

## Comparison of Competitive ELISA, PCR and Loop Mediated Isothermal Amplification of Mycoplasmal DNA in Confirmatory Diagnosis of an Outbreak of Contagious Bovine Pleuropneumonia in Eastern Rwanda

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**Abstract:** An outbreak of a respiratory disease in cattle was reported in February 2010 at the Rwanda Agriculture Board research station at Nyagatare in Eastern Rwanda. The tentative diagnosis based on clinical and postmortem signs suggested infection with Contagious Bovine Pleuropneumonia (CBPP). This paper presents a comparison of diagnostic techniques and their suitability in detecting early and asymptomatic carriers of CBPP. Serum was prepared from jugular vein blood and used to identify CBPP-infected animals using competitive ELISA (c-ELISA) assay and this was later confirmed with Loop-Mediated Isothermal Amplification (LAMP) of mycoplasmal DNA and Polymerase Chain Reaction (PCR). In order to confirm PCR positivity and enhance sensitivity, nested PCR was performed. In this study, 81 animals were examined by c-ELISA of which 52 were positive, 18 negative and 11 doubtful. Nasal swabs taken from the same 81 animals were examined by both PCR and LAMP for CBPP infections and 17 (20.9%) were found positive by all the three diagnostic techniques. c-ELISA positive and PCR-negative were 35 (43.2%) while c-ELISA- negative and PCR-positive was zero. Two samples were c-ELISA negative and LAMP positive while 35 samples were c-ELISA positive and LAMP negative. Of the 11 doubtful results by c-ELISA, LAMP technique confirmed positivity of 4 samples while PCR confirmed 2. These findings show that more false positive results were obtained with c-ELISA while LAMP indicated more sensitivity than both PCR and c-ELISA suggesting that LAMP, when used with clinical and post-mortem signs, is a suitable test for monitoring and surveillance programmes of CBPP in cattle.

**Key words:** Contagious bovine pleuropneumonia, outbreak, molecular techniques, serology

### INTRODUCTION

Contagious Bovine Pleuropneumonia (CBPP) is a respiratory disease of animals that is characterised by presence of serofibrinous interstitial pneumonia, interlobular oedema and hepatisation of the lungs coupled with capsulated lesions. In natural conditions, the disease affects ruminants of the *Bos* genus although the causative agent has been isolated from Italian buffaloes, *Bubalus bubalus* (Santini *et al.*, 1992) and from sheep and goats in some African countries and in India (Srivastava *et al.*, 2000). Wild animals do not play a role in the epidemiology of the disease. The causative agent of CBPP is *Mycoplasma mycoides* spp. *mycoides* small colony (*MmmSC*) which belongs to the phylogeny of *Mycoplasma mycoides* cluster. Transmission of CBPP occurs by direct contact between infected and healthy

animals. CBPP is associated with massive economic losses for cattle keepers; for instance, between 1949 and 1989, 178,570 cattle died of CBPP in China, causing losses estimated at 33.5 million US Dollars (Jiuqing *et al.*, 2011). While the disease was eradicated from the United States and Great Britain in the 19<sup>th</sup> Century without knowledge of the causative agent (Nicholas *et al.*, 2008), the disease persists in many African countries.

There are several established techniques for detection of the causative agent in affected animals. Post-mortem often reveals sequestra; these are encapsulated pulmonary lesions in which the content necroses progressively. Lesions are usually unilateral and the pleural cavity may contain large quantities of clear, yellow-brown fluid containing pieces of fibrin (Radostatis *et al.*, 2000). Since these animals may act as silent carriers, they represent a major transmission risk (CIRAD / Institut Pourquier,

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2010). In live animals, diagnosis of CBPP is problematic because of low sensitivity of the existing serological tests; for instance, the modified Campbell and Turner Complement Fixation Test (CFT) and competitive ELISA (c-ELISA) are based on antibody detection. Attempts to utilise serological techniques have also been used to monitor vaccination efficiency in cattle vaccinated against CBPP: for instance vaccination with strains such as T1/44 or T1-sr does not always induce detectable antibody responses (CIRAD / Institut Pourquier, 2010). Therefore, it is not possible to use CFT or c-ELISA to assess vaccination efficiency. However, as post-vaccinal antibodies do not persist after 3 months, CFT or c-ELISA can be used for the detection of natural infections even in areas where vaccination is used. Both tests are useful as herd tests and not for individual infected animal diagnosis.

In endemic countries, the disease is controlled through restriction of animal movements and vaccination campaigns using attenuated *MmmSC* strains such as T1/44 or T1-sr (Msami *et al.*, 2001). In the control of CBPP in affected animals, the use of antibiotics is theoretically prohibited in terms of clinical efficacy; however, antibiotics are widely applied in the field. Recent work has shown that antibiotic treatment of cattle may greatly reduce transmission to healthy contacts but this requires treatment of all affected cattle in the group (Hubschle *et al.*, 2004). Although new antibiotic classes have been shown to reduce clinical symptoms and post mortem lesions of CBPP, better control can be achieved when antibiotic treatments are combined with vaccinations (Hubschle *et al.*, 2006).

Due to the insidious nature of CBPP, it may go undetected for months before cattle develop noticeable clinical signs and because of this, there is a need for more sensitive tests which detect current infections. CFT is relatively sensitive in the acute phase of the disease after infection. The specificity of current serological tests is reduced due to cross-reactions with other closely related members of the *Mycoplasma mycoides* cluster. Currently, there is no single test that is sufficient for the diagnosis of CBPP during all its clinical stages. This is a weakness in the diagnosis of CBPP that results in spread of the disease, unless a number of tests are used at the different stages of the disease. This, therefore, requires new techniques such as PCR and LAMP for detecting *Mycoplasma* antigens in blood and body fluids, especially in asymptomatic cases (Egwu *et al.*, 1996). The main objective of this study was to evaluate different diagnostic tests in their suitability of detecting individual infected animals in categories of cattle that showed clinical signs and those that showed no clinical signs but were in the same herd as infected ones.

## MATERIALS AND METHODS

**Animals:** The investigation covered cattle from former Institut des Sciences Agronomiques du Rwanda (ISAR)

now known as Rwanda Agriculture Board (RAB), Nyagatare research station located in the Eastern Province of Rwanda after an outbreak of a disease of the respiratory system suspected to be CBPP in February 2010. Eighty one cattle of all ages and sex suspected to be carrying the disease were examined for CBPP, first using a serological technique, c-ELISA and later confirmed by two molecular techniques, LAMP and PCR.

**Preparation of serum:** Blood was taken from the jugular vein into vacutainers containing no anticoagulant; from each animal 3-5 mL of blood were drawn and allowed the clot to separate for a period of 12 h. Separated serum was decanted into cryovials and stored at -20°C until required.

**Preparation of samples for LAMP and PCR:** Two nasal swabs were collected from nostrils of each suspected animal. One swab was transferred into one tube containing 1 mL Phosphate Buffered Saline (PBS) and the second swab was transferred to the second tube containing 1 mL of distilled or PCR water. Both samples were stored at -20°C, until analysis.

**Sample preparation for LAMP:** The DNA sample for LAMP test was prepared by mixing 50 µL of sample in PBS and 150 µL of distilled water in a 1.5 mL micro centrifuge tube and boiled in a water bath at ≥100°C for 5 min. After cooling for 2 min, the mixture was centrifuged for 5 min at 14,000 rpm (≥10,000×g). The supernatant was recovered and stored into a new tube while the tube with the pellet was discarded.

**DNA preparation for PCR:** Purified DNA extracts for analysis by PCR were prepared according to the Chelex-100® method as described by Walsh *et al.* (1991) and Solano *et al.* (2005). Briefly, to each sample (250 µL) from nasal swab in glass distilled water equilibrated to room temperature, was added 250 µL of lysis buffer (0.32 mM sucrose, 0.01 M Tris-HCl, pH 7.5, 0.05M MgCl<sub>2</sub>, 1% Triton X-100). The mixture was centrifuged at 12,000 × g for 2 min and the supernatant discarded. The pellet was washed 3 times with lysis buffer and each time re-centrifuged at 12,000 × g for 2 min. The pellet was re-suspended in 250 µL of PCR water containing Proteinase K (50 mg) and the mixture incubated in a water bath at 56°C for an hour followed by heating at 95°C for 10 minutes. 250 µL of 5% Chelex-100® was added and the mixture was incubated at room temperature for 15 min. Thereafter, it was centrifuged at 8,000×g for 5 min to remove the Chelex. The resulting supernatant was placed into a new tube and 1/10 volume of 3 M sodium acetate (pH = 5.2) and 2 volumes (500 µL) of absolute ethanol were added to precipitate DNA. The contents of the tubes were centrifuged at 10,000×g for 5 min. The pellet was washed with 200 µL of 70% v/v ethanol. Centrifugation was repeated at 12,000×g for

3 min. The resulting DNA pellet was dried at 37°C overnight. The pellet was re-suspended in 20 µL of PCR water. The solution of DNA was stored at -20°C and used for molecular analysis.

**Contagious bovine pleuropneumonia LAMP procedure (CBPP-LAMP):** The LAMP reaction was performed as described by Njiru *et al.* (2007) using primers F3, B3, FIP, BIP, LB and LF. Briefly, amplification was carried out at constant temperature of 62°C for 60 min in a 25 µL reaction mixture containing final concentrations of 20 mM Tris-HCl pH 8.8 (10 mM KCL, 10 mM (NH<sub>4</sub>) SO<sub>4</sub>, 2 mM MgSO<sub>4</sub>, 0.1% Triton), 2.0 M Betaine, 2.0 µM for each FIP and BIP primers, 0.8 µM for each loop primers LF and LB, 0.2 µM for each F3 and B3 outer primers, 200 µM dNTPs, and 8 units of Bst DNA polymerase. The template was 1 µL for *Mycoplasma* DNA or 2 µL of supernatant from boiled samples. The LAMP test was carried out in GeneAmp 9800 Biosystem thermocycler, and in water bath and terminated by increasing temperature to 80°C for 4 min. One drop (2 µL) of SYBR Green dye was added, shaken thoroughly and colour change from yellow to green after mixing indicated positive reaction.

**Contagious bovine pleuropneumonia PCR procedure (CBPP-PCR):** A PCR assay using primers (MM 450: 5'-GTA TTT TCC TTT CTA ATT TG-3' and MM 451: 5'-AAA TCA AAT TAA TAA GTT TG -3') specific for *Mycoplasma mycoides* (SC) was conducted as described by Solano *et al.* (2005). PCR amplification was performed using 50 ng of DNA extracted from purified *Mycoplasma mycoides* (SC) as positive control DNA; test DNA comprised of 4 µL of DNA extracted from nasal swab fluid field samples. The DNA amplification was performed in 25 µL reaction mixture containing final concentrations of 10 mM Tris-HCl, pH 8.2, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 200 µM each of dATP, dCTP, dGTP, and dTTP, 10 pM each primers and 2.0 units of Taq DNA polymerase. PCR was performed using a GeneAmp PCR System 9700 from Applied Biosystems with the initial denaturation for 5 min at 94°C, followed by 40 cycles involving denaturation for 1 min at 94°C, annealing at 53°C for 1 min, extension at 72°C for 1 min; and a final extension for 5 min at 72°C. The absence of contaminants was routinely checked by inclusion of negative control samples in which the DNA sample was replaced with sterile water. Twenty microlitre of each sample were electrophoresed in a 2% w/v agarose containing 1 µg/mL ethidium bromide. The amplified products of 574 bp were observed in positive samples by ultraviolet transillumination.

A second PCR with the same amplification conditions was done in which 2 µL of the first PCR was used as template in the second PCR in order to improve

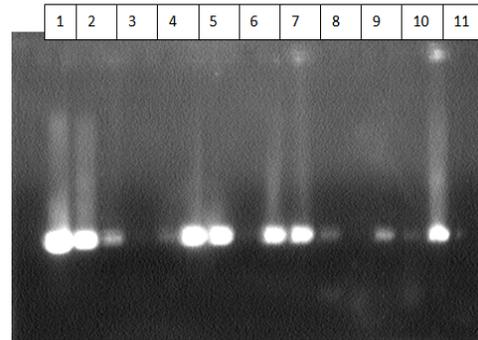


Fig. 1: Typical bands obtained from CBPP-PCR products run on 2% w/v Agarose gel

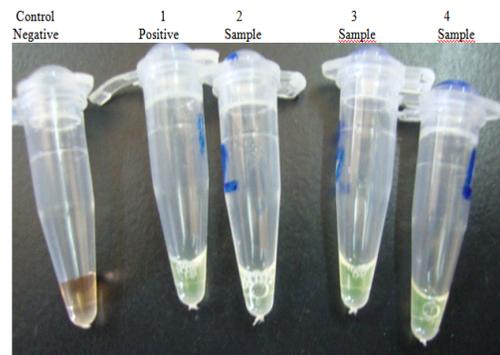


Fig. 2: Representative results of CBPP-LAMP Test with positive control and test samples. Tubes: Negative control, Positive control using 1 uL of *Mmycoides* Sc-PG1 from Denmark as standard and positive samples 2 to 4

on the sensitivity. Twenty microlitres of each PCR product sample alongside control and marker DNA were electrophoresed in 2.0 % w/v agarose gel containing 0.5 µg/mL ethidium bromide and later viewed under ultraviolet light.

## RESULTS

The results of CBPP-LAMP and CBPP-PCR are presented in the Table 1 alongside c-ELISA results. Typical PCR bands are shown in Fig. 1 and LAMP products showing positive and negative samples in Fig. 2. A total of 81 animals were examined by ELISA assay of which 53 were positive, 18 negative and 11 had doubtful results. Nasal swabs were taken from all the 81 animals for LAMP and PCR assays; results obtained contrasted sharply with those of c-ELISA indicating low number of animals positive for CBPP as follows: LAMP detected 23 positive samples, 57 negatives. PCR detected 19 positive samples and 62 negatives. Of the 81 cattle examined by all the three techniques (ELISA, PCR and LAMP),

Table 1: Identification of CBPP infection status in individual cattle by c-ELISA, PCR and LAMP

ID	Tag No	Sex	Age	Status by Method			ID	Tag No	Sex	Age	Status by Method		
				ELISA	LAMP	PCR					ELISA	LAMP	PCR
1	ANF020	F	A	D	-	-	42	AAA248	F	A	D	-	-
2	AAA306	F	A	+	-	-	43	AAA254	F	A	D	-	-
3	AGF004	F	Y	-	-	-	44	AAA321	F	A	-	-	-
4	AAA155	F	A	-	-	-	45	AAA116	F	Y	-	-	-
5	AAA170	F	A	+	+	+	46	AAA292	F	A	-	-	-
6	AAA310	F	A	-	-	-	47	AAA249	F	A	+	-	-
7	ANIF003	F	A	D	+	+	48	CNIF018	F	Y	+	++	+
8	ANIF015	F	Y	-	-	-	49	AAA132	F	A	+	++	+
9	AAA319	F	A	-	-	-	50	AAA131	F	A	+	-	-
10	AAA232	F	A	+	+	++	51	ANIF023	F	Y	+	-	-
11	AAA135	F	A	D	+	-	52	ANF024	F	Y	+	-	-
12	AAA222	F	A	D	-	-	53	CNF020	F	A	+	+	+
13	ANIF021	F	Y	-	ND	-	54	AAA277	F	A	+	-	-
14	AAA270	F	A	+	-	-	55	CNIM017	M	A	-	-	-
15	AAA242	F	A	+	-	-	56	AAA009	F	Y	+	+	+
16	AJF012	F	Y	-	-	-	57	AAA253	F	A	+	-	-
17	AAA120	F	Y	D	++	+	58	ANIF043	F	A	+	+	+
18	CNIF	F	Y	+	-	-	59	ASJ345	F	A	+	-	-
19	AAA326	F	A	-	-	-	60	ASJ256	F	Y	+	-	-
20	ANIF008	F	Y	-	-	-	61	AAA289	F	A	D	-	-
21	AAA082	F	Y	+	+	+	62	AAA198	F	A	+	-	-
22	AAA238	F	A	+	-	-	63	CNIF014	F	A	+	-	-
23	AAA228	F	A	+	+	+	64	AAA221	F	A	+	-	-
24	AAA303	F	A	+	+	+	65	AAA252	F	A	+	-	-
25	AAA188	F	A	+	-	-	66	AAA251	F	A	+	-	-
26	AAA288	F	A	+	+	+	67	AAA224	F	A	D	-	-
27	AAA220	F	A	+	-	-	68	ANIF018	F	Y	-	-	-
28	AAA311	F	A	+	+	+	69	AJF007	F	Y	+	+	+
29	AAA322	F	A	-	+	-	70	AAA200	F	A	+	-	-
30	AAA182	F	A	+	-	-	71	AAA269	F	A	-	-	-
31	AAA320	F	A	+	+	+	72	AJF009	F	Y	+	-	-
32	AAA231	F	A	+	-	-	73	AAA215	F	A	D	-	-
33	AAA260	F	A	+	-	-	74	934260	F	A	+	-	-
34	AAA267	F	A	+	+	+	75	AAA299	F	A	+	-	-
35	AAA294	F	A	+	-	-	76	AAA177	F	A	+	-	-
36	AAA229	F	A	+	-	-	77	AAA316	F	A	+	-	-
37	AAA245	F	A	+	+	+	78	AAA363	F	A	+	-	-
38	AAA264	F	A	-	+	-	79	AAA140	F	A	-	-	-
39	AAA173	F	A	D	+	-	80	ASJ343	F	A	+	-	-
40	ANIF047	F	Y	+	-	-	81	AJS351	F	A	+	-	-
41	AAA134	F	A	+	++	+							

F: Female; M: male; Y: Calf; A: Adult; D: Doubtful case; ND: Not done; +: Positive; ++: Strongly positive

17(20.9%) were positive by all the three techniques, while ELISA positive and PCR negative were 35 (43.2%), ELISA negative and PCR positive was zero.

### DISCUSSION

Several DNA-based assays which are less cumbersome than immunological tests have been developed for the efficient detection of *M. mycoides* subsp. *mycoides* SC. These include Southern blotting (Frey *et al.*, 1995; Pilo *et al.*, 2003) and conventional PCR tests (Vilei and Frey, 2001; Miles *et al.*, 2006) and real time PCR assays (Firtzmaurice *et al* 2008). Despite considerable successes, there is need to develop cheaper

and field friendly tests for the detection of CBPP in carrier and asymptomatic animals which remain as sources of infection, a phenomenon that renders control of the disease in the field very difficult. For routine field use, immunological tests and PCR are sufficient to detect active CBPP infections in cattle, but in some instances, false positives may be obtained. In such cases, biochemical tests can be performed but these are usually done in specialised laboratories and involve cloning of at least three colonies (Al-Aubaidi and Fabricant, 1971). Once the biochemical tests are complete, any one of the immunological tests like disk growth inhibition test, fluorescent antibody and dot-immunoblotting tests on filter paper can be done. The nucleic acid-based tests for

*M. mycoides* cluster (Taylor *et al.*, 1992) and for MmmSC (Dedieu *et al.*, 1994; Meserez *et al.*, 1997) have been developed and are sensitive and highly specific. Serological tests can be performed but are valid for herd use only: tests on individual animals can be misleading because animals are either in early stage of the disease before specific antibodies are produced or it may be the chronic stage when few animals are seropositive.

The CFT is widely used where infection occurs (Provost *et al.*, 1987) and is suitable for determining freedom from disease and is prescribed for international trade. These tests, however, are not suitable for screening individual cattle against CBPP, necessitating development of cheaper and field-friendly tests for detection of CBPP in carrier and asymptomatic animals which remain sources of infection, making control of the disease difficult. Therefore, the search for appropriate diagnostic techniques capable of differentiating animals in the acute phase of the disease from those in carrier state should continue to differentiate disease states in different set ups and risk factors. For effective control of CBPP in sub-Saharan African countries, post mortem should be incorporated as a diagnostic as well as a monitoring tool of the disease. Post mortem done at slaughter houses in Zambia (Muuka *et al.*, 2011) in 797 cattle in the CBPP affected area of Kazungula, showed a high sensitivity (67.5%) compared to 52.6% by CFT, 59.0 by c-ELISA and 44.0 % by LppQ ELISA.

In a previous study at the Department of Biochemistry, Makerere University, Enyaru *et al.* (2003) showed that when c-ELISA was compared with CFT as a gold standard, its sensitivity was found to be low (71.0%) which indicated that quite a number of infected animals are misdiagnosed. Lower sensitivity for c-ELISA assay in CBPP endemic area in Zambia at 59.0% has also been reported by Muuka *et al.* (2011); in the same study, CFT sensitivity was 52.6% while post mortem examination of lung tissue was higher (67.5%).

In the present study, LAMP and PCR results were compared with c-ELISA results; the c-ELISA results indicated more infected animals than both LAMP and PCR on samples from the same animals. It is noted that 17 (20.9%) of the 81 cattle examined were positive on all the three techniques. However, ELISA positive and PCR/LAMP negatives were 35, indicating high number of false positives compared to ELISA negative and PCR/LAMP positive. LAMP picked up 5 more positives than PCR indicating high sensitivity of LAMP and confirmed 4 doubtful serological results as positives. Currently, there is no single test that is sufficient for the diagnosis of CBPP during all its clinical stages. This, therefore, allows a loophole in CBPP diagnosis which

results in spread, unless panels of tests are used at the different stages of the disease. This calls for a continued search for a test that can detect CBPP during all its stages. Future prospects for eradication of CBPP depend on its accurate diagnosis, which requires new techniques such as PCR for detecting mycoplasmal antigens in blood and body fluids, especially asymptomatic cases (Egwu *et al.*, 1996), and the newly developed Loop Mediated Isothermal Amplification (LAMP) DNA technique. It is noted that the causative agent of CBPP, *M.m mycoides* SC, was among the first species for which PCR assays were developed based on CAP-21 gene fragments which were useful as screening tests to rule out the presence exotic Mycoplasmas.

Further studies at the Livestock Health Research Institute (LIRI), now the National Livestock Resources Research Institute (NaLIRRI), Tororo, Uganda, showed that PCR is very sensitive in diagnosing the Ugandan strains of *M. mycoides* subsp. *mycoides* SC using primers from the Pleurotrap Kit, (AMRAD Biotechnology, Australia (Enyaru *et al.*, 2003). Despite some good results obtained by PCR in differentiating *M.mycoides* cluster from other exotic mycoplasmas, the rate of adoption of molecular diagnostic DNA technology by laboratories in developing countries appears to be limited due to costs of reagents and equipment coupled with low availability of trained personnel. The recently developed Loop Mediated Isothermal Amplification (LAMP) appears to be a good alternative because of its rapid reaction within 60 minutes. It is simple and requires a water bath/heat block to amplify DNA at a constant temperature and produces large amounts of stained DNA which can be visualised by a naked eye (Fig. 2).

## CONCLUSION

Results from this study show that CBPP-LAMP is an ideal alternative molecular diagnostic technique for the detection and confirmation of CBPP in rural areas in resource-poor developing countries and for creating CBPP free areas which can be zoned for beef production for export. Further, Loop Mediated Isothermal Amplification (LAMP) technique is more sensitive than ELISA in the diagnosis of Mycoplasma and it is recommended that both LAMP together with clinical and post-mortem signs are suitable tests for monitoring and surveillance programmes of CBPP in cattle.

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