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Competitive ELISA: its validation and use in monitoring Contagious Bovine Pleuropneumonia (CBPP) antibody in Mbarara District.

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Abstract

Diagnosis is of CBPP presents one of the major difficulties in the control of the disease. As a way of improving diagnosis, a competitive ELISA has been developed and a study was carried out to test, validate, and assess its field performance. A cross-sectional epidemiological sero-survey was carried out in Bukanga and Isingiro counties of Mbarara district, to determine the pre-study prevalence of CBPP in the two counties. Cluster sampling based on villages as well as multistage, purposive and simple random sampling were used in site selection. For analysis, blood samples were collected from 10% of all the presented herds. Epidemiological data were collected for herd and individual animals. All animals sampled were examined clinically for signs of CBPP. Our findings indicate that sero-prevalence by ELISA was always higher than that by CFT, which confirmed the epidemiological status of the study locations that were first obtained through focus group discussions. The graphical illustrations of the distribution of the negative and positive populations depict normal distribution hence a good laboratory performance of the test. Results from this study indicate that the relative specificity of c-ELISA is satisfactory when results are interpreted at the individual animal level.

Introduction

CBPP is caused by *Mycoplasma mycoides* subspecies *mycoides* SC. The disease is restricted to bovine species and the organisms are host dependent, therefore spread of infection depends on live animals (Losos, 1996). CBPP is manifested by anorexia, fever and respiratory symptoms such as dyspnoea, polypnoea, cough and nasal discharges. With the near eradication of rinderpest, the disease has become one of the most important epizootics of cattle in Uganda. Despite the extensive vaccination programmes throughout the country, CBPP is still endemic in some foci. The disease once confined to a few sporadic cases in Karamoja has now spread and is now endemic in most districts of Uganda (Twinamasiko et al 1998). CBPP may appear as an epidemic, but is often insidious and may not be detected for several weeks or months after infected animals have entered an area. Besides, the disease may persist long after overt clinical signs have disappeared.

Detection of outbreaks is a prerequisite for the success of CBPP control policies (Le Goff and Thiacourt, 1998) but, the control programme lacks an efficient disease surveillance system to define the distribution and prevalence of the disease, which is important for implementing efficient control measures. The main problems for the control or eradication are the frequent occurrence of sub-acute or symptom-less infections and the persistence of chronic carries after the clinical phase. The assessment of true prevalence of CBPP is necessary for defining the most cost-effective animal health policy (Le Goff and Thiaucourt, 1998).

Diagnosis of CBPP requires a technique that is more specific than those previously in use. Indirect ELISAs failed in this regard and efforts were invested towards building a competitive ELISA (c-ELISA) based on the

use of a monoclonal antibody (Le Goff and Thiaucourt, 1998). The CF test, which is the recommended test with a specificity of 99.5%, can detect nearly all sick animals with acute lesions, but a rather smaller proportion of the animals in the early stages of the disease or animals with chronic lesions. Other interventions may also increase the number of false negative reactions. However for herd application, CFT is capable of detecting practically 100% of infected groups. Besides low sensitivity, CFT is quite difficult to standardize because of the use of antigens or RBCs of various qualities and requires skilled technicians. Furthermore, the antibodies detected by CFT wane rapidly, with the number of positives declining rapidly when the outbreak has occurred more than 3 months before sampling (Le Geoff and Thiaucourt, 1998). Since vaccination elicits very low titres that usually return CFT results to negativity after 3 months, this factor could be of an advantage. It can permit measurement of the incidence of the disease in a country by detecting the herds that have suffered from an outbreak during the 3 months before sampling. But since it will not permit detection of all infected animals, CFT cannot be recommended as an individual testing method for import restrictions. Despite the high specificity of the test (99.5%), false-positive results can occur (less than 0.5%) due to cross reactions with other mycoplasmas. Efforts have therefore been made to find a serological method that detects antibodies that persist longer than those detected by CFT. A monoclonal antibody (Mab) based competition ELISA has therefore been developed and results from its validation are here reported. The performances of cELISA and CFT at the herd and individual animal levels based on a cross-sectional and longitudinal study are also compared.

Materials and Methods

Study area

A cross-sectional epidemiological sero-survey was carried out to determine the pre-study prevalence of CBPP on the Uganda/Tanzania border. It was carried out in the in Bukanga and Isingiro counties.

Sample selection

Cluster sampling based on villages as the sampling units was applied, while a multistage procedure was used to select the villages. First, two sub-counties in Bukanga and one sub-county in Isingiro were purposively selected. The two sub-counties in Bukanga were selected because of their proximity to the Tanzania border and were known to have had cases of CBPP in the past. The sub-county in Isingiro was selected because of a rumour of an outbreak of CBPP.

The second stage involved determination of the number of villages to be sampled. In Bukanga the number of villages was determined using a model. The final stage was a simple random selection of villages. A total of twenty-five villages out of 85 were selected for sampling. However due to logistics only 21 were sampled.

In Isingiro the villages were purposively selected. The village with the outbreak and three neighbouring villages were sampled.

At each village crush, ten percent of all the presented herds were sampled. A percentage was chosen due to the unevenness of herd sizes.

Epidemiological data and sample selection

During a baseline survey, focus discussions were held in the villages to try and get the opinion of the veterinary officials and stock owners about the prevalence of CBPP in the locality. Three sets of herds were selected based on CBPP epidemiological status, i.e. high, medium and low CBPP prevalence.

Villages that had overt CBPP outbreaks at the start of the study (Isingiro/Nsyenyi) were categorised as high prevalence areas. Those that had had the disease in the last two years were and had evidence of chronic cases (Bukanga 1/Rwambaga) were categorised as moderate/ medium while those that had experienced CBPP in the past and had had no clinical case for more than 3 years were taken as having low CBPP prevalence.

All the herds in the 3 epidemiological zones are communal. Additional herds were purposively selected on the basis of previous disease status (CBPP free) and location (nearest to already selected herds).

All samples collected were accompanied with epidemiological data of the sampled herds and that of the sampled individuals.

The herd data covered livestock types, husbandry system, CBPP history and CBPP control measures. Individual animal data comprised of the age, sex and clinical status of the animal.

All animals sampled were also examined for clinical signs of CBPP according to the guidelines (FAO-EMPRES, 1996).

Fifteen herds were later selected from three villages and followed periodically on individual animal basis. All the animals in these herds were sampled every three months between April 2000 and January 2001 to assess the prevalence of antibodies to *Mycoplasma mycoides* subspecies *mycoides*, Small Colony (MmmSc).

Collection of serum samples

Collection, transportation and storage of serum samples were carried out as described (Thrusfield, 1995). Non-heparinised blood was taken from the jugular veins using glass vacutainers with nineteen-gauge needles, held over night at four degrees centigrade to separate the serum that was decanted into micro-cryovials, transported on ice to the laboratory and stored at –20°C.

Complement fixation test (CFT)

Complement fixation test was performed as described by Campbell and Turner (1953). The standard operating procedure for micromethod of complement fixation test for contagious bovine pleuropneumonia as adapted by Le Goff and Thiaucourt (1998) was followed.

ELISA test

The ELISA technique was performed according to CBPP competitive ELISA kit (Le Goff and Thiaucourt, 1998) as modified S.O.P supplied with the kit.

Evaluation of ELISA

Some individual animals with known clinical history were picked from the herds being studied for the evaluation of c-ELISA in repeated samples. Samples from these animals were obtained at 3 month intervals. The samples were tested using both c-ELISA and CFT to depict the periodic kinetics of antibody titres of these animals.

Results

Analysis of baseline survey data at the individual (animal) level:

It was found that the c-ELISA individual animal reactor rate for the Mbarara serum samples collected during the cross-sectional study was 5.4% (reactors being animals with Percent Inhibition (PI) readings >50).

In an effort to contribute further to the validation of the c-ELISA (performed for this batch at CIRAD) using field samples, the test properties and diagnostic performance measures, i.e. sensitivity, specificity, apparent prevalence, true prevalence, positive and negative predictive values, likelihood ratios, were calculated. For this purpose it was assumed that clinically suspected CBPP cases in the field were considered CBPP diseased and, thus, presenting the "gold standard". However, in doing we could have over-estimated the number of true CBPP cases in the field.

Table 1: Herd level analysis

No. of herds	No. of herds positive by ELISA	No. of herds "positive" clinically	Test Properties	
			Sensitivity (Se)	Specificity (Sp)
17	3 (16.6%)	11 (64.7%)	27.3 (45%)	100%

It was found that under these assumptions the c-ELISA has an extremely low sensitivity (3.66%) but a very high specificity (94.36%) if interpreted at the individual animal level (IAEA, 2001). The calculation of test agreement beyond chance (using the same assumptions) resulted in an even negative Kappa-value, i.e. no agreement at all between the clinical diagnosis (suspected CBPP) and the ELISA results.

During c-ELISA performance at CIRAD the results of 16 sera were interpreted as "dubious", i.e. had PI values >40 - <50%.

Analysis of baseline survey data at the herd (aggregate) level:

Out of the 17 herds with c-ELISA results positive reactors were found in 3 herds thus, giving a CBPP herd

Table 2. Test agreement calculations for c-ELISA and CFT for CBPP, Uganda 2000

Test agreement

		Standard test (CFT)		Alternative test (c-ELISA)	
	+	+	-		
	+	8	7	15	all T+
	-	13	479	492	all T-
		21	486	507	N
Observed proportion of agreement:				0.961	
Expected proportion of agreement:				0.963	
Observed minus chance agreement:				0.029	
max. poss. Agreement beyond chance:				0.069	
				LL	UL
KAPPA:	0.425	0.339	0.510	0.044	Se (0)
		0.217	0.632	0.106	Se (1)

infection rate (as determined by c-ELISA) of 16.6%, compared to 11 herds with clinical suspected CBPP (64.7%). With-herd c-ELISA determined infection rates were 1.9%, 3.3%, and 36.2%.

Also at herd level (herd being the unit of analysis) the test properties were calculated. As expected, both the sensitivity (now 27.3%) as well as specificity (100%) increased; if herds with "dubious" reactors (PI values >40 - <50%) are also considered positive for CBPP, then sensitivity increases even up to 45%.

The herd Nshenyi 1 (high prevalence) had encountered a previous CBPP outbreak. 12 of the 16 "dubious" reacting sera came from Nshenyi 1 and only 2 animals were suspected of clinical CBPP.

However, CBPP reactor rates as well as clinical disease prevalences are by no means representative; they are rather characteristic only for the herds and animals investigated with the objective of c-ELISA test validation!

Data with both CFT and c-ELISA readings from Mbarara, as described in Table 3, allow the determination of agreement between the two serological tests. Though being aware that both tests do not measure the same immunological response, c-ELISA results are reported to compare favourably with results from CFT (Le Goff and Thiaucourt 1998), which is the OIE prescribed test for CBPP; thus, the Kappa value for test agreement has been calculated using WinEpiscope (Ortega et al, 1996).

Table 3. 'Relative' test performance of c-ELISA versus CFT for CBPP, Uganda, 2000

	CFT	+CFT-	TOTAL	
C-ELISA T+	8	7	15	*all T+*
C-ELISA T-	1	479	492	*all T-*
TOTAL	**21** *all CFT+*	**486** *all CFT-*	**507**	**N**
		95%CI LL	UL	
Se	38.1	17.33	58.87	
Sp	99.7	97.5	99.62	
TP	4.1	2.41	5.88	
AP	3.0	1.48	4.43	
PPV+	53.3	28.09	78.58	
PPV-	97.4	95.94	98.78	

Results of quarterly monitoring (Longitudinal Study)

CFT and c-ELISA tests for longitudinal quarterly monitoring were only performed for samples of April 2000. CBPP prevalence in the Isingiro was shown to be higher (8%) than in Bukanga county (0.8%), further confirming the data provided by the stock owners during the focus group discussions.

The resulting Kappa value (Table 2) is to be interpreted as moderate agreement. Though Kappa is not independent of the prevalence in a population, one test should not replace the other and therefore, as stated by Le Goff & Thiaucourt (1998), a combination of both serological methods is to be applied in apparent enzootic regions of Uganda.

Under the assumption that CFT would constitute the "Gold Standard" the following 2x2 table could be set up and 'relative' test performance parameters calculated.

Contrary to Le Goff & Thiaucourt (1998) the relative sensitivity of c-ELISA as performed on the Uganda sera was lower (0.381) whereas the relative specificity was higher (0.997) (Table 3).

Prevalence of CBPP (April 2000)

Samples collected

The total Number of samples collected is shown in table 5.

Table 4: Number of samples and Test results showing CBPP Prevalence at animal level

	Bukanga	Isingiro
No of Villages	*21*	*4*
Number of samples	826	304
Number tested	671	304
c-ELISA Positive	4	46
c-ELISA Prevalence	0.6%	15%

The analysis of the individual animal and herd results for the same period in the two counties show higher prevalence at the herd level as compared to analysis at individual animal level.

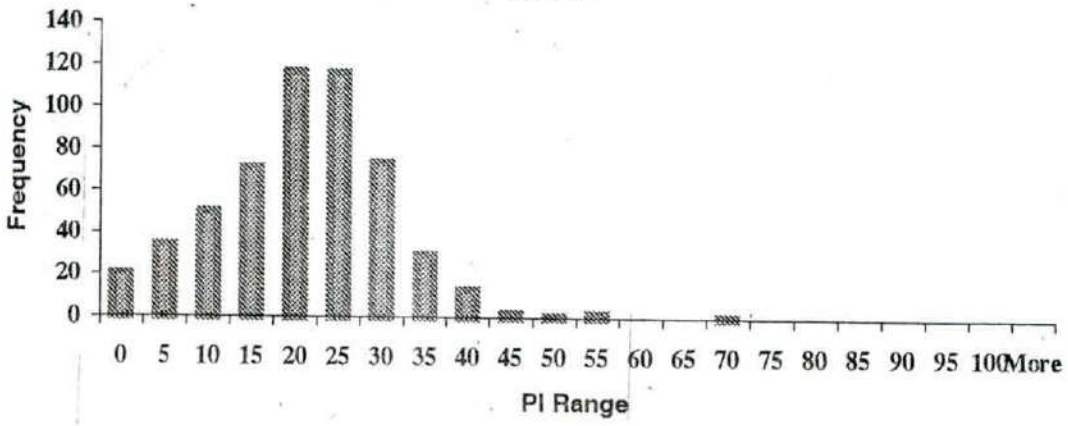
c-ELISA prevalence was higher than CFT prevalence of CBPP in the two counties in July 2000, further confirming the trend that was observed in April 2000.

Discussion

Although CFT and c-ELISA both measure different immunological response, results from the two tests are reported to compare favorably (Le Geoff & Thiaucourt, 1998). Although CFT is still the OIE prescribed test for

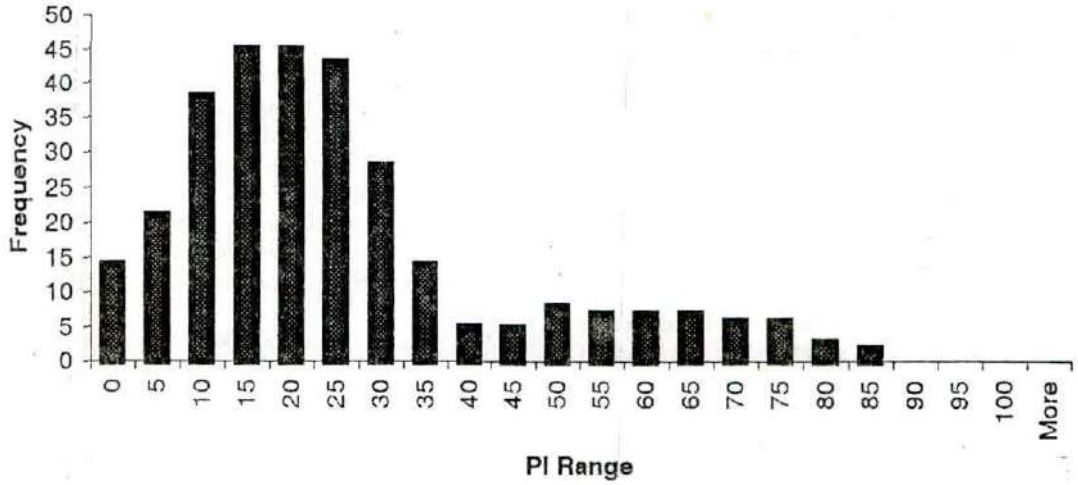
The distribution antibody titres of the animal population is demonstrated in the figure 1. below:

Fig 1. PI Frequency Distribution; Bukanga and Isingiro, April 2000



The few ELISA positive reactors from Bukanga show low antibody titres with a narrow range (50-60%) as compared to those from Isingiro (Fig.2).

Fig 2. Isingiro: Overall PI Frequency distribution



The c-ELISA positive reactors from Isingiro show a characteristic spread of antibody titres from 50 – 80%.

CBPP, it has some major shortcoming such as poor detection of chronic cases, low specificity and difficulties in standardization.

This study has shown that comparison of the two tests at herd (aggregate) level has the effect of improving the properties of c-ELISA, with the value of both sensitivity and specificity going up. Besides, c-ELISA appears to detect more positive individual animals over a period of time than the CFT. This is due to the fact that the antibodies detected by CFT tend to wane rapidly. In the analysis of the c-ELISA results of the baseline data for the Mbarara study, it was found that the sensitivity (Se) of the test was very low and there was no agreement between c-ELISA and the clinical diagnosis. This analysis assumed clinical diagnosis to be the "Gold standard". This led to over estimation of prevalence hence explaining the low Se and the lack of agreement. However, analysis of the same results at the herd level led to a marked improvement of sensitivity to 27.3% and the specificity to 100%. Clinical diagnosis and c-ELISA results comparison have been done at herd level and not individual animal level. CBPP reactor rates and clinical disease prevalences are not representative; they are rather a characteristic only for the herds and the animals investigated.

The resulting kappa value obtained from the comparison of the two tests is interpreted as moderate. Though kappa is not independent of the prevalence in a population, one should not substitute the other and therefore, as mentioned by Le Geoff and Thiaucourt (1998), a combination of both serological methods is to be applied in apparent enzootic regions of Uganda.

The PI frequency distributions of the test results from Bukanga and Isingiro counties show normally distributed populations in both the positive and negative categories indicating good laboratory performance of the test.

Conclusion

Since CBPP is an insidious disease, its control requires an understanding of the herd status of the animal population at risk. Although CFT is the recommended OIE test, some of its limitations require that an alternative test which can detect antibodies that persist longer than 3 months, which is the longest period CFT can detect antibodies to CBPP be used to complement diagnosis. CFT will detect acute cases while ELISA can be used in the detection of chronic carriers thereby necessitating the use of both tests in enzootic situations.

This study further confirmed that CBPP was highly prevalent in Isingiro county while it was of moderate prevalence in Bukanga.CBPP being a herd problem, it is also beneficial to interpret test results at a herd level for appropriate intervention. On the other hand, c-ELISA has demonstrated a superiority in detecting more positive individual animals than CFT hence better at sero-surveillance.

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