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Research Article

Development and Evaluation of an Immuno-Capture Enzyme-Linked Immunosorbent Assay to Quantify the *Mycoplasma capricolum* subsp. *capripneumoniae* (Mccp) Protein in Contagious Caprine Pleuropneumonia (CCPP) Vaccine

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An effective contagious caprine pleuropneumonia (CCPP) vaccine is essential for the increased production of healthy goats in a cost-effective manner and the prevention of animal-to-animal transmission for both domestic animals and wildlife. Quality control of this vaccine ensures that a reliable supply of pure, safe, and potent batches is obtained. As part of this control, in vitro quantification of *Mycoplasma capricolum* subsp. *capripneumoniae* (Mccp) protein in the final vaccines is required before the CCPP vaccine undergoes batch release and certification. The current method used for quantification is based on the measurement of total protein using the bicinchonic acid (BCA) test. This method quantifies the total amount of protein in the vaccine including contaminant protein from media, which can lead to overestimation of the quantity of Mccp protein, resulting in reduced vaccine immunogenicity. An immuno-capture ELISA (ICE) was developed for specific detection and quantification of the Mccp antigen in the CCPP vaccine. As the ICE detects and measures the amount of antigen between two layers of antibodies, capture and detecting antibodies are required. Mouse monoclonal antibodies (mAbs) that detect the Mccp antigen were produced and characterized. One of these mAbs, Mccp-25, was used to develop the ICE as an unlabelled capture antibody and horseradish peroxidase conjugated detecting antibody. The ICE was standardized and evaluated using an internal reference sample, experimental CCPP vaccines and commercial CCPP vaccines. A comparison between the polymerase chain reaction (PCR) and ICE showed good correlation between the two assays. Also, an *in vitro* ICE method correlated well with an in vivo sero-conversion in goats that were vaccinated with selected test vaccines. The sensitivity of the ICE was estimated at 30 ng/ml.

1. Introduction

Contagious *caprine pleuropneumonia* (CCPP) is an infectious disease in goats caused by *Mycoplasma capricolum* subspecies *capripneumoniae* (Mccp). Originally known as Mycoplasma F38, it was first isolated in Kenya from the lungs of goats with pleuropneumonia [1, 2]. Subsequently, Mccp has been isolated in other countries, such as Chad, Ethiopia, India, Oman, Sudan,

Tunisia, Turkey, and Uganda [3]. CCPP is also now known to affect some species of exotic ungulates, particularly wildlife species, such as gazelles and gerenuks [4, 5]. The disease is highly contagious and is transmitted by close contact through inhalation of respiratory droplets [2]. Clinically, CCPP affects the respiratory tract and is characterised in its acute form by fever, anorexia, and severe respiratory distress with coughing, nasal discharge, dyspnoea, polypnoea, and fibrinous pleuropneumonia with straw-coloured pleural fluid [6–8]. CCPP symptoms are similar to those of other small ruminant diseases and a differential diagnostic has to be made from *Pasteurellosis* and Peste des Petits Ruminants (PPR). CCPP causes major economic losses in goat production in the infected African, Asian, and Middle Eastern countries where the disease is endemic [7, 9]. Morbidity and mortality rates can reach 100% and 60–70%, respectively, particularly in naive flocks [7, 9]. The distribution of CCPP disease is not well established, but the clinical disease has been reported in more than 30 countries in Africa and Asia [10, 11]. Control of the disease is mainly done by vaccination. It was demonstrated that protective immunity in goats can be induced by using sonicated Mccp antigens in Freund's complete and/or incomplete adjuvants for an immunity duration of at least six months after vaccination [12]. The CCPP vaccine is an inactivated preparation of F38 mycoplasma containing saponin as an inactivating agent and adjuvant [13]. Evaluation of the amount of lyophilised F38 mycoplasma antigen plus saponin showed that the optimum formulation was 0.15 mg of antigen in 3 mg/ml of saponin solution [13, 14]. Saponin inactivates the mycoplasma and provides the adjuvant effect necessary to stimulate a protective immune response. A single immunisation with the optimum formulation produced a protective immune response in goats that lasted for longer than one year [13]. The lyophilised F38 mycoplasma can be stored for 14 months at either 4°C or 22°C without losing its immunising potential [13]. Currently, two types of CCPP vaccine formulation are received by the African Union Pan African Veterinary Vaccine Centre (AU-PANVAC) for quality control: a freeze-dried form which must be reconstituted before injection to the animal and the already inactivated CCPP vaccine liquid form containing saponin. The liquid form makes this formulation less stable than the freeze-dried form. For both forms, quantification of the Mccp antigen is used for vaccine batch potency evaluation. The optimum dose of antigen was established to be 0.15 mg of Mccp protein and 3 mg of saponin. The current assay used for Mccp antigen quantification in the CCPP vaccine is the bicinchonic acid (BCA) protein assay. This assay is used for quantitation of the total protein in a sample protein test and is not specific to the Mccp antigen. Contaminants from the medium that are present in the final product may affect the amount of Mccp antigen evaluated in the vaccine. This constitutes as one of the challenges for the evaluation of the potency of the CCPP vaccine [15, 16]. To specifically detect and quantify Mccp protein antigens in the vaccine, an alternative assay, such as the enzyme-linked immunosorbent assay (ELISA) is needed. Similar approaches have been developed for vaccines against human rabies and hepatitis B [17–20]. In this study, the development of an ICE assay using a mAb as a capture and detecting antibody for the detection and quantification of the Mccp protein in the CCPP vaccine is reported.

2. Materials and Methods

2.1. Preparation of Mycoplasma capricolum Subspecies Capripneumoniae Antigen. The Mccp F-38 strain used as the CCPP vaccine seed [21] in the AU-PANVAC vaccine seed repository was used for the mycoplasma culture, which was done according to the protocol for CCPP vaccine production. Briefly, the vaccine seed was reconstituted in 5 ml of Pleuropneumonia-Like Organisms (PPLO) media (Difco, MD21152, USA) containing 20% horse serum (Sigma-Aldrich, St. Louis, M063103, USA) and filtered through 0.45 μm filters. The reconstituted seed was then inoculated in 95 ml of PPLO media, placed in a 200 ml bottle and incubated at 37°C with no agitation or air supply for an average of 10–15 days until the desired turbidity was observed. Three passages were realised to expand the culture to the desired scale of 10 litres. Each passage was done with inoculation at a ratio of 1 : 10. Each culture passage passed to the next stage if the desired pH of 6.65–6.95 was obtained, as previously described [22], and if the culture was free from any bacterial contamination. Harvesting of the culture was carried out according to a previously described protocol [23] with a slight modification. Briefly, the culture sample was aseptically distributed in 50 ml falcon tubes and centrifuged at 10,000 ×g for 30 minutes at 4°C. The supernatant was discarded, and the pellets were washed three times with sterile 0.01 M Phosphate-Buffered Saline (PBS) at pH 7.2. Aliquots (1 ml) of the supernatant from the washing step were collected, and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Invitrogen, Carlsbad, USA) was performed to evaluate the quality of the washing. After the last washing, the Mccp pellet was reconstituted in sterile PBS and three inactivation steps were realised using 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, M063103, USA), 0.1% beta-propiolactone (Sigma-Aldrich, St. Louis, M063103, USA), and 0.2% formaldehyde (Lab Chemie, Mumbai, India). The inactivation steps with Triton (Mccp-T), beta-propiolactone (Mccp-βPL), and formaldehyde (Mccp-F) were incubated at 4°C for 24 h and aliquots were cultured to detect possible viable *mycoplasmas*.

2.2. Molecular Characterisation of the Culture of Mycoplasma capricolum subsp. Capripneumoniae. Mccp DNA extraction was performed from the culture following the protocol of the DNeasy kit (Qiagen, Hiden, Germany). Two different PCR reactions were performed. The first PCR reactions were done with DNA using the *Mycoplasma* genus-specific primers: upstream primer GPO-3, 5′-gggagcaaacaggattagataccct-3′ and downstream primer MGSO, 5′-tgcaccatctgtcactctgttaacctc-3′. These were used to amplify a 280 bp fragment [24]. In the second PCR reaction, the Mccp specific primers Mccp MCCPF 5′-atcatttttaatcccttcaag-3′ and MCCPR 5′-tactatgagtaattataatatatgcaa-3′ were used for amplification of a 316 bp fragment sequence [25, 26]. Amplicons were visualised on 1.5% (w/v) agarose gels.

2.3. Generation and Characterisation of mAbs. Eight-week-old BALB/c mice (Origin-Charles River, France) with an average weight of 20 g were immunised by intraperitoneal

injection with 200 μl of Mccp protein at a concentration of 0.3 mg/ml emulsion mixed 1 : 1 with Freund's complete adjuvant. Three booster injections were given at three weeks intervals: the first two boosts with incomplete Freund's adjuvant and the last boost without any adjuvant. Four days after the last boost, the spleen cells were collected and fused with Ag8 myeloma cells (received from FAO/IAEA Laboratories, Austria). The selection and cloning of hybridoma cells were performed in one step, following the protocol of the ClonaCell™-HY Kit (StemCell Technologies, Canada). The hybridoma cells colonies were collected after 14 days and cultured in the appropriate medium. Indirect ELISA (iELISA) tests were performed to screen for positive clones producing mAbs using the Mccp protein antigen. Briefly, ELISA plates (NUNC-MaxiSorp, Denmark) were coated with the antigen solution (100 μl/well) and incubated at 4°C overnight. The plates were then washed thrice with washing buffer (WB) of 0.002 M PBS containing 0.05% Tween 20 (PBS-T) to remove unbound antigens. The remaining free binding sites in the plate wells were blocked using 200 μl of PBS-T containing 5% skimmed milk (PBS-T-Milk) and incubated for 30 minutes at 37°C. The blocking solution was removed, and 100 μl of medium from hybridoma cell cultures was added to each well. Plates were incubated at 37°C for 30 minutes and washed again, as above. Then, 100 μl of antimouse immunoglobulin peroxidase-labelled conjugate (DAKO Company, Denmark) diluted 1 : 1000 in PBS-T-Milk was distributed into each well. The plates were further incubated at 37°C for 30 minutes. Plates were washed again with PBS-T, and 50 μl of the chromogenic substrate *3,3′,5,5′*-tetramethylbenzidine (TMB) (Thermo Scientific, Rockford, IL61101, USA) was added to each well and incubated for 10 minutes at 37°C. The colour development was stopped with 50 μl of sulphuric acid (1 M) and the optical density (OD) of each well was read with a spectrophotometer reader with the filter at a wavelength of 450 nm.

The characterisation of mouse mAb isotyping (immunoglobulin class, subclass and light chain) was performed using the Rapid ELISA Mouse mAb Isotyping Kit (Thermo Scientific, Rockford, IL61101, USA).

2.4. Precipitation and Horseradish Peroxidase (HRP) Conjugation of Mccp mAbs. Positive hybridoma cells producing Mccp mAb were propagated in serum free medium EX-CELL® 610-HSF for Hybridoma Cells (Sigma-Aldrich, St. Louis, M063103, USA), and used to precipitate immunoglobulins (Igs) in 50% Saturated Ammonium Sulfate (SAS) (Thermo Scientific, Rockford, IL61101, USA) at +4°C for overnight incubation. Immunoglobulins were pelleted at a centrifugation speed of 10,000 rpm for 30 minutes and dissolved in PBS buffer for dialysis using Slide-A-Lyzer™ Dialysis Cassettes (Thermo Scientific, Rockford, IL61101, USA) with the membrane at an exclusion limit of 20 kDa. The Ig of each mAb was quantified by BCA (Thermo Scientific, Rockford, IL61101, USA) protein quantification methods and then conjugated with Horseradish Peroxidase (HRP) following the EZ-Link Plus Activated Peroxidase kit protocol (Thermo Scientific, Rockford, IL61101, USA).

2.5. Development of ICE

2.5.1. Selection of Capture and Detecting Antibody. The mAbs were first selected based on the reactivity with the Mccp antigen determined by the optical density (OD). Briefly, ELISA plates (NUNC-MaxiSorp, Denmark) were coated with Mccp antigen (1 μg/well) for overnight incubation at 4°C. The plates were washed and blocked for 30 minutes at 37°C. After that, 100 μl of each HRP-conjugated mAb at 1 : 1000 dilution was added to the wells for 45 minutes incubation at 37°C. The plates were washed, and 50 μl of TMB was distributed to all wells. The colour development was stopped after 15 minutes incubation in a dark room with 50 μl of sulphuric acid (1 M) and the ODs were read as indicated above. The mAbs presenting high OD were selected for further evaluation as capture and detecting antibodies.

Each unlabelled Ig of the selected mAbs was used to coat ELISA plates (Nunc-Maxisorp, Denmark) for overnight incubation at 4°C. The plates were washed the next day with PBS-T and divided in two. One hundred microlitres of Mccp protein solution at 1 μg/ml prepared in dilution buffer (DB: PBS-T containing 2% milk) was added into the wells of the first half of the plate, while 100 μl of DB (representing the negative antigen) was added to the wells of the second half. Plates were incubated at 37°C for one hour to allow the corresponding Mccp protein to be trapped by each mAb. Plates were washed, and 100 μl of all HRP-conjugated mAbs at 1 : 500 dilutions was prepared in DB and added into the wells of each plate for 45 minutes of incubation at 37°C. Plates were washed, and 50 μl of the TMB substrate was added to all wells for 15 minutes and incubated at 37°C. Colour development was stopped by adding 50 μl of 1 M sulphuric acid (Sigma-Aldrich, St. Louis, M063103, USA), into the wells, and ODs were read as indicated above. The binding ratio of each HRP-conjugated mAb was determined.

2.5.2. Internal Reference Standard and Vaccine Samples. The internal reference standard (IRS) of the Mccp antigen and vaccine samples were prepared or selected to evaluate the ICE. The IRS was prepared as described above using 0.1% Triton X as the inactivating agent. The purity evaluation and quantification of the IRS was also performed as described. The IRS free contaminant was subsequently used as the reference standard in the ICE for specific quantification of the Mccp protein.

Three in-house experimental CCPP vaccines were prepared using Saponin as inactivating agent and adjuvant. Thirteen commercial CCPP vaccine batches from four manufacturers (A, B, C, and D) were tested for the development of the new ICE.

2.5.3. Design of the ICE. One mAb (Mccp25) among the list of the generated Mccp mAbs was selected as the capture and detecting antibody for the detection and quantification of the Mccp antigen. Titration of Mccp25/unlabelled and Mccp25/HRP-labelled were performed to determine the optimal dilutions of each antibody for the ICE. Serial dilutions of each antibody mAb were prepared and used to bind Mccp proteins (1 μg/well) previously coated in microplates. The antimouse immunoglobulin peroxidase-labelled conjugate

(DAKO Company, Denmark) was used to reveal the binding of Mccp25/unlabelled. The binding of Mccp25/HRP-labelled was directly revealed by the addition of TMB.

The Mccp antigen was quantified using the ICE designed with unlabelled and HRP-labelled mAb Mccp25. For each antibody, the optimal dilution was determined. ELISA plate (Maxisorp, Nunc) wells were coated with 100 μl of Mccp25/unlabelled at a saturate dilution of 1 : 100. The plates were incubated at 4°C overnight. The next day, a washing step to remove the unbound antibody was conducted. Serial two-fold dilutions of IRS were performed in PBS-T containing 2% milk, and 100 μl of each dilution was distributed into the plates for one hour of incubation at 37°C. A dilution buffer of PBS-T containing 2% milk (Sigma-Aldrich, St. Louis, M063103, USA), was included in the plate as a negative control. After a washing step to remove unbound Mccp antigen, 100 μl of Mccp25/HRP-labelled dilution at 1 : 100 was added to all wells for one hour of incubation at 37°C. Following a last washing step, 50 μl of TMB substrate was added to each well and incubated for 10 minutes at 37°C. Colour development was stopped with 50 μl of sulphuric acid (1 M), and the optical density (OD) of each well was read with a spectrophotometer reader with the filter at a wavelength of 450 nm.

2.6. BCA, Mycoplasma Specific PCR, and ICE Methods for Quality Assessment of CCPP Vaccines. A total number of eighteen samples (thirteen commercial CCPP vaccine batches, three in house experimental CCC vaccine batches produced at AU-PANVAC, one PBS and the IRS) were all quality assessed using BCA and the newly developed ICE for protein quantification. Mycoplasma specific PCR was used to detect the DNA.

2.7. Quantification of Mccp Protein with ICE and In Vivo Seroconversion. Fifteen local goats were used to correlate the estimated quantity of Mccp protein using ICE with the level of antibody production in these animals. These goats were then placed in five groups with three goats in each. Commercial vaccines were tested in ICE to quantify the amount of Mccp protein; vaccine codes P019 and P137 and vaccine codes P135 and P206, which showed good and no amount of specific Mccp proteins, respectively, were used to vaccinate four group of goats. One unvaccinated group was used as a negative control. Blood samples were collected for serum preparation on day 0 (prior to the vaccination). Then, at days 5, 7, 14, 21, 28, 31, and 38 postvaccination blood samples were collected for testing with the commercial CCPP competition-ELISA, as previously described [16, 27].

3. Results

3.1. Culture and Molecular Characterisation of Mycoplasma capricolum Subspecies Capripneumoniae. The growth of Mccp was observed by the presence of filaments and the turbidity of the media. The monitoring of the pH of the Mccp culture media showed a slight acidity compared to the pH of the normal media (Figure 1). The noninoculated PPLO broth

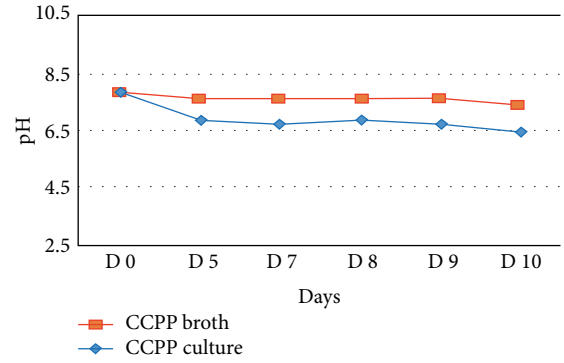

Figure 1: pH in media culture during the growth period of Mccp.

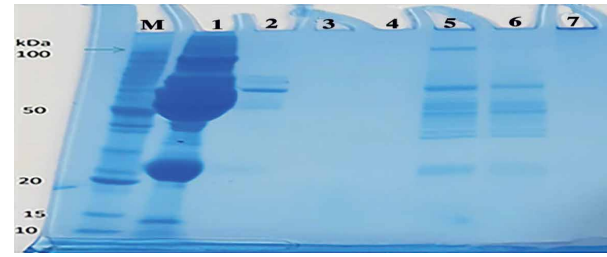

Figure 2: Evaluation of the effectiveness of washing of the culture twice or thrice with PBS. Lane M-Ladder, lane 1 represents 1st spin, 2–4 represent three consecutive supernatants that were collected after washes, lanes 5–7 represent the pellet that was treated with triton-X, βPL, and F, respectively.

remained alkaline, indicating no growth of the organism and contamination. The molecular characterisation of the Mccp culture using the PCR set of primers GPO3/MGSO and MCCPF/MCCPR showed fragments at 280 and 316 bp, respectively and represented the universal *Mycoplasmas* genus and *Mccp* genus-specific primers, respectively (data not presented).

3.2. Preparation of Mycoplasma capricolum Subspecies Capripneumoniae Antigen and the Internal Reference Standard. The washing of *Mycoplasma capricolum subspecies capripneumoniae* culture and the inactivation processes were evaluated. Buffers were collected following triple washing, and the inactivated pellets (Mccp-T, Mccp-βPL, and Mccp-F) were monitored to evaluate the presence of proteins using SDS-PAGE (Figure 2). The electrophoresis gel showed that the washing process of the pellet removed most of the soluble proteins derived from the culture media, as no protein was detected in washing buffers 2 and 3. The gel electrophoresis data also showed that the triton-X and beta-propiolactone inactivation processes maintained good protein levels, while with formaldehyde, most proteins appeared degraded.

3.3. Screening of the mAbs and Selection Capture Antibody for the ICE. Eight hybridoma cell colonies showing stable production of mAb reacting with Mccp antigen by indirect ELISA (iELISA) were identified (Figure 3). Three groups

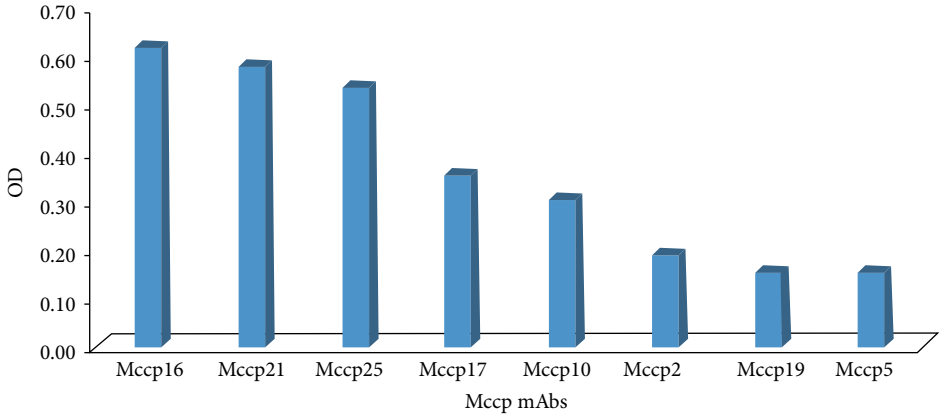

Figure 3: Reaction of Mccp mAbs on Mccp antigen coated in microplate.

Table 1: Classification of generated Mccp mAbs by mouse isotyping method.

mAbs	Subclass	Light-chain
Mccp2	IgG2a	Kappa
Mccp5	IgG2a	Kappa
Mccp10	IgG1	Kappa
Mccp16	IgG1	Kappa
Mccp17	IgA	Kappa
Mccp19	IgG1	Kappa
Mccp21	IgG1	Kappa
Mccp25	IgG2a	Kappa

Class, subclass, and light-chain of Mccp mAbs generated.

Table 2: Combinations of unlabelled mAbs and HRP-labelled mAbs for ELISA on Mccp proteins.

mAbs	Mccp protein/PBS	HRP-labelled Mccp-25 (O.D value)	HRP-labelled Mccp-21 (O.D value)	HRP-labelled Mccp-16 (O.D value)
Unlabelled Mccp-16	Mccp protein	0.10	0.16	0.30
	PBS control	0.04	0.12	0.27
	Binding ratio	**2**	**1**	**1**
Unlabelled Mccp-21	Mccp protein	0.11	0.21	0.43
	PBS control	0.06	0.21	0.37
	Binding ratio	**2**	**1**	**1**
Unlabelled Mccp-25	Mccp protein	0.53	0.22	0.38
	PBS control	0.05	0.16	0.32
	Binding ratio	**11**	**1**	**1**

Cross-reacting these mAbs to HRP-labelled mAbs on Mccp protein showed that Mccp-25 mAb captured Mccp protein.

of mAbs were identified depending on the level of optical density, indicating the expression level of the protein or epitope:

(i) Group 1: Mccp16, Mccp21, and Mccp25 detect a high expression level of the protein or epitope.

(ii) Group 2: Mccp10 and Mccp17 detect a medium expression level of the protein or epitope.

(iii) Group 3: Mccp2, Mccp19, and Mccp5 detect a low expression level of the protein or epitope.

Isotyping of the generated mAbs is presented in Table 1.

3.4. Selection of Capture and Detecting Antibodies for the Design of ICE. mAbs Mccp16, Mccp21, and Mccp25 presenting high OD for detection of Mccp protein in iELISA were selected for propagation in serum free medium (SFM). Each mAb was precipitated and evaluated as a capture or detecting mAb for the design of the ICE. Data from the cross-reaction of homologous mAbs on the captured protein by each mAbs indicate that the combination of unlabelled Mccp25 as the

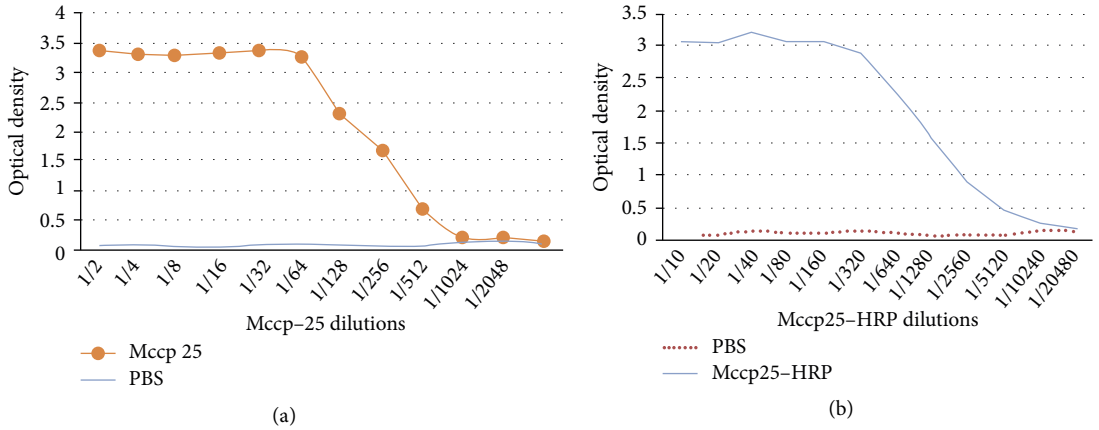

Figure 4: Titration of mAb (a) represents the titration of Mccp-25 on Mccp antigen followed by addition of antimouse conjugate (Dako), (b) represents the titration of detection antibody (Mccp25-HRP conjugate) directly to the optimal concentration of Mccp protein.

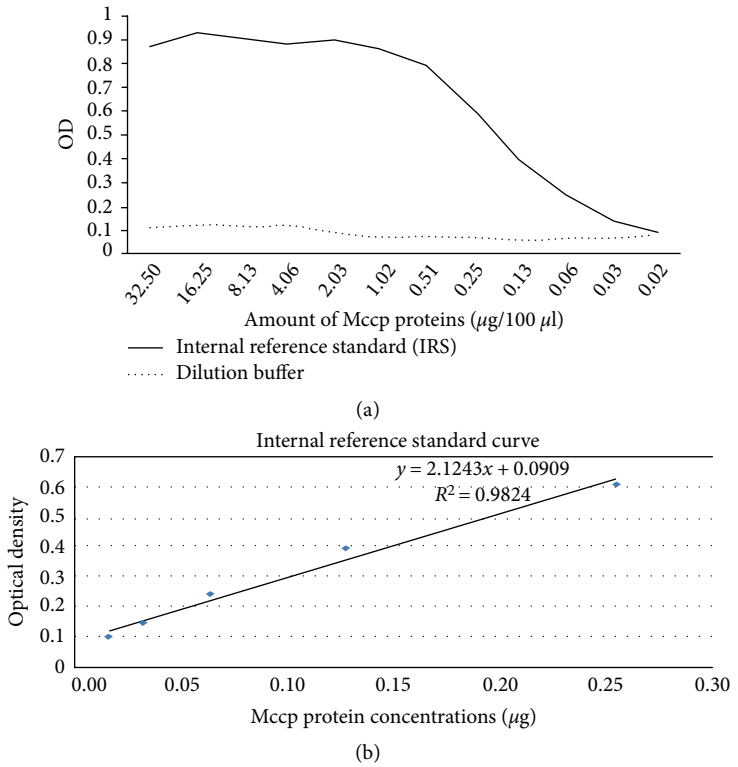

Figure 5: Determination of internal reference curve (a) represents the titration of IRS, (b) represents the standard curve derived from internal reference sample from which estimates of other tested vaccines were calculated.

capture antibody and HRP-labelled Mccp25 as the detecting antibody presents the high binding ratio for the detection of Mccp protein (Table 2).

The mAb Mccp25 unlabelled and HRP-conjugated were used as capture and detection antibodies, respectively, to design the ICE. The dilution for capture antibody was found to be 1 : 50 dilution, while detection antibody could be used from 1 : 500 to 1 : 1000 dilutions (Figure 4).

3.5. Design of the Mccp ICE

3.5.1. Internal Reference Standard and Experimental Vaccine Batches. The Mccp pellet washed and treated with Triton X-100 was chosen to prepare the IRS to be used as a reference in the ICE for specific quantification of the Mccp antigen.

Three experimental vaccine batches were prepared. Two batches, EXPV1 and EXPV2, were formulated using saponin (3 mg/ml) as the adjuvant and inactivation agent for the Mccp pellet. Another batch, EXPV3, was formulated with formaldehyde 0.2% to inactivate the Mccp pellet and then adjuvanted with saponin at 3 mg/ml. The three batches were quantified by BCA with 463, 327, and 290 μg/ml protein, respectively. The minimum required dose of CCPP vaccine was fixed at 0.15 mg/ml in the OIE Manual of Standards for Diagnostic Tests and Vaccines.

Table 3: Detection of CCPP using BCA method quantification, PCR, and ICE tests.

S/N	Vaccine code	Quantification by BCA (μg/ml)	Specific Mycoplasma PCR	ICE (μg/ml)
1	P063	490.00	+	351.00
2	P206	374.00	−	0.00
3	P268	170.00	−	0.00
4	P269	450.00	−	0.00
5	P291	1500.00	+	616.30
6	P067	395.00	−	0.00
7	P135	180.00	−	0.00
8	P136	990.00	+	210.92
9	P137	990.00	+	234.95
10	P138	990.00	+	238.00
11	P019	1500.00	+	225.88
12	P224	3000.00	+	0.00
13	P167	380.00	+	35.00
14	EXPV1	463.30	+	452.10
15	EXPV2	327.00	+	339.81
16	EXPV3	290.00	+	0.00
17	IRS	400.00	+	373.97
18	PBS	0.00	−	0.00

3.5.2. Estimation of Mccp Protein with the ICE. An IRS concentration ranging from 5.08 to 0.64 μg/ml was tested in the ICE and used to generate the internal standard curve with a R^2 value of 0.9838 (Figure 5). This showed a good correlation between the OD obtained and the protein amount. The ICE limit for detection of the Mccp protein was determined to be 30 ng/ml. The regression formula derived from the standard curve was used to estimate the protein concentration in the samples.

3.6. Comparison of the BCA, Mycoplasma Specific PCR, and ICE Methods for Quality Assessment of Vaccines. Among the 18 samples tested using the three test methods, 17 samples contained detectable levels of protein, as measured by the BCA method, whereas only 12 and 10 samples contained vaccine-specific proteins, as indicated by *Mycoplasma*-specific PCR and ICE respectively. Thus, the BCA method tended to overestimate the protein content compared to that measured by ICE. Most of the samples that were negative in the *Mycoplasma*-specific PCR were also found negative using the ICE. However, the vaccine that was inactivated by formaldehyde (EXPV3) was found positive using the *Mycoplasma*-specific PCR but negative with the ICE. The Mccp standard vaccine was used as a positive control, whereas PBS was used as a negative control (Table 3).

3.7. Quantification of the Mccp Protein in CCPP Vaccine by ICE and In Vivo Seroconversion. The amount of Mccp protein in the vaccines, as determined by ICE, was used to evaluate the seroconversion in vaccinated animals. In vivo seroconversion on vaccinated animals was compared using selected commercial vaccines; vaccine codes P019 and P137 had over 0.15 mg/ml of specific Mccp proteins, while vaccine codes P135 and P206 showed absence of Mccp proteins with ICE. The serum samples collected from each group of animals before and after vaccination (at various days) were tested in CCPP cELISA, and the data results are presented in Figure 6.

4. Discussion

The quality control of inactivated CCPP vaccine is based on total protein quantification using the BCA protein quantification method, which is not specific to the protein of interest. Thus, any nonspecific protein from the culture medium that is present in the final product can be measured and give inaccurate levels of Mccp proteins.

Quality assessment of this vaccine is crucial for batch release and to ensure its efficacy and batch consistency. To improve the quality control of CCPP vaccine, an ICE was developed to detect and specifically quantify the Mccp protein in CCPP vaccine. This ICE is based on use of mAb against Mccp antigen produced from the Mccp F-38 vaccine strain. Previous study [20] used the same approach and developed an ICE for quantification of hepatitis B surface antigen (HBsAg) in hepatitis B vaccine. ICE is a simplified procedure that measures the amount of antigen between two layers of antibodies (i.e., capture and detection antibody). Thus, the antigen to be measured must contain at least two antigenic sites capable of binding to an antibody, since at least two antibodies act in the sandwich.

For the development of the ICE, mbAs were generated for specific interaction on epitopes of the Mccp antigen. Eight mAbs were generated and evaluated to design the ICE. The number of mAbs obtained are slightly higher than those reported [28]. These mAbs were selected based on the optical density on the antigen obtained in iELISA. Three mAbs were then selected for further evaluation. The criteria for their selection was based on the expression level of the protein or epitope and high immunoreactivity on antigen as indicated in iELISA [29, 30].

The binding ratio represents the detection level of antigens trapped by the capture antibody. This study showed that the use of unlabelled (capture antibody) and HRP-labelled (detecting antibody) mAb Mccp25 presents a binding ratio higher (11-fold) than all other combination of mAbs. Therefore, the detected protein seems to have repetitive epitopes for the mAb Mccp25, as the first reaction of unlabelled mAb Mccp25 on the target protein did not affect the secondary reaction of the same HRP-labelled mAb Mccp25.

Unlabelled and HRP-labelled mAb Mccp25; Ig G1 type were then used as capture and detecting antibodies, respectively, to design the ICE as previously reported [20]. The minimum amount of target protein to be detected by the ICE test was set at 0.30 μg/ml. The internal standard curve generated with the IRS showed good correlation between the amount of protein and the OD obtained as the R^2 value was 0.9838. The repeatability of the Mccp ICE was determined by estimating the Coefficient of Variation (CV) of tests performed in-house tests (AU-PANVAC laboratory). Data showed a CV value of 6% indicating good repeatability of the assay as previously reported [31]. The R^2 correlation, measuring the

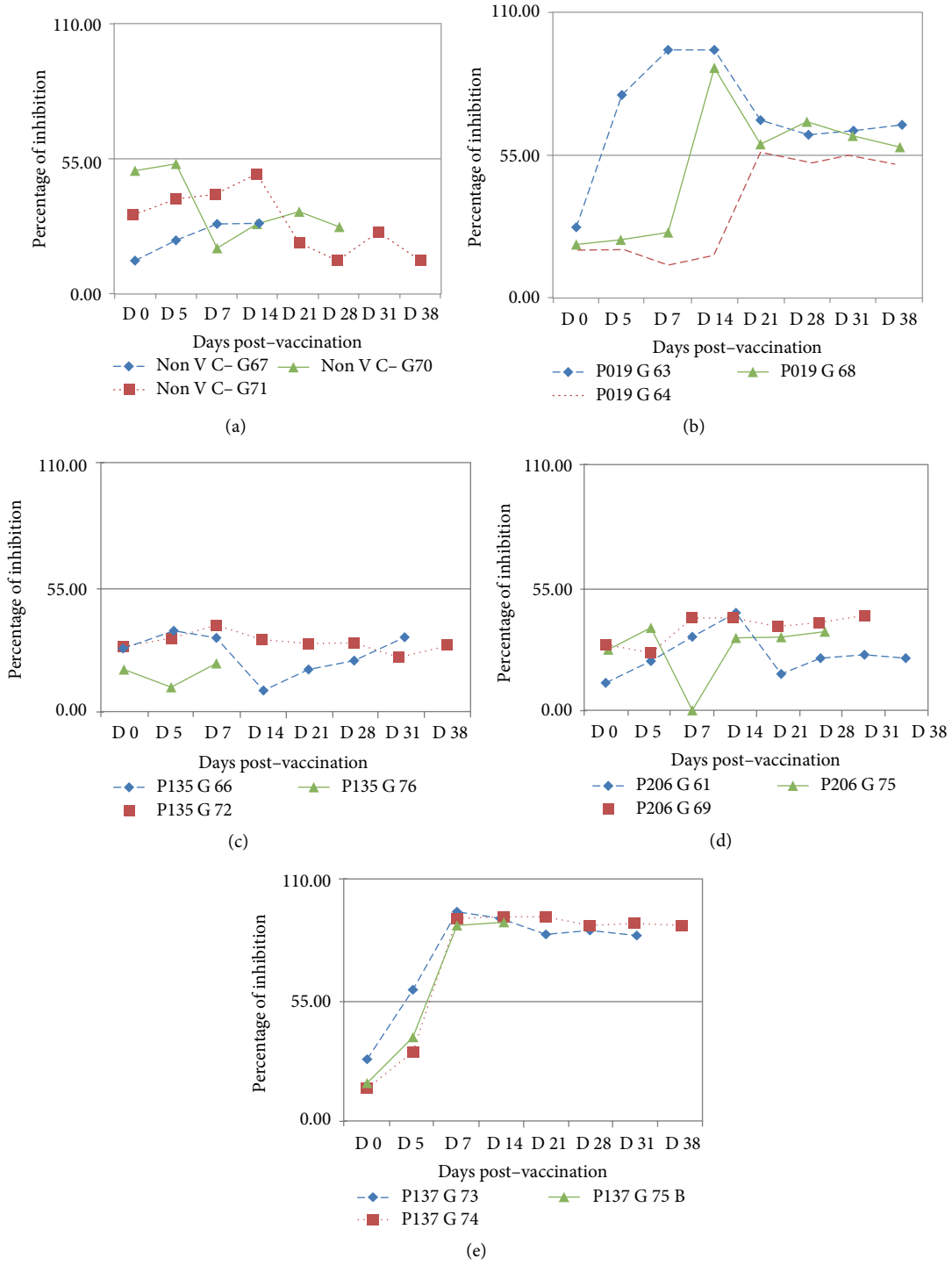

FIGURE 6: Kinetic of antibody development (expressed in percentage of inhibition) in vaccinated goats using P019, P137, P135, and P206 from Day 0 to D38 post-vaccination by CCPP c-ELISA. The cut-off of value for this test is 55% percentage of inhibition.

strength of the linear relationship between the protein amount tested and the OD generated in the ICE, was also determined. Seven repeated quantification tests were performed, and a R^2 value between 0.95 and 0.98 was obtained, indicating a good relationship between the amount of protein tested in the ICE and the OD values obtained. This is similar to the correlation (R^2) reported [32] in G-protein based ELISA as potency test for rabies vaccine.

The Mccp ICE test was then compared to the BCA method that is currently used for quality assessment for the CCPP vaccine. The 16 samples tested with the BCA method showed a protein concentration greater than 0.15 mg/ml. This suggests that all CCPP commercial and experimental vaccines contain the required amount of protein [33]. However, by testing these samples with the new ICE test, only six commercial and two experimental vaccines showed concentrations higher than the

amount of protein required (0.150 mg/ml) [34]. The two assay results showed a discrepancy for the quantification of proteins in CCPP vaccine which can be explained by the fact that the BCA method measures the total protein in the sample, while the ICE targets a specific antigen of Mccp.

It was also found that the ICE test was not able to detect and quantify proteins from inactivated Mccp with formaldehyde. This indicates that the target protein may have been degraded or that a change in conformation that could impact the epitope bound by the mAb may have occurred [35, 36].

A seroconversion study to assess the correlation between protein quantification results obtained with the ICE and antibody production was conducted. Antibody detection was carried out by a commercial c-ELISA (IDEEX) which uses a mAb that is different from the Mccp-25 mAb. The results of seroconversion of vaccinated goats correlates with the ICE results for selected CCPP vaccines containing equal or greater than 0.15 mg/ml of protein amount. Therefore, the result of this study indicates that the newly developed ICE is specific to Mccp protein and could be used as alternative test for quantification of Mccp protein in CCPP vaccine. However, further studies using this ICE should be conducted to determine the minimum amount of Mccp antigen that induces antibody production and correlates with degree of protection through vaccination and challenges.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Ethical Approval

All applicable international, national, and institutional guidelines for the care and use of animals were strictly adhered to. AU-PANVAC is a specialized technical agency of the African Union Commission with a host country agreement with the Government of the Federal Democratic Republic of Ethiopia. Consequently, all laboratory activities were conducted in accordance with the laws and regulations of Ethiopia. Animal manipulations were conducted under the AU-PANVAC Quality Management System.

Conflicts of Interest

The authors declare that they have no conflicts of interest regarding the publication of this paper.

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