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Production of Modified Rapid Serum Agglutination Antigen for Sero-diagnosis of Contagious Bovine Pleuropneumonia (CBPP)

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Authors' contributions

This work was carried out in collaboration between all authors. Author RAHO designed the study, wrote the protocol and managed the literature searches. Authors GEM and FMH managed the analyses of the study. Author EIE performed the statistical analysis. Authors GEM and EAM revised the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: This work was conducted to develop rapid serum agglutination test for Contagious Bovine Pleuropneumonia (CBPP) serodiagnosis.
Study Design: Production of two types of antigen, one from T1/44 reference strain of *Mycoplasma mycoides* sub sp. *mycoides* (Mmm) and the other from local strain (RH).

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Place and Duration of Study: This study was carried out at Mycoplasma department Central Veterinary Research Lab (CVRL) in Sudan during 2016-2018.
Methodology: The antigens have been developed using standard methods and evaluated using standard reference serum, c.ELISA and Latex Agglutination test. Statistical analysis using Chi-square test and Kappa agreement were applied.
Results: The sensitivity of the developed stained antigen from T1/44 strain (SAT1) was 100% in comparison of 92% and 95% of SAT2 (RH local strain) and LAT, respectively, The developed SAT2 antigen revealed satisfied specificity results (70%), which is higher than SAT1 (31%) and LAT (59.6%).
Conclusion: From these results we found that SAT2 which is produced from local strain- proved to be more specific and highly sensitive. The SAT2 combines low cost and high specificity and easy applicable in field, without need for any specialist training or equipment.

Keywords: Contagious; bovine; Pleuropneumonia; Mycoplasma mycoides subsp mycoides (Mmm); competitive ELISA; latex agglutination test; sensitivity; specificity.

1. INTRODUCTION

Contagious Bovine Pleuropneumonia (CBPP) is a highly contagious acute, subacute or chronic respiratory disease primarily of cattle caused by *Mycoplasma mycoides* subsp. *mycoides* (*Mmm*). CBPP is one of the most severe diseases affecting cattle in most countries of sub-Saharan Africa [1]. Cattle infected with (*Mmm*) can develop acute, subacute, or chronic disease. The acute form is manifested by anorexia, fever and respiratory signs, such as dyspnoea, polypnoea, cough and nasal discharges. In the case of acute outbreaks under experimental conditions, the mortality rate may be as high as 50% in the absence of antibiotic treatment. The disease has major economic concern in the affected countries, not only due to the morbidity and mortality but also due to restrictions on cattle trade imposed by international regulations. Therefore, control of the disease is a priority for countries in which it is endemic, in order to eradicate the disease as quickly as possible after outbreaks and to avoid it's spreading, as well as for countries which are free of CBPP, in order to keep their status [2] In general control strategies of the disease rely heavily on the detection of endemic areas, quarantine measurement, diagnosis, stamping out policy and vaccination. In the absence of better vaccines, improved diagnostic assays for recurrent testing combined with isolation and treatment of positive animals represent an option for CBPP control in Africa [3] Diagnosis and control can be hampered due to long incubation periods when cattle may express no overt disease symptoms. A number of diagnostic tests currently exist, but most are difficult to use in situ, lack sensitivity, or require resources unavailable in many countries affected by the disease [4].

Serological tests for CBPP are valid at the herd level only because false positive or false negative results may occur in individual animals [4]. Tests on single animals can be misleading, either because the animal is in the early stage of disease, which may last for several months, before specific antibodies are produced, or it may be in the chronic stage of the disease when very few animals are seropositive. Different serological tests were used in CBPP diagnosis including Tube Agglutination Test [5], Slide agglutination test [6], Complement fixation test [7], Precipitation test whether in tubes [8] or agar plates [9] and Enzyme linked immuno sorbant assay (ELISA) [10,11]. All these tests appear to be sensitive but may lack specificity. A competitive ELISA (cELISA) was developed at CIRAD EMVT, Montpellier [12], Latex agglutination test (BoviLAT) [13] and Immunoblotting tests (IBT) [14]. In Sudan after eradication of Rinderpest, CBPP has become the disease of prime concern in terms of epizootics that affect cattle in the country. Recent study which directed to estimate the prevalence of CBPP in IGAD countries (Surveillance of Trade Sensitive Diseases project STSD, 2016) revealed 8.7% prevalence using ELISA test (708 positive samples out of 8121 serum samples).The disease is concentrated in Central Darfur (26.7%), North Darfur (26 %) and Al Gadaref (12%).The available diagnostic tests are expensive; which led us to develop ease, high sensitive and cheap slide agglutination test; using reference and local strain.

2. MATERIALS AND METHODS

2.1 Production of Working Seeds

The T1\44 reference strain was obtained from Pan African Veterinary Vaccine centre (PANVAC)

reference laboratory (Ethiopia) as vaccine strain. The antigen was produced from the working seed in broth medium and RH-Local strain (RH) which isolated from animals show typical CBPP clinical signs and the strain confirmed by conventional and serological technique. They were cultivated in Gourlay´s broth medium, and then incubated at 37°C for 48 hours. This culture was used for the production of stained antigens. Sterility, purity, identity and viable count tests were carried out as quality control tests [4].

2.2 Preparation of Mycoplasma-stained Color Antigen

The preparation of the stained antigen was guided by Newing and Field [15], Morgan et al. [16] and Arefin et al. [17] methods. The reference and local strain were seeded at 1:10 in Gourlay´s broth medium and incubated at 37°C for 8-10 days to reach to the optimum growth, Then Rose Bengal stain (1%) was added (1 ml of diluted stain to every 100 ml of culture) and Phenol saline (0.5%) was added 5ml/L for inactivation. The flasks were agitated and incubated for 24 hours at 37ºC. After that they were centrifuged at 4,000 rpm for 60 minutes.

2.3 Titration of the Antigen

The supernatant fluid was decanted and the pellet was washed twice; using PBS (pH 7.2) the packed cells were titrated by dilution with different percentage using PBS (pH 7.2). The concentrated cells were diluted to reach to the best result: 90%, 70%, 50%, 30% and 10%, respectively. The suspension was vortexed vigorously and transferred to another Eppendorf tube. A dense suspension of deeply stained cells resulted. For testing the prepared antigen, 20 µl of stained antigen and 20 µl bovine sera were placed on an enamel plate using microtitre pipette and mixed thoroughly by gentle shaking. The results were read within 2-3 minutes.

2.4 Assessment of the Antigen

The Latex agglutination antigen (ENGLAND) and c. ELISA (IDEXX –France) were used in the antigen assessment. A total of 222 bovine serum samples were collected randomly from dairy farms at Khartoum state-Sudan. They were tested using the c.ELISA, the latex agglutination test (ENGLAND) and the new developed antigens. The validity of the test was defined by using characteristics like sensitivity and specificity, and parameters like predictive value positive (PPV) and negative (NPV).

2.5 Antigen Stability

The developed antigen was checked for efficacy in serodiagnosis when preserved at different temperature i.e. at 4ºC and 37ºC.

3. RESULTS

3.1 Production of Working Seeds

A single colony of *Mmm* was inoculated into Gourlay´s broth formed confluent growth that showed turbidity and sliminess. The local strain culture was sterile, pure and the viable count was 10^7CCU/ml.

3.2 Preparation of Stained Antigen

The Rose Bengal -stained antigen showed pink and the agglutination result was clearly seen. For proper mixing, shake the antigen before use. The stain that used for the preparation of antigen is sensitive to light so it kept away from the light.

3.3 Titration of the Antigen

It was found that 40% concentration is the most clearly visible concentration. As shown in Fig. 1. In positive case, granules (agglutinates) formed rapidly due to combination of homologous antigen and antibody, which was seen during rocking. The agglutinin was pink with purple background (Fig. 1). The reaction is graded as follows: rapid coarse agglutination complete with in 15-30Sec = three-plus, definite reaction but far from complete= two plus, very fine agglutination recognisable in a good light = one plus. Finally no agglutination reported as negative (Fig. 2).

3.4 Assessment of Antigen

Reference positive and negative control sera brought from (APHA) were used to assess the modified antigen. The results revealed typical reaction as shown in Tables 1, 2 and 3.

3.5 Stability of the Antigen

The modified antigen was found to be stable for 12 month at refrigerator and for 2 days at 37ºC.

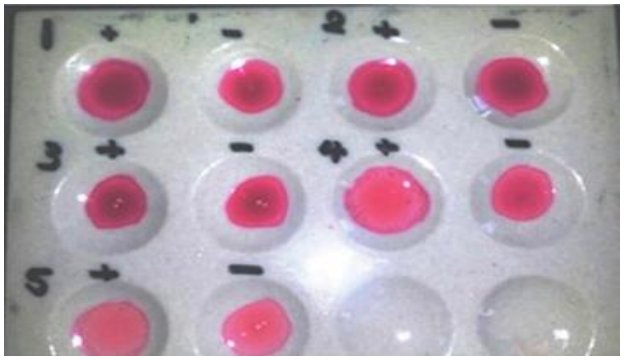

Fig. 1. Titration of the modified antigen 1:90%, 2:70%, 3:50%, 4:30%, and 5:10%

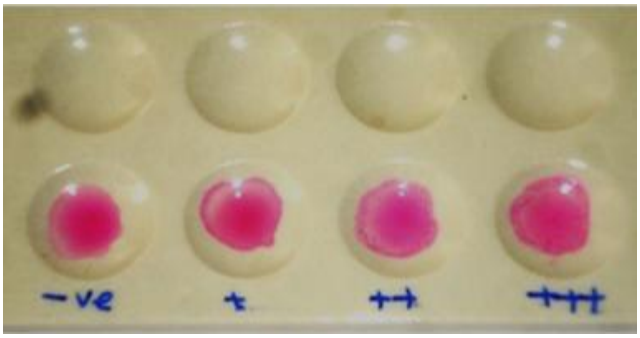

Fig. 2. Grades of *Mycoplasma* strained antigen

Table 1. The sensitivity and specificity of LAT* comparing with c.ELISA

		ELISA		Sen.%	Sp. %	PPV%	NPV%
		Positive (%)	Negative (%)				
Latex	Positive	39 (34.8)	73 (65.2)	95	59.6	34.8	98
	Negative	2 (1.8)	108 (98.2)				
Total		41 (18.5)	181 (81.5)				

*Mc Nemar Test =.001; Kappa Agreement = 0.3; *Latex=Latex agglutination test*
Sen. = Sensitivity; Sp. = Specificity; PPV = Positive predicted value; NPV= Negative predicted value

Table 2. The sensitivity and specificity of SAT1 comparing with c.ELISA**

		ELISA		Sen.%	Sp.%	PPV%	NPV%
		Positive (%)	Negative (%)				
SAT1	Positive	41 (24.7)	125 (75.3)	100	31	24.7	100
	Negative	0 (0.0)	56 (100)				
Total		41 (18.5)	181 (81.5)				

McNemar test = 0.001; Kappa agreement = 0.1
***SAT1= Slide agglutination test produced from T1/44 strain*

Table 3. The sensitivity and specificity of SAT2* comparing with c.ELISA**

		ELISA		Sen.%	Sp.%	PPV%	NPV%
		Positive (%)	Negative (%)				
SAT2	Positive	38 (41.3%)	54 (58.7%)	92	70	41.3	97.7
	Negative	3 (2.3%)	127 (97.7%)				
Total		41 (18.5)	181 (81.5)				

McNemar Test = 0.001; Kappa Agreement = 0.3.
****SAT2= Slide agglutination test produced from Local strain*

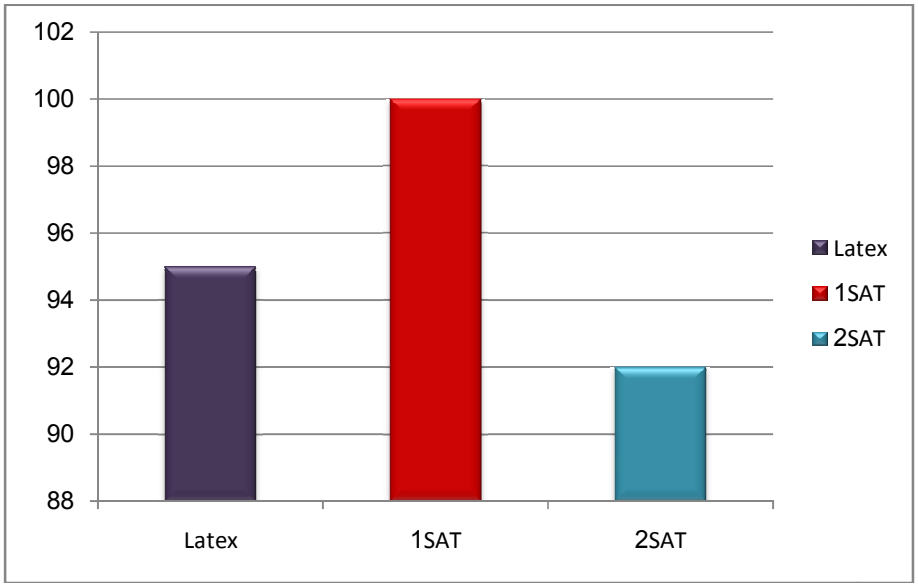

Fig. 3. Comparison of the sensitivity of the three tests, using cELISA as a golden test

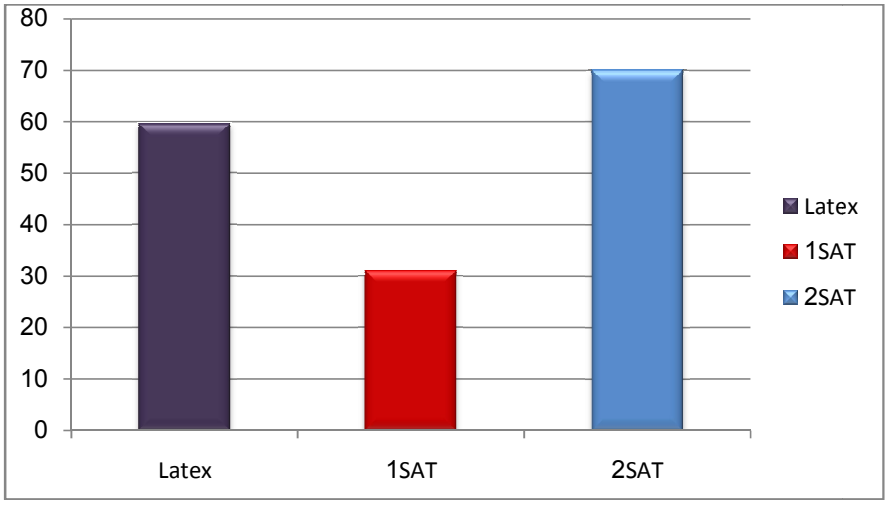

Fig. 4. Comparison of the specificity of the three tests, using c ELISA as a golden test

4. DISCUSSION

CBPP is one of the major infectious diseases which affecting cattle in Africa. This situation necessitates continuous surveillance and data collection in Sudan to eliminate quickly positive reactors and hence, restricting the disease. CBPP- Stained agglutination test antigen (SAT) have been developed from reference and local strain. Sensitively and specificity are two features used to quantify the performance of any diagnostic laboratory test. While the sensitivity of the test is defines as the ability to correctly identify diseased animals and therefore gives an indication of how many false negative results can be expected, the specificity is the ability of the diagnostic tests to correctly identify non diseased animals and gives an indication of how many false positive results can be expected [18]. In that respect, the sensitivity of the developed stained antigen from T1/44 strain (SAT_1) was found 100% in comparison of 92% and 95% of SAT_2 (RH local strain) and LAT respectively using c.ELISA. In this study we reported (95%) sensitivity of LAT using CFT test, which is less than 62% reported by March et al. [19].This may be due to different gold standard used by those authors, as stated by Mohamed Babiker [20], who

mentioned that there were obvious variations in the obtained results between two serological tests (CFT and C-ELISA). Also Amanfu et al. [21] reported that the c-ELISA test is more sensitive than CFT in terms of sensitivity for chronic carriers but less sensitive than CFT for the detection of acute cases .Our serum samples were collected randomly without focusing on the stage (acute or chronic) of the diseased. The Specificity of the developed SAT_2 antigen revealed satisfied specificity results (70%), which is higher than SAT_1 (31%) and LAT (59.6%), this high specificity percentage is due to close antigenic relationship between the local strain and antibodies circulating in the local animals so this encourage us to use autologous antigens in serodiagnostic assays to increase sensitivity of the tests.

At the herd level, the SAT_2 test should allow rapid and early recognition of a CBPP infection with high sensitivity which allows appropriate control measures to be swiftly implemented, for example, quarantine or movement restriction.

The test allows rapid and inexpensive primary herd screening prior to confirmatory laboratory diagnosis test e.g. (isolation of the organisms) so it is useful in the field. In contrast to other diagnostic tests, no expensive equipment or hazardous reagents are required (e.g., ELISA plate readers, PCR machines, gel electrophoresis equipment, enzymes, etc.), and results can be obtained within minutes.

5. CONCLUSIONS AND RECOMMENDATIONS

The stained *Mycoplasma* antigen from T1\44 reference and RH local strains was successfully modified.

The antigens are sensitive and reliable for detection of animals harbouring the disease (acute). It is better to use SAT_2 firstly on field samples to detect positive ones, because it is highly sensitive and then check the positive serums using CFT or C.ELISA for confirmation; this can decrease the cost of the kits. Further research should be tackled to produce a purified specific antigen from our isolates to reduce the cross reaction.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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