

Full Length Research Paper

A sustainable laboratory approach for contagious bovine pleuropneumonia (CBPP) monitoring in Nigeria: Comparison between two serological tests in an endemic area complimented with post mortem lesions

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A study was carried out to develop a sustainable laboratory approach for contagious bovine pleuropneumonia (CBPP) monitoring using two serological tests and examination of lungs from slaughtered cattle in Maiduguri, Nigeria. A total of 500 lungs from 340 males and 160 females, aged between 1 and 5 years were examined at post-mortem (PM) for pneumonic lesions characteristic of CBPP. Out of this number, 92 (18.4%) showed typical lesions of CBPP. From the 92 lungs of cattle affected with CBPP, 36 (39%) and 56 (61%) were in the acute and chronic stages respectively. In order to determine the serological prevalence of CBPP using dot blot and complement fixation tests (CFT), 150 serum samples were collected from cattle with unaffected lungs, whilst 36 and 56 serum samples were obtained from those with acute and chronic lesions. Of the 150 serum samples from unaffected cattle, dot blot detected more positives 23 (15.3%) than CFT 18 (12.0%). Also dot blot detected more positive cases 35 (97%) and 41 (73.2%) than CFT in the acute and chronic stages of cattle with CBPP, respectively. The geometric mean (GM) CFT serum titres of 35.2, 40.2 and 63.5 from unaffected, acute and chronic stages of disease respectively, indicated a significant ($P<0.05$) sero-conversion and persistence of antibodies in the chronic stage (GM=63.5) than the other clinical stages. It is concluded that abattoir post mortem surveillance backed with a reliable, affordable and cheap diagnostic test, could improve the detection of this disease in CBPP endemic countries like Nigeria.

Key words: *Mycoplasma mycoides*, cattle, postmortem, complement fixation tests (CFT) and immuno-blot assays.

INTRODUCTION

Nigeria with an estimated cattle population of around 14 million (Bourn et al., 1994) is constantly under the threat of contagious bovine pleuropneumonia (CBPP). CBPP is

an endemic disease characterized by respiratory difficulty, bronchopneumonia and high mortality during outbreaks and it is increasing in Nigeria (Nawathe, 1992; Aliyu et al., 2000; Danbirni et al., 2010). The disease CBPP caused by *MmmSC* has persisted in Africa to the present century (Windsor, 2000). The economic impact of this disease is due to its alarming spread in Africa particularly in Nigeria, where the disease is presently

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causing huge losses in cattle (Foluso, 2004; OIE, 2010a, 2010b; Tambi et al., 2006). The diagnosis of CBPP can proceed via serology, pathogen isolation, post mortem (PM) examination, histochemical evaluations and molecular techniques (Persson et al., 1999; Blanchard and Browning, 2005; OIE, 2008). The isolation of *MmmSC* from infected animals is essential for the successful diagnosis of CBPP. However, *MmmSC*, like most mollicutes, is nutritionally very fastidious and is dependent on its host for a large variety of organic nutrients such as vitamins, nucleic acid precursors, lipids, fatty acids and amino-acids (Razin, 1985; Persson et al., 1999; Defra, 2007).

For accurate diagnosis of CBPP, gross pathological changes in the affected organ(s) and tissues (Nunes – Pesticia et al., 1990) backed with sensitive and specific serological reactions (Egwu et al., 1996) are required. The use of more sophisticated tests such as DNA hybridization, polymerase chain reaction (PCR) and DNA insertion sequences have their limitations in developing countries of Africa as is the case in Nigeria, where molecular tools cannot be utilized routinely because they are too expensive. For these reasons, there is need for reproducible, sensitive and reliable but cheap tests; this in addition to gross pathological lesions of affected organs and tissues obtained during PM examination, could be far more effective and reliable in the diagnosis and surveillance of the disease in these countries.

This study was undertaken to ascertain whether the combined use of PM examination of lungs from slaughtered cattle and two serological tests would likely improve the detectable level of CBPP.

MATERIALS AND METHODS

Lung samples

A total of 500 lungs from slaughtered cattle at the Maiduguri municipal abattoir were examined for pneumonic lesions during the study period of 13 months. Both halves of the lungs including the various lobules were examined for typical lesions of CBPP.

Lungs from both sexes of cattle (340 males) and (160 females) age approximately between 1 and 5 years were examined. Prior to PM examination, lungs were graded as unaffected or apparently normal (408), acute (36) and chronic stages (56) of disease.

Serum samples

A total of 150 blood samples were collected from cattle with apparently normal lungs, whilst 92 were collected from cattle with acute (36) and chronic (56) stages of CBPP respectively. 10 ml of blood were collected aseptically using vacuainers® tubes from the jugular veins of slaughtered animals. They were brought to the laboratory and centrifuged at 1500 rpm for 6 min; and approximately 2 ml of the serum was harvested and stored at -20°C until assayed.

Propagation of *Mycoplasma* antigen for CFT

Stock *Mycoplasma mycoides* subspecies *mycoides* SC was sub-

cultured on modified Eaton's mycoplasma basal agar and broth media respectively. The methods of cultivation, subculture and storage were essentially those described by Boughton and Thorn (1993).

Serological assay

Sera were assayed by CFT and dot enzyme immuno-assay (dot blot).

Preparation of *Mycoplasma* antigen for CFT

100 ml *Mycoplasma mycoides* subspecies *mycoides* SC was inoculated into 4 ounce bottles containing mycoplasma broth medium. This was incubated at 37°C in 5% CO₂ for 24 to 48 h. The logarithmic broth culture was harvested and centrifuged at 1500 g. The deposits were pooled and washed with Phosphate buffered saline (PBS) and diluted to a McFarland Standard tube No.2 containing approximately 10⁵ colony changing units (CCU). The final volume was then made to contain at least 1/10th of the original volume. The contents were homogenised using a whir mixer® and stored at -20°C until used.

Preparation of sheep erythrocytes

Blood was collected from the jugular vein of the animal and stored at 4°C. The sheep red blood cell could be stored for up to one week provided no haemolysis was evident. Before use, the cells were washed three times in Alsever's solution and centrifuged at 2000 rpm on each occasion. After each washing, the buffy coat of the white blood cells (WBC) was removed with a pipette, and the final suspension centrifuged for 15 to 20 min to concentrate the cells.

Alsever's solution and Veronal buffer

This was prepared by dissolving 0.5 g citric acid, 0.8 g sodium citrate, 4.2 g sodium chloride and 20.5 g dextrose in 100 ml distilled water. The solution was stored at 4°C until used. Veronal buffer was prepared by dissolving one CFT tablet (Oxoid, Basingstoke, UK) in 100 ml distilled water.

Assay of test serum samples using CFT

Serum samples were diluted 1:5 Serial two-fold dilutions in 25 µl amounts along the row of a microtitre plate. This was followed by the addition of 25 µl of the antigen. Then 25 µl of 2.5 HD50 (haemolytic dose) was added to each well and plates allowed to stand for 60 min at 37°C. Twenty five microlitres of the haemolysin was then added to each well and plates incubated at 37°C for 30 min.

The plates were brought out and read 1 to 2 h at room temperature. The titre of the positive serum was expressed as the highest dilution allowing the fixation of enough complement to prevent 50% haemolysis. Antigen, antigen-anti complementary activity and serum controls were set up in the same microtitre plate.

Dot enzyme immuno-assay (dot blot)

Dot enzyme immuno-assay was performed as described previously (Egwu and Aliyu, 1997). Briefly, *MmmSC* was washed twice in 10 ml PBS, PH 7.2, and resuspended in 20 µl of same diluent PBS. Two microtitre of each of 3 (1:10, 1:40, 1:160) dilutions were placed

Table 1. Number of lungs samples examined within each age group of cattle with acute and chronic lesions of CBPP.

Age range in years	Number of lungs samples examined		Number of lungs with lesions		Number of lungs unaffected
	(Male)	(Female)	(Acute)	(Chronic)	
1-2	22	2	-	-	24
3-4	84	58	8	18	116
5	234	100	28	38	268
Total	340	160	36	56	408

Unaffected: Normal architecture of the lung is maintained with no obvious pneumonic lesions. The lungs (a piece) could still float when suspended in water and both pleura were clear and distinct. Acute: There is pleurisy, reduced marbling, with pin-point focal necrotic lesions. There is loss of architecture of the lung parenchyma. Chronic: Typical marbling, consolidation and thickening of interlobular septa. Large areas of focal necrosis with massive *sero-sanquinous* haemorrhagic fluid.

Table 2. Number of serum samples collected from unaffected and affected cattle with CBPP lesions to group according to age.

Age range in years	Number of serum samples collected		
	Unaffected lungs	Affected lungs	
		(Acute)	(Chronic)
1-2	8	-	-
3-4	42	8	18
5	100	28	38
Total	150	36	56

on nitrocellulose stripes. When dried, the membrane was treated (blocked) with 5% skimmed milk solution in PBS (pH 9.2, 0.5% Tween 20) for 1 h at 37°C and washed 5 times in PBS. Test sera, diluted 1:400 and 1:800, were incubated with stripes for 1 h at 37°C. This was then followed by 5 washes in PBS, and incubated with anti-bovine conjugate® (1:5000 Amersham, U.S.A) linked with alkaline phosphates for 1 h at 37°C. Five further washes of the stripes in PBS was followed by detection of the adherent antibodies with 5 bromo-chlorindoly phosphate (BCIP)/nitroblue tetrazolium (Dynatech, U.K). Interpretation of the test was by specific dilutions of antigen compared with known positive and negative sera.

Statistics

CFT titres were converted to geometric mean (GM) and values compared by t-test using $P=0.05$ as the level of significance.

RESULTS

A total of 500 lung samples from slaughtered cattle were examined at post mortem for pneumonic lesions of CBPP. Also 150 blood samples were collected from cattle with apparently normal lungs; 36 and 56 serum samples from cattle with acute and chronic lesions of CBPP respectively, and tested for complement fixing antibodies and dot blot tests against *MmmSC*.

Table 1 shows that of the 500 cattle lungs examined, 160 represented females and 340 were males. Out of this number, 92 (18.4%) cattle lungs examined had lesions of CBPP, with 36 (7.2%) and 56 (11.2%) showing acute and

chronic lesions of CBPP, respectively. Cattle aged 3-4 years were moderately affected with CBPP, whilst those >4 years showed more number of affected cases (Table 1), but there was no significant ($P > 0.05$) difference in the rate of occurrence of CBPP between these two age groups.

Table 2 shows the number of serum samples collected from cattle with apparently normal lungs and from those with acute and chronic lesions of CBPP. 150 serum samples were collected from unaffected cattle, whilst 36 and 56 sera were collected from acute and chronic cases of CBPP respectively.

Table 3 shows the number of serum samples positive for dot blot and complement fixing antibodies (CFT) to *MmmSC*. Out of the 150 serum samples from unaffected cattle tested at parallel (same time) with dot-blot and CFT, 41 (27.3%) were positive to both tests, of which 23 (15.3%) and 18 (12.0%) were positive for dot-blot and CFT, respectively. From the acutely affected cattle, 35/36 (97.2) were positive and only 2.7% negative for dot-blot, whilst 29/36 (80.6%) were positive and 19.4% negative for CFT (Table 3). Out of the 56 serum samples obtained from chronically affected lungs with CBPP, 41/56 (73.2%) were positive and 26.8% were negative for dot-blot. Also 32.1% of the serum samples at the chronic stage were positive and 67.9% were negative for CFT.

The GM titres of serum samples collected from unaffected cattle and those with acutely and chronically affected lungs with CBPP lesions were 35.2, 40.2 and

Table 3. Number of samples positive for dot blot and compliment fixation test and the geometric (reciprocal) CFT with the clinical stage of affected lungs with CBPP.

Parameter	Number of serum samples tested		
	Unaffected lungs N=150	Affected lungs	
		(Acute) N=36	(Chronic) N=56
Number positive for both dot blot and CFT tested simultaneously	41 (+)	35(2+)	56 (3+)
Number positive for dot blot	23	35	41
Number positive for CFT	18	29	18
Mean of natural log CFT	3.56 ± 0.65	3.70 ± 0.57	4.45 ± 0.88
Antilog of geometric mean (GM) titre	35.2	40.4	63.5

± Mean and standard deviation, +, Range of intensity of dot blot stains.

63.5 respectively. The mean GM titre (63.5%) of sera collected from chronically affected cattle was significantly ($P<0.05$) different from those of acutely affected (40.4%) and unaffected (35.2%) lungs. However, the dot-blot detected more positive sera from unaffected, acute and chronically affected lungs of cattle with CBPP as visualized from the intensity of staining (Table 3).

DISCUSSION

The inherent obstacles against the successful control of CBPP particularly in the sub-Saharan regions such as Nigeria could be linked to inadequate abattoir surveillance and reliable diagnostic tests (Egwu et al., 1996). Moreover, the inability of the CBPP endemic countries to implement the test and slaughter policy, coupled with enshrined nomadic practices, as well as vaccination failures, has further exacerbated the spread of this disease Masiga and Domanech (2004).

Varying clinical syndromes and post-mortem lesions were manifested by cattle affected by CBPP, which perhaps could be due to differences in breed susceptibility (Regalla et al., 1984). The problems inherent in cultural (microbiological) isolation of *MmmSC* from various clinical sites including the lungs, as well as the inability of most developing CBPP endemic countries in Africa to implement the test and slaughter policy, has necessitated the use of PM examination in combination with affordable serological diagnostic tests, for the surveillance of CBPP in developing countries (Nicholas et al., 1996; Danbirni et al., 2010).

No sex or age predisposition was particularly significant to the development of CBPP lesion in this investigation. However, cattle aged 3-4 years were more prone to acute

and chronic CBPP lesions. This observation was similar to those reported by Windsor and Masiga (1977). Fewer animals aged 1-2 years compared to those aged >4 years were slaughtered during the present investigation, most of which were older males. The reason for this may be associated with increasing cattle trade particularly around the borders with Chad, Niger and Cameroon Republics. This may also not be unconnected with the urge and pressure on families to sell off their unproductive cattle to meet other financial demands of the family.

The increased prevalence rate of 18.4% in the present study showed that CBPP is fast spreading in Nigeria, which is likely due to inadequate vaccination coverage or vaccine failures as previously highlighted (Nwanta and Umoh, 1992). This finding further corroborates the observations of Provost et al. (1987) and Nicholas and Palmer (1994) on the spread of CBPP in other endemic areas of Africa and the world in general.

Complement fixation test (CFT) is known to detect acute and sub-acute cases of CBPP (Poumarat et al., 1991), but its limitation in detecting early and chronic cases has been documented (Nicholas and Palmer, 1994). The use of dot blot has been well evaluated as a reliable diagnostic screening test for CBPP, and this has been shown to correlate with similar ELISA tests in previous studies (Nicholas et al., 1996).

The present study has shown that dot blot and CFT tests were able to detect specific antibodies to CBPP in unaffected and affected cattle. Though dot blot detected more positive sera in unaffected and in the acute and chronic stages of the disease in cattle, the combination of the two tests as diagnostic tool, could improve the detection of the prevalence of this disease as corroborated by the findings of previous workers

(Nicholas and Palmer, 1994; Nicholas et al., 1996) and in the current study.

The detection of positive sera in unaffected cattle using both tests (dot blot and CFT), in the absence of overt clinical PM lesions, further confirms the salient nature of CBPP, and the likely implication of some of these positive animals to act as carriers (Provost et al., 1987). Furthermore, the need to buttress PM examination of cattle during CBPP surveillance, backed up with a reliable single or combination of serological tests cannot be over-emphasized, if the effective control of CBPP is to be achieved.

The CFT detected less positive cases of CBPP in the chronic stage compared to the acute stages, thus the need to scale up or buttress the test for better detection of CBPP positive animals. The geometric mean of 63.5% in the chronic stage of CBPP is significant ($P < 0.05$) when compared with 40.4% for acute and 35.2% for unaffected. This shows that more animals sero-converted at this stage, and perhaps anti-bodies tend to persist for longer periods during this stage of infection.

Sequestration was not observed in the lungs of CBPP affected cattle in the present study, which is in contrast to previous observations in Europe (Nicholas et al., 1996). Paradoxically, this observation confirms the complexity of the pathogenicity of *MmmSC*. However, the serological evidence provided in this study, in addition to PM examination, coupled with good surveillance, might provide the necessary tools needed for better monitoring and detection of this disease. This position is in support of a recent report in Nigeria (Danbirni et al., 2010).

CONCLUSIONS AND RECOMMENDATIONS

This study has shown the need to combine more reliable and cheap serological tests with PM examination of lungs of cattle with or without CBPP lesions. The use of two serological tests and PM lesions is a better means of improving the detection of CBPP especially during surveillance. It is therefore recommended that since many cattle from various sources usually converge on the cattle market, attempts should be made to "trace back" (herd-tracking) such positive animals to their original herds. This will in no small measure provide details of their vaccination history, previous outbreaks, so as to assist policy makers in instituting adequate control and immuno-prophylactic measures.

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