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Research Article

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Preliminary Findings of Lipoprotein B in Detecting Cattle Chronically Infected with Contagious Bovine Pleuropneumonia

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Abstract

Contagious Bovine Pleuropneumonia (CBPP) caused by *Mycoplasma mycoides* subsp. *mycoides* is an important disease of cattle affecting international trade. Complement Fixation Test (CFT) and competitive Enzyme Linked Immunosorbent Assay (c-ELISA) are the only serological tests recognized for diagnosis of CBPP. The performance of CFT depends on the quality of the antigen and the combination of reagents used and only detects CBPP during the acute phase of the disease. There is a need to develop a more sensitive and specific test. Therefore, one mycoplasma protein previously identified as being a potential diagnostic antigen, Lipoprotein B (LppB), was tested for its usefulness to detect infected cattle in an indirect ELISA (i-ELISA). Samples obtained from pre-challenged and challenged cattle were compared using CFT and an i-ELISA based on lipoprotein B. The i-ELISA developed with the new antigen, detected more positive samples than the CFT, which is considered a gold standard. Therefore, the LppB recombinant protein could be explored further as target for screening cattle infected chronically with CBPP.

Keywords

CBPP; M*ycoplasma mycoides*; CFT; LppB; Sensitivity; Specificity

Introduction

Mycoplasma mycoides subsp. *mycoides* (*Mmm*) is the causal agent of contagious bovine pleuropneumonia (CBPP) that causes serious economic losses in cattle in Africa [1]. It is a disease that mainly affects domestic cattle and symptoms range from acute, to sub-acute and chronic forms [2]. Up to date, no single test is able to detect all clinical stages.

Serological tests recommended by the Office of International Epizootics (OIE) for serological diagnosis of CBPP employs either complement fixation test (CFT) and/or competitive enzyme linked immunosorbent assay (c-ELISA). These tests have drawbacks necessitating the need to develop more robust tests. CFT has a high specificity but takes long to perform and requires more elaborate training of staff. Additionally, it is less effective at diagnosing animals with chronic lesions [3]. The c-ELISA test on the other hand, is simpler to carry out. However, validation studies are still insufficient to make good conclusions about its use in surveillance studies where vaccinations are undertaken in enzootic areas of infected zones. In addition, the test is sensitive but its specificity is uncertain [4]. There is a need for development of another test as sensitive as c-ELISA and as specific as CFT.

Preliminary studies have been carried out to introduce surface proteins of the mycoplasma *Mmm* strain to develop sensitive and specific diagnostics and efficacious vaccines [5-7]. In this study, a few serum samples that had been tested and confirmed positive by CFT were re-tested against LppB recombinant protein to determine if there are differences in antibody responses, sensitivity and specificity between the two tests.

Materials and Methods

All cattle experiments from which the samples were derived were carried out in accordance with the Kenya Agricultural and Livestock Research Organization (KALRO), Veterinary Science Research Institute (VSRI)-Institutional Animal Care and Use Committee (IACUC), numbers: KALRO/VSRI/IACUC/1/29092009 and KALRO/VSRI/IACUC/2/00122010.

The cattle used in the study were purchased from Kakamega in western Kenya, a CBPP-free zone. The samples were obtained following challenge by contact transmission using a field isolate of *Mmm* referred to as strain B237 from Thika, Kenya [8].

Expression and purification of lipoprotein B

Inoculated 5 ml Luria-Broth (LB) medium containing 30 µg/mL kanamycin with a single colony of BL21DE3 STAR cells containing a pETite expression construct of LppB. Shaken overnight at 220 rpm and 37^0 C in shaker incubator. The following morning, 0.5% glucose was added before addition of 5 ml to 250 ml LB media plus kanamycin. The cultures were grown in a shaker incubator until the optical density (OD at 600 nm) was between 0.5-0.7, before inducing with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4 h. Cells were centrifuged at 14,000 x g for 30 min at 15^0 C, then purified under denaturing conditions as described by Miltiadou et al. [7].

Antibody responses as determined by CFT and LppB

Serum samples were selected on the basis of CFT results and the presence or absence of lesions. They were tested using an indirect ELISA (i-ELISA) based on the LppB antigen. An analysis was then carried out to compare antibody responses, sensitivities and specificities of the two tests. The CFT was carried out according to Campbell et al. [9].

The LppB assay was performed as follows: Wells of polysorp 96-micro-well plates (Nalge International, Roskilde Denmark) were pre-coated with 150 µl of 1.2 µg/µl LppB recombinant antigen in phosphate buffered saline (PBS) pH 7.4 for 2 h at 37^0 C. The wells were blocked with 300 µl of blocking buffer (0.2% casein in PBS) for 20 min at 37^0 C and washed 3 times in washing buffer (PBS + 0.1% Tween 20). Serum test samples were diluted 1:400 in 1% skimmed milk in PBS, 150 µl added in duplicate on the plate and incubated at 37^0 C for 40 min before washing 5 times in washing buffer. One hundred and

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fifty microlitres of horse reddish peroxidase (HRP)-conjugated anti-bovine IgG (Svanova, Sweden) were added at a dilution of 1:10, 000 in PBS and the plate incubated at 37^{0}C for 30 min. The diammonium salt 2, 2'-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid (ABTS) was used as chromogen and H_2O_2 as substrate for peroxidase to detect bovine anti-LppB antibodies. The plate was incubated in the dark with gentle shaking for 30 min before OD readings were obtained using an Immunoskan ELISA plate reader at 405 nm.

Determination of sensitivities and specificities between tests

Determination of sensitivities and specificities between tests were done using a 2 × 2 contingency table as shown in Table 1 [10]. All cattle were assumed to be positive after challenge and negative before challenge.

Statistical analysis

Data was entered into Microsoft excel 2010 and SPSS 22.0 where Cohen's Kappa test was used to determine if there was an agreement between CFT and LppB serological tests in detecting positive and negative serum samples.

Results

Antibody responses as determined by CFT and LppB

Table 2 shows results of pre and post-challenge sera, tested with the two assays. Post-challenge sera were further split into sera from animals without lesions, with sequestrae or from early acute cases. These post-challenge sera were selected from a total of 36 cattle, where $^{20}/_{36}$ had no lesions, $^{9}/_{36}$ had sequestrae and $^{7}/_{36}$ had acute infection. The eight pre-challenge samples were samples collected 21 days before challenge. Acute sera were collected within 1 month after challenge while sera from animals with sequestrae and no visible lesions were collected between 3 to 12 months after challenge. The negative and positive sera in various disease stages were collected at same time points. LppB-ELISA was very efficient in detecting serum samples from infected animals; in particular, from animals with sequestrae (100%), and just over half of sera from animals without lesions. However, it did not identify any sample from acutely infected cattle. It also detected one of the pre-vaccination serum samples.

Determination of sensitivities and specificities between different assays

Table 2 shows CFT and LppB results on post-challenge serum samples for animals without lesions, with sequestrae and early acute cases. *Mmm* SC was isolated from lungs of all challenged cattle. Upon challenge, fever was first recorded on the 40th day post-contact. Eight out of nine animals with sequestrae presented with fever. The longest period with fever recorded was 14 days in one animal while the shortest fever period recorded lasted 2 days in two animals. All 7 animals with early acute lesions presented with fever. The highest fever recorded lasted 13 days in one animal while the least fever recorded lasted one day in one cattle.

Calculation of sensitivities and specificities

Sensitivities of the assays was calculated as follows: it was assumed that all infected animals were positive, including those that had no lesions since *Mmm* SC was isolated from lung tissues of all cattle. Specificity was calculated using pre-challenge samples only since these were assumed to be true negatives. In Table 3 below, sensitivity of LppB was found to be higher than that of CFT while specificity of CFT was higher.

Table1: Calculation of Sensitivities and Specificities.

Type of test	Tested (+)	Tested (-)
Test (+)	T+	F-
Test (-)	F+	T-
Sensitivity (Se)= $\left(\frac{T-}{(T-)+(F+)}\right)X100\%$		Specificity (Sp)= $\left(\frac{T-}{(T-)+(F+)}\right)X100\%$

T+: True positive; T-: True negative; F-: False negative; F+: False positive

Table 2: Number of Serum Samples Testing Positive Pre-Challenge and at Various Disease Stages Post-Challenge.

Test	Positive serum samples of animals			
	Pre-challenge (n = 8)	Post-challenge (n = 36)		
		Without lesions	With sequestrae	With acute infection
CFT (KALRO)	$^{0}/_{8}$ (0%)	$^{2}/_{20}$ (10%)	$^{3}/_{9}$ (33.3%)	$^{1}/_{7}$ (14.3%)
LppB (i-ELISA)	$^{1}/_{8}$ (12.5%)	$^{14}/_{20}$ (70%)	$^{9}/_{9}$ (100%)	$^{0}/_{7}$ (0%)

Table 3: Results of Sensitivities and Specificities of Two Tests.

Type of test	CFT	LppB
Se (%)	17	64
Sp (%)	100	87.5

Table 4: A Comparison of Similarities and Differences between Different Tests.

T	Categories	Kappa	P
Acute Lesions (N=7)	CFT & LppB	-0.167	0.659
Sequestrae (N=9)	CFT & LppB	0.000[a]	0.000
No Lesion (N=20)	CFT & LppB	-0.212	0.023

a. No statistics are computed because the test is a constant.

Determination of agreement between CFT and LppB

Table 4 below presents the frequency counts for positive and negative serum samples as assessed using CFT and the indirect ELISAs based on LppB recombinant antigen. Although there was no agreement between CFT and LppB in detection of antibodies in no lesions and sequestrae cases respectively, there was significant agreement between CFT and LppB in detection of antibodies in acute cases ($p = 0.659$).

Discussion

The observed differences between the sensitivities obtained in our study and previous studies [10,11], are probably due to the disease status of the animals after challenge and the time point at which sera were collected. Animals in our study were infected by in-contact challenge, and there is always uncertainty on the time point at which a particular animal gets infected [12]. In our study, CFT had a significantly lower sensitivity, 17%. In our study the test was performed on serum samples at the end of 3rd week for acute cases, end of 4th month for cases with sequestrae and end of also 4th month for cases with no lesion. In addition, the immune status of the individual animal seems to play a role in the time course and level of specific antibody production [13]. During the acute phases of the disease, CFT detects both IgM and IgG1 that bind complement but not IgG2 [14]. Antibodies detected by CFT decrease early and the number of positive results decreases dramatically when a blood sampling has occurred 3 months later in an outbreak. This possibly explains the low sensitivity of the CFT assay, which depends on complement binding antibodies, mainly IgM [15]. In contrast, c-ELISA strongly detects IgG2 and therefore will identify positive animals in later stages of disease compared to CFT [14,16].

The sensitivities of the i-ELISA with LppB, detecting mainly specific IgG, was 64%. This figure is comparable to the sensitivity of an assay based on another recombinant protein, LppQ [11], at 69% but with a specificity of 100%. It is known that LppQ induces a very high antibody response [17], and is also specific for *Mmm*. However, our i-ELISA assays detected the highest number (100%) of positive serum samples in cases with sequestrae, while 70% of animals without lesions (Table 2). Progress of CBPP in animals with the acute form of disease is rapid and it is possible that such animals have not had time to produce high enough IgG antibody titres to the proteins. High levels of mycoplasma antigens in the acute form will mop up any specific antibodies generated early in the infection, resulting in negative diagnosis, as suggested before [18]. In contrast, animals with sequestrae have been infected for a longer time and therefore IgG antibody titres may have had time to build up in the blood. In animals without lesions, antigen is relatively low compared to animals with sequestrae, so the number of positive samples detected is intermediate.

Conclusion

The two serological tests showed different results. The LppB-based test detected more positive serum samples than CFT. Since LppB detected all animals with sequestrae, it holds the possibility that a diagnostic test could be developed to identify animals with carrier status.

Authors' Contributions

All authors contributed to conception and design of the study; HOL, JN and HOW wrote the paper.

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