. They all followed a gaussian curve with: 1) for the high positive control a mean of 77, acceptable lower control limit (LCL) at 68, upper control limit (UCL) at 78 (figure 2a); 2) for the low positive control a mean of 48, LCL at 38, UCL at 58 (figure 2b); and 3) for the negative control a mean of 10, LCL at 0, UCL at 25 (figure 2c). With these settings for LCL and UCL there was a single plate that fell outside the limits because of a value below LCL for the slight positive control.

Another approach to control the reproducibility of the test was to analyze the distribution of the variation between duplicates. This value was calculated systematically by the EDI software that showed the absolute value of the difference between the two

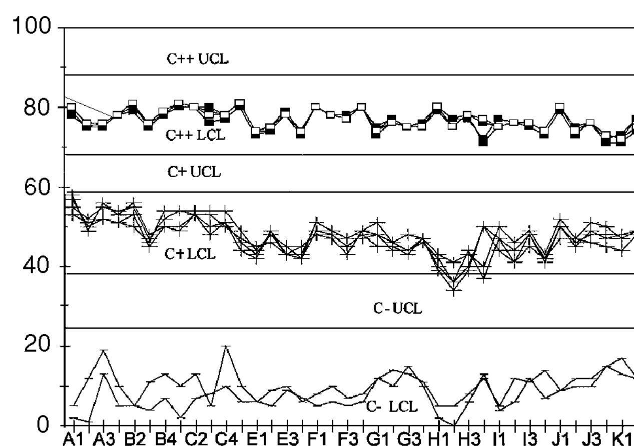

***Figure 1**: Values of the three serum controls obtained in ten consecutive days by two technicians. The lower (LCL) and upper (UCL) control limits are respectively: C- (0 to 25%), C+ (38 to 48%) and C++ (68 to 78%)*
C- = negative control, C+ = intermediate control, C++ = highly positive control.

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duplicates for each serum. The distribution was obtained by pooling 400 results from negative and positive sera (figure 3). It showed a unilateral distribution with only one value above 10, which is currently considered as the limit that shows that an individual result is acceptable.

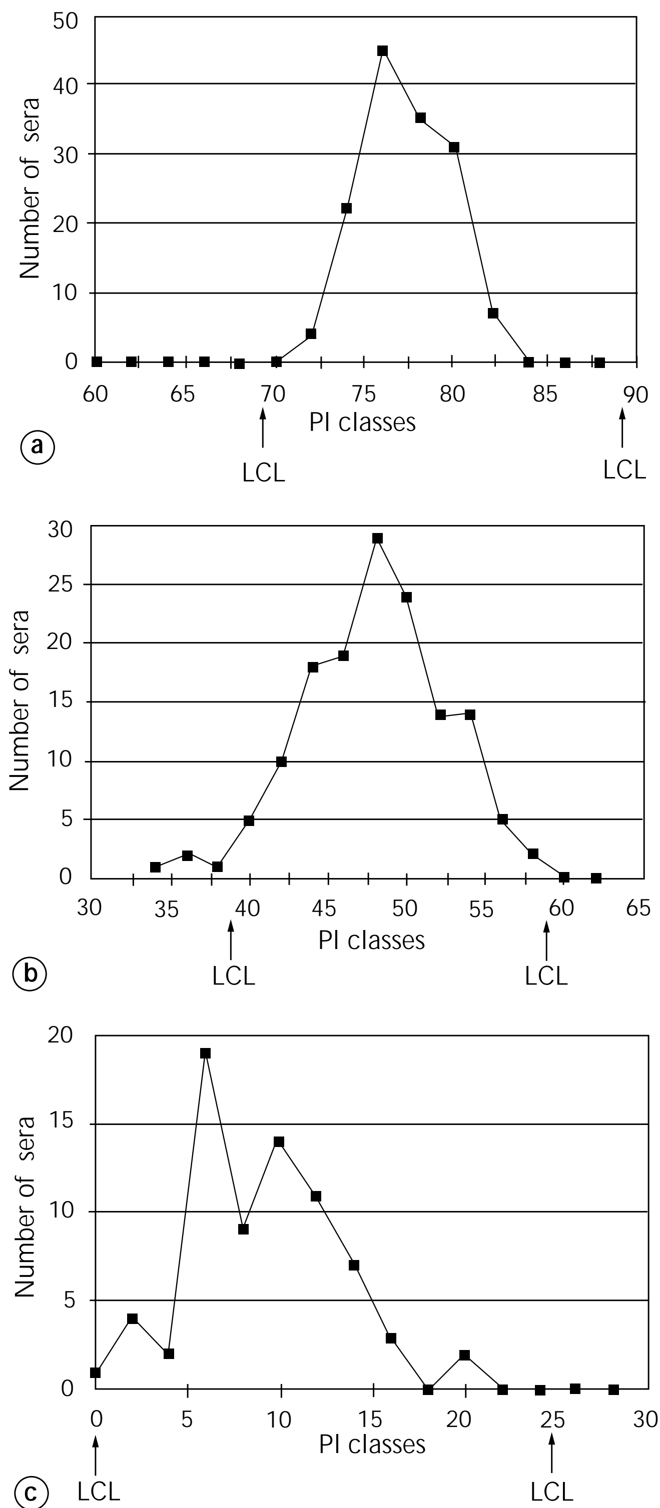

Figure 2*: Distribution of the values obtained with the serum controls at the National Veterinary Laboratory, Gaborone.*
Figure 2a: C++ (N = 140); figure 2b: C+ (N = 140); figure 2c: C- (N = 70)
C- = negative control, C+ = intermediate control, C++ = highly positive control.

Specificity

The true specificity of the cELISA was evaluated with a representative sample of Botswana cattle (n = 895) that were known to be free of CBPP. The distribution of the cELISA results followed a gaussian (figure 4) curve with a mean of 20.8 and a standard deviation of 10.3. Only one serum had a cELISA result above 50 which is the cutoff of this test. It showed that the specificity of the test approximates 99.9%. It has to be noted however that the titer of the only serum to exceed the cutoff point was 50.3, which is in no way comparable to the values obtained during the outbreak.

Sensitivity

The sensitivity was assessed by analyzing 320 sera from a known CBPP outbreak. The distribution of the results was strikingly different from that of the negative population with half of the results being above the cutoff point (figure 4).

Further comparisons were made on a subset of 48 sera that were obtained when the animals were slaughtered and necropsied. Serological results could then be compared with the actual gross lesions and true sensitivity and specificity could be determined.

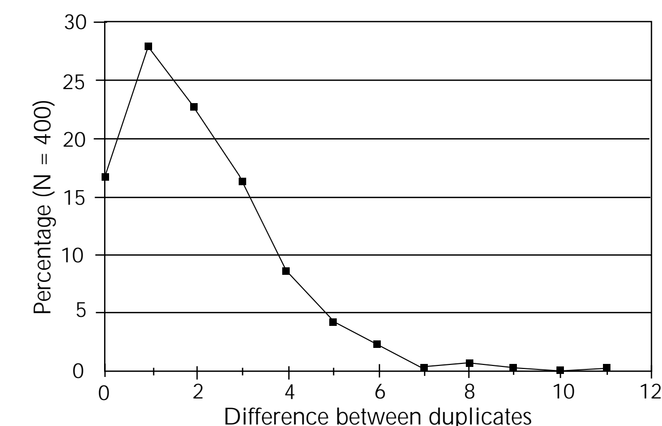

Figure 3*: Distribution of the absolute value of the difference between duplicates. An individual result is accepted if this value is less than 10. Here, all but one result were acceptable and more than 95% had differences below 5.*

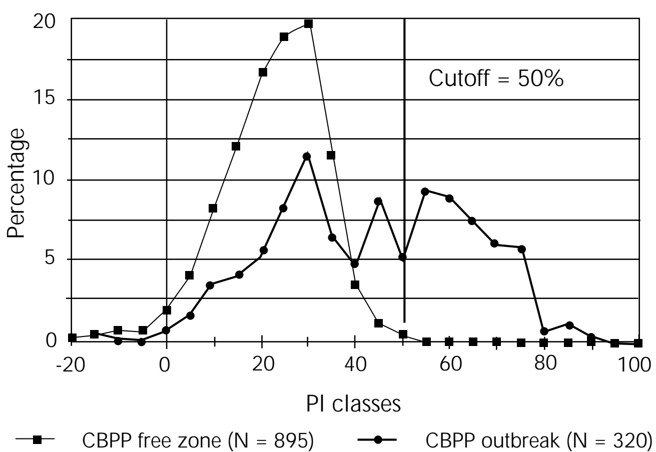

Figure 4*: Distribution of the cELISA results from a negative population of Botswana in 1998 (N = 895) and distribution of sera from an epizootic zone in 1995. The cutoff value at 50% is materialized by a vertical line. All but one negative sera were below the cutoff point (specificity = 99.9%). Many sera collected during an outbreak had values above the cutoff.*

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Of the 16 animals without lesions, there was complete agreement between CFT and cELISA with a single animal being positive (table I). As the same serum was also positive in SAST it can be assumed that this animal was truly infected. This may be due to a complete healing of the lesions. SAST seemed less specific as it detected two other positives, although with slight agglutinations (rated 1 on a scale of 1 to 4).

Of 32 animals with lesions, CFT and SAST gave 30 positives (93%) and cELISA 28 (87%) (table II). The slightly lower number of positives by cELISA could be explained by the fact that these animals were slaughtered during the acute stage of the outbreak and therefore were more likely to have high IgM titers which are complement fixing and agglutinating. It must be noted that the difference between the three tests is not significant. Agglutinations with SAST were sometimes weak, which in fact could be considered as dubious, taking into account that only high positives would have greatly reduced the sensitivity but improved the specificity.

Table I

Serological results in 16 animals without lesions

	CFT	cELISA	SAST
Positive	1	1	3
Negative	15	15	12

Table II

Serological results in 32 animals with lesions

	CFT	cELISA	SAST
Positive	30	28	30
Negative	2	4	2

■ DISCUSSION

The results obtained at the National Veterinary Laboratory show that this cELISA is a very effective test for CBPP sero-surveillance. The experience gained on Botswana cattle shows that the cutoff at 50%, that was primarily set with bovine sera from different origins, is still valid. It gives a specificity of 99.9%, which should enable CBPP free countries such as Botswana to minimize the number of serologically false positive results. The reduction of this percentage is of great economical importance as the detection of positives should always be followed by conservatory control measures such as quarantine and limitation of animal movements in the suspected area until field investigations rule out the presence of CBPP. The use of cELISA will certainly decrease the occurrence of such events.

Good performance in terms of repeatability and user-friendliness are two of the main advantages of this new serological tool when compared to CFT. The analysis of numerous plates showed that the variations between duplicated wells were minimal, normally less than 10% and less than 6% in 95% of the cases. The analysis of the variations between duplicates is therefore a good indicator of the technician's abilities and should be taken into account for the acceptance of the results at the individual level (difference less than 10) or at the plate level. This test requires minimal training as long as the laboratory has all the facilities to perform ELISA techniques. The use of uniform protocol and software gives the opportunity to compare easily the results obtained in various laboratories.

With the sera analyzed in this study, cELISA had apparently a slightly lower sensitivity than CFT at the individual level. This might be due to the presence of IgM that exist at the early stages of the immune response, these antibodies being complement fixing but with low affinities. The sensitivity of cELISA is however expected to compare well with that of CFT, when sera are collected long after the outbreak as bovine immunoglobulins of the IgG2 subclass are unable to fix guinea pig complement (5). In any case, these serological tests for CBPP are to be used at the herd level and numerous examples have shown that the sensitivity of both tests was similar with the advantage of the cELISA being very specific. In the near future, it is expected that cELISA will permit to assess the real prevalence of CBPP in various regions of Africa and thus provide accurate information for decision makers to define the best cost-effective control strategies.

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Résumé

Amanfu W., Sediadie S., Masupu K.V., Benkirane A., Geiger R., Thiaucourt F. Validation de terrain d'un test ELISA de compétition pour la détection de la péripneumonie contagieuse des bovins au Botswana

Le test ELISA de compétition, récemment décrit pour détecter la présence de la péripneumonie, a été utilisé au Laboratoire national vétérinaire du Botswana à Gaborone. L'échantillonnage de sérums a compris un nombre significatif de sérums récoltés durant l'épizootie de 1995 et ensuite en 1998 après que la totalité du cheptel bovin de la zone infectée ait été abattue. Les résultats obtenus ont montré l'excellente spécificité du test avec un seul sérum négatif, sur 895, ayant un titre légèrement supérieur au seuil de positivité. La comparaison avec deux autres tests, la fixation du complément et l'agglutination sur lame, avec des sérums récoltés durant l'épizootie de péripneumonie en 1995 a montré que ces trois tests avaient des sensibilités équivalentes. Les principaux avantages de l'ELISA de compétition sont sa spécificité, sa reproductibilité et la possibilité qu'il offre de pouvoir effectuer un contrôle de qualité fiable en étant utilisé avec des logiciels développés au niveau international comme l'*ELISA Data Interchange* (EDI).

Mots-clés : Péripneumonie contagieuse bovine - *Mycoplasma mycoides* - Test ELISA - Spécificité - Botswana.

Resumen

Amanfu W., Sediadie S., Masupu K.V., Benkirane A., Geiger R., Thiaucourt F. Validación de campo de un test inmunoabsorbente ligado a una enzima competitiva (cELISA) para la pleuroneumonía contagiosa bovina en Bostwana

El nuevo cELISA para la pleuroneumonía bovina contagiosa (CBPP) se aplicó en el Laboratorio Veterinario Nacional, Gaborone, en un número significativo de sueros, colectados durante la epizootia de 1995 y 1998, después de la despoblación total de ganado en la zona de la epidemia. Los resultados confirman la excelente especificidad del test, con solamente un suero de las áreas no infectadas en 895, presentando un título ligeramente superior al valor límite. Las comparaciones con otros tests serológicos, como el test de fijación de complemento y el test de aglutinación rápida en placa, en sueros colectados durante las epidemias de 1995, también mostraron que los tres tests presentan sensitividades comparables. Las principales ventajas del cELISA residen en su especificidad, reproductibilidad y en la posibilidad de seguir un programa de control de calidad a través del uso de programas desarrollados a nivel internacional, como el *ELISA Data Interchange* (EDI).

Palabras clave: Pleuroneumonía contagiosa bovina - *Mycoplasma mycoides* - ELISA - Specificidad - Botswana.