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SUMMARY

Contagious bovine pleuro-pneumonia is a highly contagious and infectious disease of cattle and water buffaloes caused by *Mycoplasma mycoides subsp. mycoides* small colony that affect the respiratory tract of animals. Vaccination with CBPP T1-44 live attenuated strain is the most effective method of controlling disease, though the African manufacturers are unable to produce the CBPP vaccine of the recommended titre per dose of 10^8. Including a 4-(2-Hydroxyethyl piperazine-1-Ethanesulfonic Acid) (HEPES) buffer in growth media helps maintain pH and obtain optimal antigen and vaccine titre. This study aimed to evaluate the potential effects of HEPES buffer in improving CBPP T1-44 strain vaccine titre. Vaccines were produced by culturing in the PPLO media buffered with HEPES at different concentrations of 0.05M, 0.075M, and 0.1M, with Na_2HPO_4 as a control unit. Harvest pH was fixed at 6.5±0.3. The titre of the vaccine was obtained by titration whereby a total viable number of *Mycoplasma* was determined by colour change unit 50, and the titre was calculated according to Spearman and Karber's formula. Mean titre per dose were $\log_{10} 1x10^{9.3}$, $\log_{10} 1x10^{9.23}$, $\log_{10} 1x10^{9.133}$ and $\log_{10} 1x10^{6.982}$ for HEPES buffered vaccine at 0.05M, 0.075M, 0.1M and Na_2HPO_4 respectively. The post hoc test revealed the statistically significant difference between all concentrations of HEPES tested and Na_2HPO_4. Adding relatively inexpensive HEPES to most current media formulations is essential for producing a quality, efficacious, stable vaccine at a minimal cost with optimal recommended titre.

Keywords*: CBPP T1-44, CBPP disease, HEPES buffer, titre, pH*

INTRODUCTION

Contagious Bovine Pleuro-pneumonia (CBPP) is an infectious and highly contagious disease of cattle and water buffaloes. It is considered to be amongst the most important infectious diseases of these animals. CBPP is caused by *Mycoplasma mycoides subsp. mycoides* small colony (*Mmm*SC) that affect the respiratory tract of animals (Swai *et al*., 2013). Contagious bovine Pleuropneumonia (CBPP), an OIE-listed disease, is the second most crucial transboundary animal disease (cattle) after rinderpest (Tambi *et al*., 2006). The CBPP impacts both the national and local economies. The direct effects include losses from mortality, decreased milk yields, and the expenses of vaccinations, antibiotics, veterinary care, and disease surveillance (Phiri, 2006). The chronic nature of illness primarily brings on indirect costs. It includes decreasing fertility, loss of weight and working capacity of the animal, losses from quarantine, and decreased trade in cattle (Phiri, 2006).

Numerous initiatives have been undertaken to successfully control CBPP in Africa, such as the Joint Project 28 of the 1970s and the Pan African Control for Epizootics. In the context of Joint Project 28, CBPP was controlled through a mandatory mass vaccination campaign in Africa's endemic foci, which was followed by quarantine, testing, and the compensation-based slaughter of infected cattle, as well as the

reporting of disease outbreaks (Alhaji *et al*., 2020). Immunity against CBPP was shown for a maximum of one year following vaccination with the T1-44 attenuated live vaccine (Scacchia *et al*., 2007). Recently, the quality of vaccines used to control CBPP has declined due to militating factors such as inadequate handling of vaccines by inoculators during vaccination campaigns, such as poor cold chain maintenance, and usage of vaccines with sub-optimal quantities of *Mmm* strains. This is in addition to other challenges associated with mass vaccination, such as pastoralists refusing vaccinations due to post-vaccination reactions (Alhaji *et al*., 2020).

Most vaccine producers in Africa face the challenge of not meeting the required titre per dose, as the results obtained by AU-PANVAC demonstrate that no vaccine producers in Africa seem consistently able to produce the product per the recommendation of 10^8 mycoplasmas per dose (Rweyemamu *et al*., 1995). The minimum vaccine dose for cattle is 10^7 viable mycoplasmas according to WOAH/OIE requirements that take into consideration time course from production and local transport conditions that may significantly contribute to a reduction of vaccine titre during transit; that is why it is therefore recommended that production laboratories supply vaccines having titre value of at least 10^8 mycoplasmas per dose (WOAH, 2018).

Change in pH from neutral to acidic level has been the major factor that affects the viability of *Mmm* small colony (T1-44, T1-SR) (Waite & March, 2001) since it has been demonstrated that *Mmm* SC is pH sensitive (March, 2004). As *Mmm*SC grow, they physiologically produce acidic metabolites, which lower the pH of the medium. Ultimately, it affects the biological activity of Mycoplasma, which includes viability and titre value of vaccine falls down and therefore, the ideal pH range for *Mmm*SC growth is 7.4 to 8, with a pH drop to less than 6.5 resulting in the cessation of growth and rapid death of cells (Waite & March, 2001; Windsor, 1978).

Growth media that is buffered with 4-(2-hydroxyethyl piperazine-1-ethanesulfonic acid) (HEPES) buffer helps in maintaining the pH condition that will highly contribute to the optimal titre value of the antigen and vaccine (FAO-OIE, 2004). Metabolism of reducing sugar(Glucose) contributes to a dropping in the pH; thus, it is important to incorporate a strong buffer system such as HEPES and not dibasic (Na_2HPO_4) that has a poor ability to hold fluctuation of the hydrogen concentration in most of the commercial growth media used for growing of Mycoplasma (Waite & March, 2001).

This study aimed to investigate the significant effect of HEPES compared to Na_2HPO_4 in improving optimal bacteria titre during the production, transport and storage phase of the freeze-dried T1-44 vaccine strain of CBPP.

MATERIALS AND METHODS

CBPP T1-44 working seeds were obtained from the working seed bank at the Department of Quality Control-Hester Biosciences Africa Limited (HBAL) in Kibaha, Coastal region, Tanzania. Three vials were collected from each set-up (lot) of the study for each test performed during the study, i.e., titration, sterility, identity, and purity (British Pharmacopoeia Commission, 2021). Three vials from each set or category of the experimental vaccine (CBPP T1-44 vaccine) grown in HEPES buffered media of 0.1M, 0.075M, 0.05M and Na_2HPO_4 were sampled randomly and subjected to the titration test (British Pharmacopoeia (Veterinary), 2001). The treatment group was CBPP T1-44 vaccine buffered with HEPES at concentrations of 0.1M,0.075M, and 0.05M. In contrast, the control group was CBPP T1-44 vaccine buffered with Na_2HPO_4. The experiment was conducted by measuring the effectiveness of HEPES buffer versus Na_2HPO_4 on the vaccine culture of the CBPP T1-44 strain. Harvest pH was fixed at the range of 6.5±0.3. Growth features considered during the harvesting period include colour change from pink to yellowish in the vaccine culture. Following harvesting, a titration test was done to measure CBPP antigen and vaccine titre. Replicates for antigen and vaccine titration were conducted at weekly intervals. Colour Change Unit 50 (CCU_{50})

was used to calculate the titre value following titration.

Production of CBPP T1-44 and titration test

Growth media buffered with HEPES at various concentrations of 0.05M, 0.075M and 0.1M were prepared by weighing the essential ingredient of Pleuropneumonia like organism base (60g of PPLO), Yeast extract(11.24g), 0.2% DNA (52.63ml), Dextrose (Glucose) 8.99g, Glycerol 10ml, Phenol red 0.05g, Penicillin and Horse serum 20%. PPLO broth was made by dissolving all of the components into the 2000 litres of water for injection, followed by the partition of the prepared Media into four different set-ups, whereby four sterile borosilicate bottles were used to hold media with different concentrations of HEPES buffer and one that contains Na_2HPO_4. HEPES special for cell culture with a molecular weight of 238.3g/mol were used to make HEPES treated media for three different concentrations by weighing 5.9575g (0.05M), 8.9363g (0.075M), 11.915g (0.1M) respectively. Then 1.25g (0.002M). of the Na_2HPO_4 buffer was used in the PPLO media as the control unit.

Thorough mixing of the essential ingredient of each prepared medium and adding respective buffers were done with a magnetic stirrer**.** Followed by adjusting the pH of the prepared media by adding a few drops of 10M NaOH to make a final pH of 7.8 for both set-ups, whereby high volume of NaOH (9-11ml) was used for the 0.1M of HEPES and less volume of <3ml of the same NaOH 10M was used to adjust the of pH PPLO buffered with HEPES at 0.075M and 0.05M, and less than 2ml of NaOH used in the PPLO buffered with Na_2HPO_4.

Sterilization and Sterility test of the prepared Growth media

Sterilization was done by filtration through a membrane filter (syringe filter with 0.2µm pore size). The Media was sterilized using a syringe filter of 0. 2µm). Sterilized media were stored at a temperature of 2°C-8°C.

A sterility test of the media was done by inoculating each 2ml of filtered Media into soya bean casein digest broth (40ml of SCDM) for fungal detection and into Fluid Thioglycolate media (FTM) for bacteria detection (Litamoi & FAO, 1996). The sample tested for bacteria screening was incubated at 33+/-2 °C and 23+/-2°C for fungal detection. In both tests, two vaccine vials from the respective category were used. As in process sterility testing, one bottle for each FTM and SCDM was used for the respective media sample tested. After seven days following incubation, the samples from each tube were sub-inoculated and incubated for a further seven days.

Revival and inoculation of the CBPP T1-44 working seed

The working seed of CBPP T1-44 vial number 40, manufactured on 25.04.2022, was reconstituted using 1 ml of sterile Phosphorus buffer saline (PBS). From the reconstituted antigen, 100 µl was revived by inoculating into 900 µl of respectively media buffered with HEPES at 0.05M, 0.075M, 0.1M and Na_2HPO_4. 900 µl supplemented with 20% heat-inactivated horse serum, accounting for 180 µl of horse serum and 720µl of fresh media. Following inoculation into the 2ml microcentrifugation tube, a total of 1ml culture for each of the experimental units (All concentrations of HEPES and control arm of Dibasic sodium hydrogen ortho-phosphate) were incubated into walk in incubator (hot room) with the conditions of 37°C. After 48 hours of incubation, all cultures changed colour from pinkish to yellowish. Following a colour change in the first passage, it was then subcultured for the second passage.

Subculturing (Passage) of the CBPP T1-44

After successful revival and the first passage, the second passage was carried out by inoculating each of the first cultures into 9ml of the fresh buffered media with 20% Horse serum. A 50 ml sterile falcon tube was used for this transfer in the bio-safety cabinet class II. After transfer, cultures from the second passage were incubated at 37°C. Third passages were done by inoculating the second passage vaccine culture (10ml) into 90ml of the fresh media to make a total of 100ml for each of the experiment's set-ups (HEPES-0.05M, 0.075M, 0.1M and

Na2HPO4 (control)). Sterile Borosilicate glass bottles were used, and following sub-culturing, they were incubated at 37°C.

Harvest of the CBPP T1-44 culture

Following the inoculation of the third passage, pH monitoring was done on each culture, and colour change was used as a critical point for harvesting. A fixed pH range of 6.5 +/-0.3 was used, and harvest pH of 6.58, 6.67, 6.8 and 6.27 for the HEPES culture in 0.05M, 0.075M, 0.1M and Na_2HPO_4 was recorded. Following harvesting, the sample was aliquoted (10ml) from each experiment category for titration, and the remaining sample was stored at -20°C.

In the process of antigen production, harvested antigen was subjected directly to in-processing sterility testing by inoculating 0.5ml of the CBPP T1-44 culture into 40ml of Soya bean casein digest (SCDM) broth for fungal, aerobic bacteria detection as well 0.5ml from the same sample inoculated into Fluid Thioglycolate media (FTM) for bacteria detection. It was incubated at 23+/-2 °C for SCDM and at 33+/- for FTM, and the time taken for the incubation was seven days, followed by another seven days after a sub-inoculation for the fresh media of SCDM and FTM. The CBPP T1-44 strain used was identified using a PCR kit with the prime sequences of 5'-GTA-TTT-TCC-TTT-CTA-ATT-TG-3'(Forward) Sequence: 5'-AAA-TCA-AAT-TAA-TAA-GTT-TG-3' (Reverse) according to (WOAH, 2021).

The titre of CBPP T1-44 antigen culture was obtained by measuring the Median dose effect depending on the colour change unit 50(CCU_{50}), and the counts were subjected to the Spearman and Karber formula for antigen titre calculation (Litamoi & FAO, 1996). The harvested culture was stored at -20 °C for three to five days prior to further production steps. Before freezing-drying, the CBPP T1-44 antigen was titrated, and the pre-freeze-dried antigen titre was determined.

Purification and blending of the Vaccine culture

Vaccine culture from each set was purified by centrifugation using 14500 rpm for 30 minutes. After being repeated three times, supernatants from each centrifugation tube were discarded, and pellets obtained were collected and suspended into the respective fresh medium with 10% horse serum. After thorough washing, 40ml of the fresh media (PPLO) was added to each culture.

10% Skimmed milk stabilizer with an initial pH of 6.7 was used for blending. The purified vaccine culture was blended by mixing 60% of the Antigen and 40% of the stabilizer (Litamoi *et al*., 2005). Lastly, the blended vaccine culture was filed into sterile and depyrogenated glass vials using an automated hand filler and half stoppering for lyophilization, the sealing of freeze-dried vaccine, and quality control tests.

Titre determination of the CBPP T1-44 vaccine

Three vials from each set of HEPES (0.05M, 0.075M, 0.1M) and Na_2HPO_4 buffer sampled randomly were subjected to a titration process so that the viable number of Mycoplasma was determined using CCU_{50}. One vial was reconstituted with the 10ml of Phosphate buffer saline, whereby three vials from each set were pooled together, making a total of 30ml, followed by serial dilution in tenfold dilution starting from 10^{-1} to 10^{-10}. Dilution was done using the deep well method, whereby the antigen sample was diluted serially. During dilution, 75µl of the sample (respected vaccine product) was diluted into 675µl of the sterile prepared to count base media, whereby 75µl transferred serial from the highest point to the lowest, discarding the last volume from 10^{-10} tubes. Following dilution completion, 100 microliters of the prepared media of the 20% heat-inactivated horse serum was dispensed into the microtitration plate in ten consecutively columns with two columns reserved for control (positive and negative), whereby one column had 200µl of prepared media without serum as the negative control and the last control had 200 µm of the complete counting media (prepared media with serum). That was followed by pipetting 100 µl of the diluted sample starting with the dilution factor of 10^{-3} to $10^{-10,}$ respectively. Finally, plates were incubated at 37°C for ten days and observed daily for colour change as well as calculating the Colour change unit (CCU_{50}) (Thiaucourt & Dí Maria, 1992) based on the formula provided by Spearman and Karber that is useful in calculating titre value for the given

vaccine or antigen. The titer value established at both HEPES buffered vaccines, and Na_2HPO_4 was compared by looking at the significant difference between them in each tested concentration level. Titre determination and Quality tests of residual moisture determination, vaccine identity test, vacuum test, and vaccine sterility test were all conducted according to (the British Pharmacopoeia Commission, 2021; Litamoi & FAO, 1996).

Data collection and Data analysis

The specially designed laboratory Log sheets were used to capture primary data. Then, it was transferred into the personal notebook and Microsoft Excel in the computer for permanent storage prior to analysis and interpretation.

All statistical procedures were performed using Microsoft Excel 2019 and STATA (Statistical Software for Data Science, Version 14.2, College Station, Texas 77845, USA), and results were interpreted at the 5% significance level. One-way ANOVA, pairwise comparison of means, and eta square were used following the normality test of the variable.

RESULTS

CBPP T1-44 antigen titration during the harvesting process

A maximum of two days was used to harvest antigen in the Na_2HPO_4 media, while a maximum of three to four, three to five and four to five days was used to reach the harvest pH for the HEPES buffered vaccine in 0.05M, 0.075M and 0.1M respectively. The number of days used for antigen propagation in each passage was in order of increase from the lowest (0.05M) to the highest concentration of HEPES (0.1M).

Pre and Post freeze dried titre and accelerated stability titre of the CBPP T1-44 vaccine

Titration values of the CBPP T1-44 were captured before and after lyophilization of the antigen, and their findings are presented in Fig. 1A-C. From the produced CBPP T1-44 vaccine, an accelerated stability test was conducted after exposure to the vaccines at 40°C for two days (48 hours) (Fig. 1D).

Table 1. Harvest pH and titre value per ml of CBPP T1-44 Antigen culture buffered with HEPES and Na_2HPO_4

TEST	Experimental unit	Harvest pH	Titre value/ML
I	0.05M HEPES	6.78	$1\times 10^{9.4}$
	0.075M HEPES	6.8	$1\times 10^{8.6}$
	0.1M HEPES	6.83	$1\times 10^{8.6}$
	0.002M Na_2HPO_4	6.16	$1\times 10^{9.7}$
II	0.05M HEPES	6.58	$1\times 10^{9.9}$
	0.075M HEPES	6.69	$1\times 10^{9.4}$
	0.1M HEPES	6.87	$1\times 10^{9.2}$
	0.002M Na_2HPO_4	6.19	$1\times 10^{9.8}$
III	0.05M HEPES	6.59	$1\times 10^{9.4}$
	0.075M HEPES	6.74	$1\times 10^{9.6}$
	0.1M HEPES	6.89	$1\times 10^{9.4}$
	0.002M Na_2HPO_4	6.27	$1\times 10^{9.5}$
IV	0.05M HEPES	6.49	$1\times 10^{10.6}$
	0.075M HEPES	6.67	$1\times 10^{9.9}$
	0.1M HEPES	6.72	$1\times 10^{10.2}$
	0.002M Na_2HPO_4	6.18	$1\times 10^{9.3}$
V	0.05M HEPES	6.51	$1\times 10^{10.9}$
	0.075M HEPES	6.66	$1\times 10^{9.9}$
	0.1M HEPES	6.7	$1\times 10^{9.5}$
	0.002M Na_2HPO_4	6.23	$1\times 10^{9.7}$

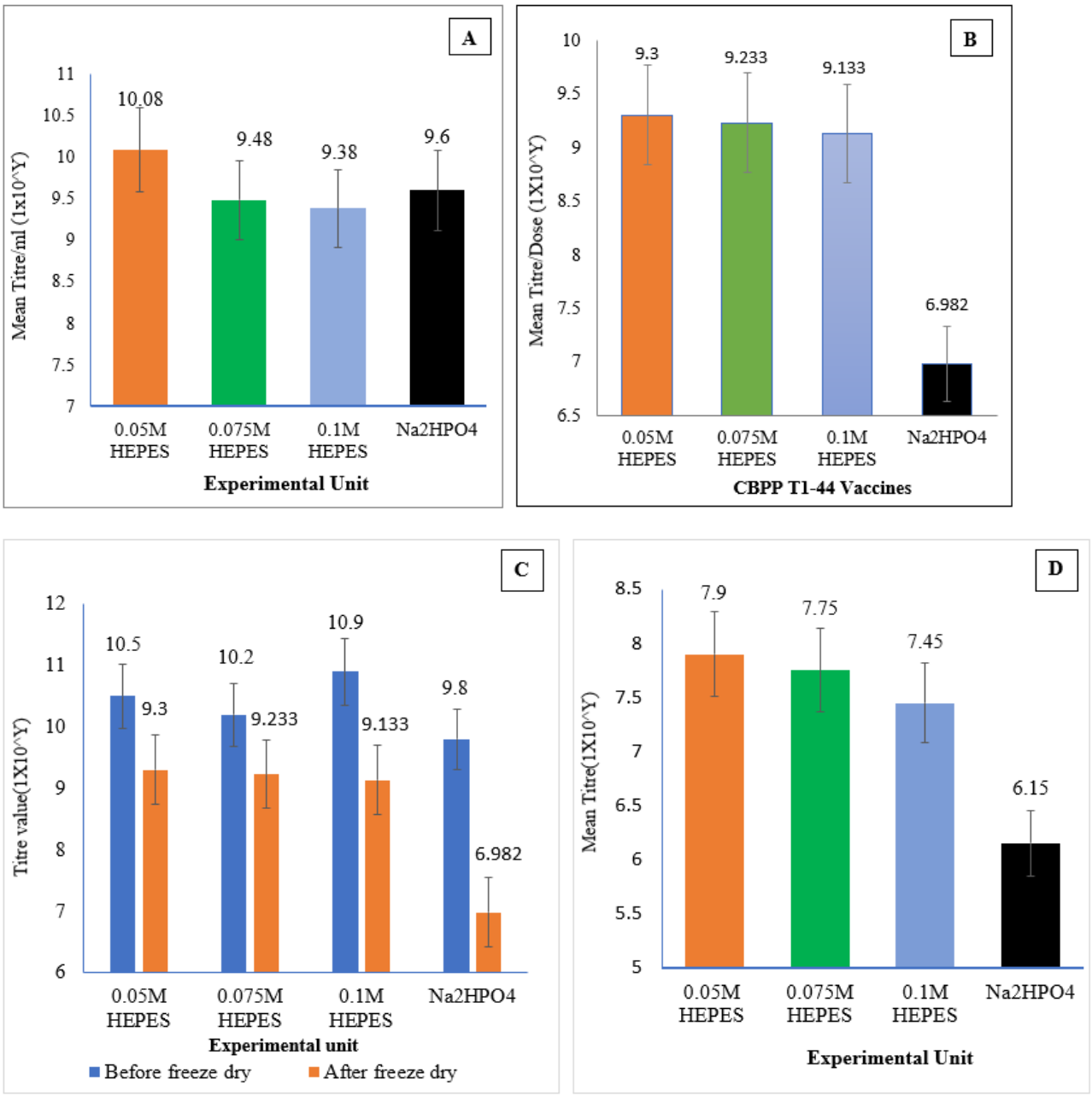

Fig. 1. Mean titre of the CBPP T1-44 antigen and vaccine (lyophilized vaccine). **A**: Mean titre value of the CBPP T1-44 vaccine in cultured pleuropneumonia like organism in broth buffered with HEPES and Na_2HPO_4 media. **B**: Overall titre of the CBPP T1-44 vaccine per dose in a vial of 100 doses after freeze drying process. **C**: Comparative titre of CBPP T1-44 vaccine, and **D**: Mean titre after accelerated stability test following exposure of the vaccine at 40°C for two days (48 hours).

DISCUSSION

The production of quality CBPP vaccines in most African laboratories or pharmaceutical companies has been challenging (Alhaji *et al*., 2020). AU-PANVAC's assessment showed that no producers or manufacturers in Africa could consistently supply the CBPP vaccine per the recommendation of 10^8 mycoplasmas per dose (Rweyemamu *et al*., 1995; WOAH, 2021). Despite of these challenges, vaccination against CBPP disease remains as the most viable control option. The vaccines which are mostly used are the freeze-dried T1-44 live attenuated strain, or streptomycin resistant variant (T1/sr) vaccine that was used in combination with rinderpest vaccine during a successful campaign of eradicating rinderpest disease (Amanfu, 2009). The persistence of CBPP disease provides some questions on the quality of the vaccine used from production to the point of usage (WOAH, 2018). The findings released by

the AU-PANVAC, gives facts of suboptimal antigen titre in the vaccine as a first source of vaccination failure against CBPP disease in Africa countries including Tanzania (WOAH, 2018).

This study found that HEPES has a high effect in improving the antigen titre of the CBPP T1-44 vaccine at the difference of $\log_{10}10^3$ as compared to Na_2HPO_4 buffer. *Table 1*; provides an overview of the potentiality of HEPES in the optimal *Mycoplasma* bacteria titre due to its strength in maintaining pH drop following metabolic activities caused by the growth of Mycoplasma as opposed to weak Na_2HPO_4 as reported by (Gourlay, 1964; Waite & March, 2001). Rapid fluctuation of pH in the phosphate buffer system from a starting pH of 7.8 to 6.16 shortly after only 48 hours of microbial activity was observed, while HEPES buffered media was found to resist pH change in increasing order of concentration from 0.05M<0.075M<0.1M and thus significantly causes an increase in the titre of the antigen and vaccine of CBPP T1-44.

In addition, the maximum CBPP vaccine titre of at least one $\log_{10}$ higher at 10^{11} CCU ml^{-1} at 24-48 h post-inoculation was maintained above 10^8 CCU ml^{-1} for up to 1 month at 37°C in HEPES-buffered Gourlay's medium as reported by (Waite & March 2001). Similar findings were observed in this study as the highest mean titre was obtained from 0.05M, 0.075M and 0.1M of HEPES buffered vaccines. Though the lowest concentration of HEPES (0.05M) was found to have a high antigen titre in the vaccine compared to the 0.075M and 0.1M, the difference in titre between these concentrations was not statistically significant with post hoc P value of 0.917.

However, HEPES buffer has demonstrated an excellent titre in the vaccine even after an accelerated stability test conducted by exposing the vaccine under the stressful conditions of a higher temperature of 40°C for 48 hours as previously reported (Waite & March 2001). This was also demonstrated in the accelerated stability study whereby the antigen titre for HEPES-treated Pleuro-pneumonia organism in the media was above the recommended dose, as shown in *Fig. 1.D*. This finding is promising due to the fact that, vaccine quality was maintained even at high temperatures and stressful conditions when the CBPP T1-44 vaccine was produced from HEPES-treated media. The vaccine was able to remain within the WOAH/OIE recommended value of 10^7 per dose.

This study demonstrates that using HEPES buffer improves CBPP T1-44 stability, and that it is possible to produce CBPP vaccine with optimal titre regardless of the stress related to propagation and storage. Importantly, the formulation of media containing HEPES requires addition of small amount of HEPES without changing other components and culturing procedures. The use of HEPES is not expected to significantly increase production cost as it has proven that even a small concentration of 0.05M had the highest antigen titer.

Lastly, the findings in this study is expected to significantly contribute to the improvement of how CBPP T1-44 vaccines are produced and overcome the existing huddle of maintaining vaccine quality and consequently, improve CBPP prevention.

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CONFLICT OF INTEREST

The authors do not have any conflict of interest.

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