Research Article

Bovine Respiratory Disease Associated *Mannheimia haemolytica* Serotype A:1 Outer Membrane Vesicles Immunogenicity

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Abstract | Outer membrane vesicles (OMVs) of *Mannheimia haemolytica* present a wide range of surface antigens. This study was designed to evaluate the immunogenic potential of *M. haemolytica* serotype A:1 OMVs (MH-OMVs). Fifteen calves were divided randomly into three groups of five calves and immunized with single-dose and booster-dose of 0.15 mg/ml vesicle preparation. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) assay of the partially purified MH-OMVs revealed approximately ten protein bands ranging from 25 to 104 kDa. Hemagglutination inhibition (HI) assay confirmed that the immune response was significantly increased in both single-dose and booster-dose immunized groups as compared to calves in the control group. Calves in booster-dose group reach its peak geometric mean (GM) $\log_{10}$ HI antibody titer (3.46 ± 0.06) at the 42nd day. Whereas calves in the single-dose immunized group reach the peak GM $1\log_{10}$ HI titer (2.79 ± 0.13) at day 28th post immunization ($p < 0.05$). Challenged calves showed the lowest mean clinical respiratory sign score in booster-dose immunized calves (1.2 ± 0.35) than in the single-dose group (4.2 ± 0.51) but the highest score was noted in the control group (6.2 ± 1.01). Hence, the current finding revealed that booster-dose-based immunization was found to induce protective level of immune response to protect calves from infection. Therefore, the booster-dose immunization schedule may be useful in inducing higher and maintaining longer protective antibody titer in calves.

Keywords | Calve, HI antibody titer, Immunization, *M. haemolytica*, Outer membrane vesicle

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INTRODUCTION

M. haemolytica is the principal bacterial pathogen to cause a severe often fatal form of pneumonia in bovine respiratory disease (BRD). The disease causes substantial economic losses to cattle producers in the world and Ethiopia due to prominent mortality, morbidity, treatment cost, and prevention measures (Fulton, 2009; Confer, 2009; Confer and Ayalew, 2018; Akalu *et al.*, 2021). *M. haemolytica* resides naturally as a commensal in the upper respiratory tract of healthy cattle. *M. haemolytica* serotype A:2 strains frequently colonizes healthy cattle, however disease is almost always caused by pathogenic isolates of serotype A:1 strains in immune-compromised animals (Cozens *et al.*, 2019). This might be due to stress, concurrent viral infection, and lung parasites that leads to sudden explosive proliferation of *M. haemolytica* serotype A:1 in the upper respiratory tract. Hence, pneumonia is caused by inhalation of aerosol contaminated with bacteria droplets into the trachea and lungs (Highlander, 2001;

Rice *et al*., 2007; Murray *et al*., 2017).

The sudden shift from commensal serotype A:2 to pathogenic serotype A:1 with in the upper respiratory tract are not clear. However, few studies demonstrated that serotype A:1 invades differentiated bovine bronchial epithelial cell by transcytosis and subsequently undergoes rapid intracellular replication before spreading to adjacent cells and causing extensive cellular damage. These findings suggest that the explosive proliferation of *M. haemolytica* serotype A:1 that occurs within the bovine respiratory tract prior to the onset of pneumonic disease is potentially due to bacterial invasion and rapid proliferation within the mucosal epithelium (Griffin *et al*., 2010; Singh *et al*., 2011; Cozens *et al*., 2019).

M. haemolytica possess multiple virulence factors which are responsible to colonize the lung. These include outer membrane proteins (omp), adhesins, leukotoxin (lkt), lipopolysaccharide (lps), capsular polysaccharides (cps), protectins, hyaluronidase, ruminant-specific repeats in toxin (rtx), and iron-binding proteins (Rice *et al*., 2007; Panciera and Confer, 2010). Thus, the interaction of LKT and LPS with bovine leukocytes results in the activation of leukocytes to undergo oxidative burst and release proinflamatory cytokines such as interleukin (IL-1β, IL-6, IL-8) and tumor necrosis factor (TNFα) as well as the proinflammatory chemokine CXCL8 are produced by differentiated bovine airway epithelial cells (AECs) during BRD (Singh *et al*., 2011; N'jai *et al*., 2013). These conditions lead to accumulation of inflammatory cells in the lung, evade the host defense mechanism, and stimulate immune responses. Besides, the virulence factors are important to induce immune response and are targets to be considered as potential candidates in vaccine design.

Prevention of BRD infection involved vaccination, comprehensive measures of prophylaxis, and therapy (Confer and Ayalew, 2018; Kurćubić *et al*., 2019). Vaccines against *M. haemolytica* are commercially available to prevent respiratory infections in cattle but not all vaccines have consistent efficacy to control the disease (Fulton, 2009; Griffin *et al*., 2010; Nagai *et al*., 2019). Treatment of *M. haemolytica* infected cattle with antibiotics is widely practiced and repeated use of antibiotics may lead to multi-drug resistance. Hence, studies have focused to search and evaluate potential immunogenic agents to design an efficacious vaccine. Hence, continuous development of a new vaccine or improvement of existing vaccines is indispensable to produce an effective vaccine against *M. haemolytica*.

Bacterial OMVs recently have gained the interest in vaccine design and targeted drug delivery (Ellis and Kuehn, 2010; Wang *et al*., 2019). OMVs are enriched with bioactive proteins, toxins, and virulence factors, and play a critical role in the bacteria-bacteria and bacteria-host interaction. Besides, these nanoparticles are non-infective, non-replicating, and present a wide range of surface antigens. Moreover, uptake by immune cells, self-adjuvation, and immunogenic properties of OMVs make them suitable in the application of vaccine design (Van *et al*., 2015; Jan, 2017; Qing *et al*., 2019). Thus, the use of OMVs can be expanded by conveying heterologous antigens. Bacterial vesicles are a potential vaccine candidate for various pathogens of gram-negative bacteria. The immunogenic potential of MH-OMVs was proved in different studies (Roier *et al*., 2013; Ayalew et al., 2013). Therefore, considering the heterogeneous mixture of antigenic properties an attempt was made to evaluate the immunogenic potential of *M. haemolytica* serotype A:1 OMVs in single-dose and booster-dose immunization protocol in calves model.

MATERIALS AND METHODS

Bacterial growth

M. haemolytica serotype A:1 was kindly provided by the National Veterinary Institute (NVI), Ethiopia. Colonies were grown onto brain heart infusion (BHI) agar and incubated overnight at 37°C. Colonies were transferred into a BHI broth of 270 ml and sub-cultured into two-liter BHI broth containing 10% horse serum and 0.5% yeast extract. Bacterial cell pellets were harvested by centrifugation (5,000 x g) from the broth culture. The cell pellets were transferred into ten-liter BHI broth and were grown for 13 hrs at 37°C with agitation to the late exponential phase of OD_{600} nm of 1.0. This procedure was based on previous experiments with slight modification (Roier *et al*., 2012; Kothary *et al*., 2017).

OMVs isolation

Centrifugation

The bacteria culture was pelleted by two successive centrifugation steps to remove bacterial cells at 6,000 x g and 10,000 x g in a Beckman (J2-MI) centrifuge. The supernatant with MH-OMVs was filtered through 0.45 µm and 0.22 µm pore size filters to ensure the complete removal of bacterial cells. Finally, 0.5 ml of the filtrate was cultured onto BHI agar and incubated for 48 hrs to confirm the absence of viable bacteria.

Supernatant ultrafiltration

The supernates containing MH-OMVs were concentrated with centrifugal filter devices to approximately 500 ml. FlexStand Benchtop hollow-fibers-cartridge system with membrane surface area of 1.10/1.15m^2 (GE Healthcare, USA) at 100 kDa nominal molecular weight cutoff (NMWC) was used to concentrate the pellet.

Ultracentrifugation

The supernate was ultracentrifuged at 140,000 x g using JXN-30 centrifuge (Beckman coulter, USA) and the supernatant was discarded. Harvested pellets were re-constituted in 5 ml PBS (pH 7.4) and stored at -20°C for further analysis.

Bicinchoninic acid (BCA) assay

BCA assay was conducted to determine the protein concentration. Briefly, the working reagent was prepared by mixing 50 parts of BCA (tartrate in alkaline carbonate buffer) and 1 part of 4% copper sulfate pentahydrate (Cu SO_4.5H_2O) solution. 25 µl of standard and/or blank and MH-OMV pellet suspension was pipetted into a microwell plate. 200 µl of working reagent was added to each well and mixed thoroughly. The plate was covered, incubated at 37°C for 30 min, cooled at room temperature, and absorbance was measured at 562 nm.

SDS-PAGE analysis

Protein profile of the partially purified MH-OMVs was analyzed using Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 4.8% stacking and 12.0% separating gel. The vesicles sample was boiled in SDS gel loading buffer (PH 7.2). The sample was electrophoresed with a standard protein marker 10 – 200 kDa (Fermenta, Germany) at 20 mA constant current until the bromophenol blue dye appeared at the bottom of the resolving gel (~120 mins).

Challenge bacterial strain preparation

M. haemolytica serotype A:1 isolated from field outbreak cases of BRD were used as a challenge strain. The isolate was recovered by culturing onto BHI agar with horse serum (10%) and incubated at 37°C for 18–22 hrs. Isolated colonies were sub-cultured into BHI broth and incubated for 16 – 18 hrs at 37°C. Bacterial culture was centrifuged (3,200 x g), washed 3x in sterile PBS (PH 7.4), and re-suspended in BHI broth. Ten-fold (1:10) serial dilutions were prepared and the dilution corresponding to 3 x 10^8 colony forming units (CFU)/ml was used as a challenge dose and pathogenicity was validated on mice.

Pathogenicity assay

M. haemolytica serotype A:1 pathogenic strain was validated in six Balb/c mice. Three mice were challenged with 0.1 ml of culture (3 x 10^8 CFU) via the intraperitoneal route. Fresh PBS preparation was used to inject control group mice. All mice were kept *adlibtum* and observed for 24–36 hrs for fatality rate. Lung and liver samples were collected from dead mice and cultured onto 10% sheep blood agar and incubated at 37 °C for 24 hrs. Colonies were confirmed by mPCR assay.

mPCR

Primers targeting the *M. haemolytica* virulence-associated *PHSSA* gene and *Rpt2* gene were used in the PCR assay. Briefly, PCR was conducted in 25 µl final volume of 3 µl RNase free water, 2 µl (5 pm/µl) forward and reverse primers, 10 µl IQ supermix (Bio-Rad, USA), and 3 µl template DNA (Table 1).

Immunization and challenge

Fifteen calves were divided randomly into three groups of five calves. Five calves in the first group were immunized subcutaneously (0.15 mg/ml) of MH-OMVs preparation at day 0 (single dose group). Five calves in the second group were immunized at day 0 and day 21 (booster-dose group). Five calves in the third group were injected with PBS (control group). All calves were challenged with 20 ml (3 x 10^8 CFU/ml) of *M. haemolytica* serotype A:1 strain at day 84 post-immunization and observed for 7 days. Daily temperature and clinical signs scores were recorded during the experiment period and at the end of the experiment, calves were treated with injectable florfenicol.

Serum collection

All calves were observed during the experiment period and a blood sample (5 ml) was collected from the jugular vein of calves at days 0, 14, 28, 42, 56, 70, and 84 days post-immunization.

HI assay

Fresh sheep red blood cells (SRBCs) were collected, centrifuged, washed with PBS (pH 7.0), and adjusted to a final dilution of 0.65%. A two-fold serial dilution (25 µl) of serum samples was prepared with PBS and 4 HA units (25 µl) of standardized *M. haemolytica* antigen was added into each well except for the serum control wells. Plates were incubated at 37°C for 60 min and kept at 4°C for 30 min. SRBCs (50 µl) of 0.65% were added and kept at 4°C for 1 hr. The presence of agglutination inhibition was recorded and the HI titer was stated as the reciprocal of the highest dilution of the serum that completely inhibited the HA activity. The mean antibody titer was converted into $\log_{10}$ values.

Clinical score

Challenged calves were observed twice per day (morning and afternoon) throughout the experiment period. Clinical respiratory signs were scored from 0 to 3 (rectal temperature, coughing, nasal discharge, lacrimation, and ear drooping) as well as sneezing and dyspnea were evaluated to diagnose BRD using the Wisconsin scoring chart (Table 2).

Ethics approval and consent to participate

Samples collection followed scientific procedures and animal handling employed with basic animal welfare protocols.

Table 1: Oligonucleotides and PCR assay condition.

Gene	Primers	Sequence (5` to 3`)	Size (bp)	PCR condition (35 cycles)				
				Initial denaturation	Denaturation	Annealing	Extension	Final extension
PHSSA	PHSSA-F	TTCACATCTTCATCCTC	325	95°C for 3 min	95°C for 1 min	48°C for 1 min	72°C for 1 min	72°C for 5 min
	PHSSA-R	TTTTCATCCTCTTCGTC						
Rpt2	Rpt2-F	GTTTGTAAGATATCCCATTT	1022					
	Rpt2-R	CGTTTTCCACTTGCGTGA						

Table 2: Evaluation parameter and clinical respiratory scoring.

Parameter	Score*			
	0	1	2	3
Rectal temperature (°C)	< 38.6	38.6 – 39.0	39.1 – 39.6	> 39.6
Cough	No cough	Induce single cough	Repeated coughing	Repeated spontaneous coughing
Nasal discharge	Normal	Unilateral cloudy discharge	Bilateral, cloudy, or excessive mucus	Copious, bilateral mucopurulent nasal discharge
Lacrimation	Normal eye	Mild lacrimation	Moderate bilateral lacrimation	Heavy lacrimation
Ear position	Normal ear	Ear flicking	Unilateral eardrop	Severe head tilt, or bilateral ear droop

*Score ≥ 5 is indicative of pneumonia.

Laboratory assay was performed following the standard bacteriological and immunological methods. BALB/c mice were used for challenge assay and calves were used for immunization in strict accordance with the recommendations for the care and use of laboratory animals at the National veterinary institute, Ethiopia. The corresponding animal protocols for laboratory animal handling (NVI-VPS-LAQ-PR-02) and postmortem examination (necropsy) procedure (NVI-VPS-LAQ-PR-01) were strictly followed during the study. Mice and calves were housed with food and water *ad libitum* and monitored under the care of full-time staff. All laboratory animals were acclimatized for 1 week before any procedure and calves were screened for any disease condition before the experiment.

Statistical analysis

Data were coded and recorded in an excel spreadsheet and analyzed using STATA software version 11. The HI antibody titers were interpreted logarithmically to $\log_{10}$ values and expressed as geometric mean (GM) ± standard error (SE). The geometric mean $\log_{10}$ values (X) were calculated using the formula $GM = \sqrt[n]{X_1 X_2 X_3 \cdots X_n}$, where n = total number of calves. The differences among groups were analyzed using the Kruska-Wallis, chi-square test, and Bonferroni correction method ($\alpha = \alpha/n = 0.05/3$) was used for pairwise and multiple comparisons among the experimental groups. The level of significance was considered at $P < .05$.

RESULTS AN DISCUSSION

Multiplex PCR and SDS-PAGE assay

Multiplex PCR assay of *M. haemolytica* serotype A:1 strain, used in the current study, confirmed amplification of primers targeting the virulence-associated *PHSSA* gene (coding serotype-specific antigen) and *Rpt2* gene (coding for methyltransferase). The BCA assay of MH-OMVs preparation revealed an initial concentration of 0.32 mg/ml of protein at 562 nm and the result was adjusted to an immunizing dose of 0.15 mg/ml. Besides, SDS-PAGE analysis of the partially purified MH-OMVs revealed the presence of approximately ten protein bands of the most abundant proteins. The estimated molecular weight of these protein bands ranges from 28 kDa to104 kDa (Figure 1).

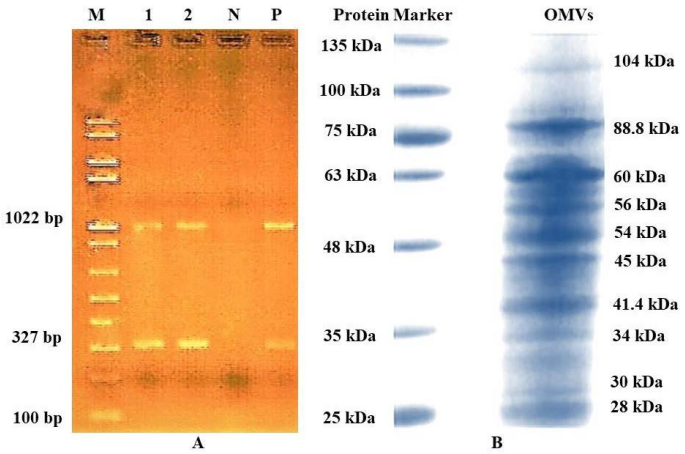

Figure 1: (A) Agarose gel electrophoresis of *M. haemolytica*; (B) SDSPAGE of MHOMV.

HI assay

Calves showed a very low level of antibody titer before immunization at day 0 in all experimental groups and control group calves while a significant rise in the antibody titer was recorded post-immunization. Immunized calves

showed a rise in the GM log_{10} HI titer value in both single-dose and booster-dose groups though the antibody titer (GM log_{10} HI value) in the control group remains constant throughout the experiment period. The GM log_{10} antibody titers in an interval time of post-immunization in both single and booster-dose groups markedly reached the protective level (≥ 1:80/ > 1.9 GM log_{10} HI titer) after the primary immunization (Table 3).

In single-dose immunized calves, the immune response reached the highest GM log_{10} HI titer of 2.79 ± 0.13 on day 28th after the primary immunization (Day 0). Whereas, boosted calves on day 14th after the primary immunization (day 0) showed their peak antibody titer (GM log_{10} HI titer) with 3.46 ± 0.06 at the 42nd day after primary immunization (i.e., 28th day after booster-dose administration). Control calves were showed constant antibody titer (GM log_{10} HI titer) throughout the experiment period. The change in antibody titer against MH-OMVs preparation was significantly higher after immunization compared to control group calves ($P < .05$; P = 0.00001). Multiple comparisons of the mean ranks of pairs were significantly different (Table 4).

Challenge assay

Challenged were deceased within 24 to 36 hrs post intraperitoneal challenge. Giemsa stain and multiplex PCR assay confirmed the presence of *M. haemolytica* A:1. Calves challenged after immunization were showed a slight rise in the mean daily rectal temperature (38.8°C - 39.7°C) in single and boosted dose calves but higher mean daily rectal temperature (39.6°C - 40.5°C) was recorded in the control group. The mean clinical respiratory sign score was showed 1.2 ± 0.16, 4.2 ± 0.26, and 6.2 ± 0.45 in booster-doses, single-dose, and control group of calves, respectively.

The present study was aimed to evaluate the immunogenic potential of OMVs derived from *M. haemolytica* serotype A:1 strain. Researches have been conducted in an attempt to develop an effective vaccine against *M. haemolytica* infection by focusing on membrane vesicles and chimeric proteins (Jan, 2017). OMVs play significant role in the bacteria-bacteria and bacteria-host interaction. Vesicles produced by *M. haemolytica* are important structures that retain immunogenic properties. Thus, OMVs are considered as appropriate candidates in vaccine design. Immunization of MH-OMVs preparation have been proved to induce a protective immune response in calves (Ayalew et al., 2013; Jan, 2017).

In this study, SDS-PAGE assay of *M. haemolytica* OMVs from partially purified preparations revealed the presence of approximately ten major protein bands ranging from 25 to 104 kDa. The estimated molecular weight of protein bands observed during the analysis includes 28, 30, 34.3, 41.4, 45, 54, 56, 60, 88.8, and 104 kDa. The current finding was in agreement with previous studies except slight difference in molecular weight (Knights *et al.*, 1990; Roier *et al.*, 2013; Ayalew *et al.*, 2017). This could be due to the *in vitro* passage levels that may lead the pathogen to express proteins of different molecular weight (Knights *et al.*, 1990). Liquid chromatography-tandem mass spectrometric analysis of vesicle components of *M. haemolytica* identified around 226 proteins (Ayalew et al., 2013; Gerritzen *et al.*, 2019). Besides, immunoproteomic analysis revealed 57 outer membrane proteins that may have a potential immunogenic character (Ayalew *et al.*, 2010).

Protein analysis of vesicles in the current study is in agreement with earlier findings of genetically related outer membrane protein A (OmpA), OmpC, OmpE, and OmpF that range from 30 kDa – 35 kDa (Ayalew *et al.*, 2011). These proteins are known to express strong immunogenic potential. Besides, OmpP2 a homologue of major omp (approximately 41.4 kDa) express weak immunogenicity. In addition, outer membrane lipoprotein protein (PlpE) of 45 kDa has been identified from *M. haemolytica* culture supernatants and characterized in different studies for its immunogenicity (Knights *et al.*, 1990; Pandher *et al.*, 1999). Moreover, OmpD15 (high-molecular-mass outer membrane) approximately 88.8 kDa is known to express weak immunogenic character. *M. haemolytica* serotype 1-specific antigen (SSA-1) 104 kDa was proved to be potentially immunogenic (Ayalew *et al.*, 2011; Gerritzen *et al.*, 2019). Hence, the proteomics analysis revealed that OMVs possess many periplasmic and outer membrane-associated proteins which play critical role in toxin and virulence factor transport to host cells, and this mechanism mediates the host immune response (Singh *et al.*, 2011; Confer and Ayalew, 2018).

Table 3: HI assay antibody titers (GM log_{10} value) of immunized calves at different days

Experiment groups	Dose	Days post-immunization and GM log_{10} HI titer ± SE						
		0	14	28	42	56	70	84
Single-dose group	150 mg/ml*	0.66 ± 0.12	2.13 ± 0.11	2.79 ± 0.13*	2.54 ± 0.15	1.96 ± 0.06	1.59 ± 0.09	1.28 ± 0.09
Booster-dose group	150 mg/ml***	0.57 ± 0.10	2.01 ± 0.12	3.16 ± 0.06	3.46 ± 0.06*	3.22 ± 0.07	2.79 ± 0.09	2.26 ± 0.06
Control group	Non	0.66 ± 0.12	0.76 ± 0.12	0.76 ± 0.12	0.57 ± 0.10	0.66 ± 0.12	0.57 ± 0.10	0.66 ± 0.12

SE: Standard error; * Peak GM log_{10} antibody titer; ** Single-dose (immunization at day 0); *** Booster-dose (primary dose immunization at day 0 and secondary (booster) dose at day 14).

Table 4: Multiple comparisons of GM log_{10} HI titer of experimental groups.

Pair	Comparison			
	Difference	H statistic	Critical value	p-value
Single dose control group	1.3	16.7683	5.7308	0.00004
Booster dose control group	2.13	14.7078	5.7308	0.0001
Single dose booster dose	0.83	6.7252	5.7308	0.0095

Serum samples HI analysis revealed a comparable GM log_{10} antibody titer of 0.57 ± 0.10, 0.57 ± 0.10, and 0.66 ± 0.12 in single, booster, and control group of calves, respectively at day 0. The rise in GM log_{10} HI titer ranks of single-dose, booster-dose, and control group of calves; pairwise comparison revealed a significant difference ($p < 0.05$). The antibody titer of GM log_{10} value of >1.9/≥ 1:80 is enough to protect calves against virulent strain *M. haemolytica* (OIE, 2021). Calves with HI titer of GM log_{10} value of <1.6/1:40 were fully susceptible and HI titer between 1.9/1:40 and 1.6/1:80 is considered to be partially protected (Ferede *et al*., 2014). Thus, the rising trend of the GM log_{10} antibody titer continued to reach the highest HI titer value (2.79 ± 0.13) at the 28th day of post primary immunization (Day 0) in the single-dose group. This group of calves maintained the protective antibody titer (1.96 ± 0.06) till the 56th day post-immunization. Whereas, the GM log_{10} antibody titer in the booster-dose group reached its peak (3.46 ± 0.06) at the 42nd days of post primary immunization or at the 28th days after the booster-dose immunization and this group of calves maintained the protective antibody titer (2.26 ± 0.06) till the end of the experiment period.

Validation of *M. haemolytica* pathogenicity revealed that challenged mice have deceased within 24–36 hrs post intraperitoneal inoculation. Colonies grown from lung, liver tissue, and Giemsa-stained smear showed bipolar organisms and mPCR assay confirmed re-isolation of *M. haemolytica*. Challenged calves post-immunization revealed a slight rise in the mean daily rectal temperature ranging from 38.8°C - 39.7°C within the first three days post-challenge in single and booster dose immunized calves while higher mean daily rectal temperature (39.6°C - 40.5°C) was recorded in the control group. The mean clinical respiratory sign score was lower in booster-dose immunized calves (1.2 ± 0.35) compared to the single-dose group (4.2 ± 0.51) however the highest score was noted in the control group (6.2 ± 1.01). Hence, the current finding revealed that booster-dose based immunization was found to induce the highest protective level of the immune response as indicated by the HI assay and protect animals from infection.

CONCLUSIONS AND RECOMMENDATIONS

In the present study, the antibody titer in the booster-dose group reached the peak GM log_{10} HI titer of 3.46 ± 0.06 at the 42nd days post-immunization. Calves immunized with booster-dose maintained a higher immune response for a longer period as compared to the single-dose immunized calves. Antibody responses against numerous periplasmic and outer membrane-associated proteins protected challenged calves. The finding of the current study indicated that the raised antibody titer was protective in boosted calves. Therefore, the booster-dose immunization may be useful to induce and maintain protective antibody titer in calves.

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NOVELTY STATEMENT

This study is the first to evaluate the immunogenic response of single-dose and booster-dose immunization of M. haemolytica serotype A:1 outer membrane vesicles in calves and analyzing the immune responses over a longer period of time. Besides, the experiment is employed on target animal with challenge test.

AUTHOR'S CONTRIBUTION

All authors participated in the conception and design of the study; V.B.M., T.A. and E.G.: supervise the current study; M.A., B.T. and T.D.: conducted the experiment on calves and performed laboratory assay; M.A.: anlalyzed the data and drafted the manuscript; V.B.M., T.A. and E.G.: revised the article. All authors read and approved the final manuscript.

Conflict of interest

The authors have declared no conflict of interest.

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