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COMPARATIVE STUDY OF THE STABILITY AND IMMUNOGENICITY OF INACTIVATED BACTERIN, ALUM PRECIPITATED AND OIL ADJUVANTED HEMORRHAGIC SEPTICEMIA VACCINE

MVSc THESIS

BY

HASSEN BELAY GELAW

DEPARTMENT OF VETERINARY MICROBIOLOGY, IMMUNOLOGY AND VETERINARY PUBLIC HEALTH

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BISHOFTU, ETHIOPIA

COMPARATIVE STUDY OF THE STABILITY AND IMMUNOGENICITY OF INACTIVATED BACTERIN, ALUM PRECIPITATED AND OIL ADJUVANTED HEMORRHAGIC SEPTICEMIA VACCINE

A thesis submitted to the College of Veterinary Medicine and Agriculture of Addis Ababa University in partial fulfillment of the requirements for the degree of Master of Veterinary Science in Veterinary Microbiology

By

Hassen Belay Gelaw

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Bishoftu, Ethiopia

Addis Ababa University

College of Veterinary Medicine and Agriculture

Department of Veterinary Microbiology, Immunology and Veterinary Public Health

As members of the Examining Board of the final MVSc open defense, we certify that we have read and evaluated the Thesis prepared by **Hassen Belay Gelaw** entitled: **Comparative study of the stability and immunogenicity of inactivated bacterin, alum precipitated and oil adjuvanted hemorrhagic septicemia vaccine** and recommend that it be accepted as fulfilling the thesis requirement for the degree of: Masters of Veterinary Science in **Veterinary Microbiology.**

_____	_____	_____
Chairman	Signature	Date
_____	_____	_____
External Examiner	Signature	Date
_____	_____	_____
Internal Examiner	Signature	Date
Dr. Bedaso Mammo (DVM, MSc, PhD Scholar, Asst. Prof)	_____	_____
Major Advisor	Signature	Date
Dr. Fufa Daewoo (DVM, MSC, PhD, Asso. Prof.)	_____	_____
Co- Advisor	Signature	Date
Dr. Nick Nwankpa (DVM, MSC, PhD)	_____	_____
Co- Advisor	Signature	Date
Dr. Gezahegn Mamo (DVM, MSc, PhD, Assoc. Prof)	_____	_____
Department chairperson	Signature	Date

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LIST OF ABBREVIATIONS

%	Percent
^{0}C	Degree centigrade
ANOVA	Analysis of Variance
APC	Antigen Presenting Cells
APHRD	Animal and Plant Health Regulatory Directorate
APV	Alum Precipitated Vaccine
AU-IBAR	Inter African Bureau for Animal Resources
AU-PANVAC	Pan African Veterinary Vaccine Centre
bp	Base Pair
CMI	Cell Mediated Immunity
CSY	Casein Sucrose Yeast Medium
DIVA	Differentiation of Infected from Vaccinated Animals
DNA	Deoxyribonucleic acid
EDTA	Ethylene Diamine Tetraacetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
g	gram
GMT	Geometric Mean Titre
G-SRBC	Gluteraldehyde fixed Sheep Red Blood Cells
HCl	Hydrochloric acid
hr	Hour
HS	Haemorrhagic Septicaemia
IBV	Inactivated Bacterin
IHA	Indirect Haemagglutination Test
km	Kilometer
LD_{50}	Median Lethal Dose
LPS	Lipopolysaccharide
mg	Milligram
MHC-II	Major Histocompatibility Complex Class II
min	Minute
ml	Mililitre
NSS	Normal Saline Solution
O/W/O	Water in Oil adjuvant
OAV	Oil Adjuvanted Vaccine

OD	Optical Density
OMP	Outer Membrane Protein
PCR	Polymerase Chain Reaction
PMPT	Passive Mouse Protection Test
RBC	Red Blood Cells
REA	Restriction Endonuclease Analysis
SRBC	Sheep Red Blood Cells
v/v	Volume Per Volume
W/O/W	Oil in Water adjuvant
xg	Gravity

STATEMENT OF AUTHOR

First, I declare that this thesis is my *bonafide* work and that all sources of material used for this thesis have been duly acknowledged. This thesis has been submitted in partial fulfilment of the requirements for an advanced (MVSc) degree at Addis Ababa University, College of Veterinary Medicine and Agriculture and is deposited at the University/College library to be made available to borrowers under rules of the Library. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate.

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Name: Hassen Belay Signature: _____

College of Veterinary Medicine and Agriculture, Bishoftu

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ABSTRACT

Haemorrhagic septicaemia (HS) is the most devastating disease of cattle and buffalo particularly to smallholder farmers where husbandry and preventive practices are poor. Due to the acute nature of the disease and the presence of carrier animals after recovery, vaccination is considered as the only efficient method of controlling the disease. Although alum precipitated vaccine is the most widely used; it has come across drawbacks including poor stability and weak immune response. Oil adjuvanted vaccine on the other hand has been emerging with better stability, longer periods of protection and stronger immune response. Therefore, this experimental study was conducted with the objectives of comparing the stability and immunogenicity of inactivated bacterin (IBV), alum precipitated (APV) and oil adjuvanted (OAV) HS vaccine candidates. To achieve this, *Pasteurella multocida* B:2 (Ethiopian isolate) was grown on tryptose soy broth (TSB) supplemented with 10% horse serum, inactivated with formalin (0.5%) and the turbidity was standardized to contain 1.5mg antigen/ml. APV was prepared by admixing aluminium potassium sulphate (1%) with the bacterin whereas OAV was prepared by mixing the bacterin with equal volume of Montanide ISA 61 VG. The inactivated culture was used as IBV. The three vaccine formulations were stored at 4ºC and tested for stability (potency and identity) once a month for 5 months. Potency test was conducted on mice while test for identity was conducted using polymerase chain reaction. On the other hand, nine calves were divided into three groups, each group vaccinated with each candidate and boosted on day 28 post primary vaccination. Sera were collected on days 0, 28, 42 and 56 to assess the immune response. The IBV and APV showed retention of stability when stored at 4°C for 30 days. The potency dropped to 0.2 $\log_{10}$ and 0.8 $\log_{10}$ for IBV and APV respectively when storage period was extended to 60 days whereas there was no difference between vaccinated and unvaccinated groups when both formulations were stored for 90 days. OAV retained stability when stored at 4°C for 90 days and potency dropped to 0.6 $\log_{10}$ and 0.2 $\log_{10}$ when storage was extended to 120 and 150 days respectively. Application of indirect hemmaglutination test on sera obtained from calves revealed that OAV induced stronger immune response with peak antibody titre of 3.4 $\log_{10}$ on day 56 while APV induced protective, but, relatively weaker immune response with peak antibody titre of 2.4 $\log_{10}$ on day 56. The IBV induced weak immunity (1.5 $\log_{10}$ on day 56) indicating the need for incorporation of a suitable adjuvant. There was a significant difference in the immune response between the vaccine groups ($P<0.05$, ANOVA). Therefore, this study revealed the weak stability as well as immunogenicity of the conventionally used alum precipitated vaccine and urges the need to produce oil adjuvanted vaccine for the control of the disease.

Key words: *adjuvant, immunogenicity, Montanide, Pasteurella multocida, Vaccine, stability,*

1. INTRODUCTION

Haemorrhagic septicaemia (HS) is a major disease of cattle and buffaloes occurring as catastrophic epizootics, resulting in high mortality and morbidity. The disease is caused by the E:2 and B:2 serotypes of *Pasteurella multocida*, a gram negative coccobacillus residing mostly as a commensal in the nasopharynx of animals (Khan *et al*., 2013). Hemorrhagic septicemia epidemics may occur as an alarming and devastating disease problems in cattle and buffaloes affecting not only the economic return of animal production but also animal traction and the harvest of vital crops (OIE, 2012).

Hemorrhagic septicemia is an important disease of cattle and water buffalo in Africa, Asia and the Middle East. Until recently type E was believed to have been the cause of HS in all countries in Africa except Sudan and Egypt where both serotype B and E have been implicated. Possibly because of the proximity of these two countries to Asia, where only serotype B:2 is involved. Outbreak of the disease in Namibia and Cameron, however, has been caused by serotype B:2, while an outbreaks in Zimbabwe in 1990 were also caused by B:2 (Dziva *et al*., 2000; Voigts *et al*., 1997; Martrenchar and Njanpop, 1994).

During 2013, hemorrhagic septicemia and other pasteurellosis outbreaks were reported to Inter African Bureau for Animal Resources (AU-IBAR) by 10 countries, with a total of 1,211 outbreaks, 14,122 cases and 2,530 deaths. Ethiopia recorded the highest number of outbreaks (1,063) followed by Niger (81) and Zambia (22), a pattern similar to that of the previous year. Outbreaks occurred most often in January and February (AU-IBAR, 2013). In Ethiopia, according to the major animal disease outbreaks reported to the Animal Production and Health Regulatory Directorate (APHRD) during 2007 to 2011, hemorrhagic septicemia is the most important animal disease with 64,011 cases in 3,857 outbreaks (APHRD, 2012).

Up to 5% of healthy cattle and water buffalo are colonized by small numbers of *P. multocida* serotype E:2 or B:2, which can be shed during periods of stress. Common stressors associated with outbreaks include high temperature and humidity, concurrent infection (blood parasites or foot and mouth disease), poor nutrition and work stress (Ahmed, *et al*., 2014). Although outbreaks can occur at any time, disease is most prevalent during the rainy season. Acute nature and short duration of the disease are the main causes of therapeutic failure in affected animals. Thus, most research activities on hemorrhagic septicemia are being focused on the control of the disease through vaccine development and vaccination (Karunasree, 2016; Tapdasan and Salces, 2016).

Immune response to the bacterin is poor and is of short duration. Immune response in the vaccinated animals is required against outermost component of the bacterial body (i.e. capsule) and the capsule of *P. multocida* is composed of mainly lipopolysaccharide and of minor fraction of proteins. Lipopolysaccharide induces B cell response and cannot be presented along with MHC-II antigen by antigen presenting cells of the animal body, and hence the responsive B cells cannot get cooperation of T cells for enhanced antibody production. The B cell response to LPS is primary and the immunity is of low level and short duration (AbdelAziz *et al*., 2015). This necessitated the use of adjuvants in the bacterin. Currently, four preparations of killed vaccines are used including inactivated bacterin and dense bacterin combined with either alum, aluminium hydroxide or oil adjuvant (Doorn *et al*., 2017; OIE, 2012). Alum precipitated vaccine is the most widely used in Africa and Asia. However, this type of vaccine not only provides short duration and less pronounced immunity, but, also causes post vaccinal shock reactions (Mudassar, 2014; Qureshi and Saxena, 2014; Petrovsky and Aguilar, 2004; Jabbari and Jula, 2002). On the other hand, significantly drastic effects of storage on the potency of the alum precipitated haemorrhagic septicaemia vaccine have been reported by Sheikh *et al*. (1995). The quality control data at Pan African Veterinary Vaccine Centre (AU-PANVAC) also shows that most of the alum precipitated hemorrhagic septicemia vaccines submitted more than three months post production have failed either for identity, potency or both (AU-PANVAC, 2016).

The use of oil as an adjuvant is reported to have several advantages over the conventional alum precipitated vaccine (Sarwar *et al*., 2015; Sivakumar *et al*., 2011;). Oil adjuvant not only reduces the toxicity of the vaccine, but also results in delayed absorption, enhanced immune response and enhanced stability of the vaccine. Tests for stability and potency have indicated that oil adjuvanted HS vaccine stored at 4 - 8 °C in a refrigerator is stable for more than six months (Afroz *et al*., 2016; OIE, 2012). Difficulty of vaccine administration and higher cost of production discourage vaccine production laboratories from producing oil adjuvanted HS vaccine. However, recent innovations described the development of oil adjuvant of low viscosity with relatively less expensive cost (Doorn *et al.*, 2015). Abdelaziz *et al*. (2015) also reported that Montanide was found to be incomplete adjuvant type of mineral oil based on complex water in oil emulsion characterized by low viscosity and easy to inject. Furthermore, Seppic (2013) reported that Montanide™ ISA 61 VG has been designed to offer a powerful adjuvant that induces a strong and long lasting immune response with a large space for antigenic media in the emulsion suggesting the possibility of longer stability.

In Ethiopia, hemorrhagic septicemia vaccine precipitated with aluminium potassium sulphate is being produced and distributed by the National Veterinary Institute for field use (Sorri *et al*, 2018). Vaccination against hemorrhagic septicemia is widely practiced in endemic areas in Africa in general and in Ethiopia in particular, however, significant outbreaks still occur (Ahmed *et al*., 2014; AU-IBAR, 2013; APHRD, 2012). This might be due to the induction of short duration of immunity by the alum precipitated vaccine and the low stability of the vaccine that does not fit with the time required for quality control and reach at field level. Though studies suggest that oil adjuvanted vaccine has an outstanding stability, safety and immunogenicity profile; comparative stability and immunogenicity of the vaccine candidates are not yet studied in Ethiopia. On the other hand, potency studies conducted so far for alum precipitated vaccine involved only a small number of animals and the results still need to be verified in a further trial.

Therefore, the objectives of this study were to:

- Compare the stability and immunogenicity of inactivated bacterin, alum precipitated and oil adjuvanted hemorrhagic septicemia vaccine candidates, and
- Determine the shelf life of inactivated bacterin, alum precipitated and oil adjuvanted hemorrhagic septicemia vaccine candidates.

2. LITERATURE REVIEW

2.1. The causative agent

2.1.1. History and Nomenclature

Bollinger (1878) first described the disease among wild boar, dear, and cattle in munch. Pasteurella is named after Louis Pasteur in recognition of his pioneering work in the 1880s (Alwis, 1999). The genus Pasteurella belongs to a large family of bacteria, the Pasteurellaceae, which includes two other important genera, Actinobacillus and Haemophilus. Two members of the genus Pasteurella, *Pasteurella multocida* and *Pasteurella haemolytica* cause a variety of important diseases in domestic, agricultural and wild animals (Kuhnert and Christensen, 2008). The two species can be differentiated by several biochemical characteristics. *Pasteurella multocida* is the causative organism causing hemorrhagic septicemia disease in the cattle. A summary of the historical evolution of the nomenclature of *Pasteurella multocida* is shown in table 1.

Table 1. History of the nomenclature of *Pasteurella multocida*

Author	Year	Name
Bollinger	1879	-
Pasteur	1880	-
Burril	1883	*Micrococcus gallicidus*
lopf	1885	*Micrococcus cho/erae-gallinarum*
Kitt	1885	*Bacterium bipolare multocidium*
Oreste and Armani	1887	*Bacillus septicaemiae*
Trevisan	1887	*Pasteurella cholerae-gallinarum*
Lehmann and Neumann	1889	*Baderium multocidium*
Sternberg	1893	*Bacterium septicaemiae haemorrhagicae*
Lignieres	1900	According to host species: *Pasteurella aviseptica* *Pasteurella boviseptica* *Pasteurella suiseptica*
Topley and Wilson	1929	*Pasteurella septica*
Rosenbach and Merchant	1939	*Pasteurella multocida*

Source: Namioka (1978)

2.1.2. Morphology and staining

Pasteurella multocida is a non-motile, non-spore forming short rod or coccobacillus, 0.2-0.4 by 0.6-2.5 mm in size. Repeated laboratory subcultures of old cultures or cultures grown under unfavourable conditions tend to be pleomorphic and longer rods and filamentous forms appear. In tissues, exudates and recently isolated cultures, the organism shows the typical coccobacillary forms (Quinn *et al*., 2002). It is a gram-negative organism and, in fresh cultures and animal tissues, gives typical bipolar staining, particularly with Leishman or methylene blue stain (Ashraf *et al*., 2011).

2.1.3. Growth characteristics and colony morphology

Pasteurella multocida grows in most common laboratory media such as nutrient agar. Special media such as dextrose-starch agar and casein sucrose yeast (CSY) medium support an abundant growth. Blood agar and CSY agar with 5% sheep blood are convenient media for routine laboratory culture. The optimum growth temperature is 35-37°C (Kamruzzaman *et al*., 2016; Ashraf *et al*., 2014).

The organism shows different types of colonies, which are related to the capsular type. Capsular type A produces the largest colonies, which are translucent, greyish in colour, and mucoid in consistency. There may be considerable variation in colony size, ranging from rounded, convex, discrete colonies with circular edges to large watery colonies with flowing margins. Colonies of types B and E may also vary in size, depending on the degree of capsulation. They will range from larger greyish colonies, when freshly isolated or when grown in media containing blood serum, to smaller colonies that give a yellowish-green or bluish green iridescence when viewed in transmitted light (Jabeen *et al*., 2013; OIE, 2012).

Rough colonies may be produced by old cultures. Production of rough colonies is the result of loss of capsular material, a process referred to as 'dissociation'. Dissociation may take place to different degrees, giving rise to intermediate colony forms. Passage of rough cultures in natural host animals or laboratory animals, or subculture in media containing animal tissues, causes reversion to the capsulated, iridescent colony forms (Jabeen *et al*., 2013; Ashraf *et al*., 2011). Dissociation also occurs during storage of stock cultures either in stock culture media or in a lyophilised form. In such instances, an animal passage should be carried out upon reconstitution of the stock culture. Colony morphology is also related to virulence. Dissociation is associated with reduction or loss of virulence and also with loss of antigenicity. Since well capsulated

cultures make good vaccines, vaccine seed cultures are passaged in laboratory animals or even in natural host species periodically (Kuhnert and Christensen, 2008).

Some of the biochemical properties are stable and consistent within the species but some characters are variable (Kamruzzaman *et al*., 2016; Ashraf *et al*., 2014). The consistent characteristics of the species are shown in Tables 2. Classification of the species into groups or biotypes based on biochemical reactions has not been successful. Although some fermentation reaction patterns have been associated with isolates from a particular host species, such relationships have not been consistent enough to form the basis for classification of the species (Table 3).

Table 2. Summary of common biochemical properties of *Pasteurella multocida*

Biochemical property		**Reaction**
Haemolysis		-
Growth on McConkey medium		-
Gelatin hydrolysis		-
Indole production		+
Hydrogen sulfide production		+
Nitrate reduction		+
Methyl red reaction		-
Voges-Praskauer reaction		-
Growth on potassium cyanide		+
Urease production		-
Catalase production		+
Oxidase production		+
Citrate utilisation		-
Malonate utilisation		-
Arginine decarboxylase		-
Lysine decarboxylase		-
Ornithine decarboxylase		+
Sugar fermentation	Glucose	+
	Fructose	+
	Galactose	+
	Mannose	+

Sucrose	+
Maltose	V+
Lactose	V-
Trehalose	V+
Arabinose	V+
Xylose	V+
Mannitol	V+
Sorbitol	V+
Inositol	V-
Dulcitol	V-
Salicin	V-

+ = Positive; - = Negative; V+ = Variable – more often Positive; V+ = Variable – more often Negative

Source:- Kamruzzaman *et al*. (2016); Ashraf *et al*. (2014); Jabeen *et al*. (2013); De Alwis (1999)

Table 3. Differentiation of *Pasteurella multocida* biotypes

Species	**Sugar fermentation**				
	Trehalose	**Xylose**	**Arabinose**	**Sorbitol**	**Dulcitol**
P. multocida subsp. multocida	V	V	-	+	-
P. multocida subsp. septica	+	+	-	-	-
P. multocida subsp. galicida	-	+	V	+	+

+ = Positive; - = negative; V = Variable

Source:- Carter (2004); Weiser *et al*. (2003); Quinn *et al*. (2002)

2.1.4. Serotyping

Roberts (1947) developed a system of serological classification based on passive protection tests in mice. He used antisera prepared in rabbits to protect mice against challenge with a wide range of strains. He was able to identify four types on the basis of mouse protection, which he designated types I, II, III and IV. This was the first classification to meet some degree of acceptance. Since all HS strains fell into Roberts type I, this designation became fairly well

established. Subsequently, Hudson (1954) added a fifth serotype. The basis of these early attempts at serological classification and the types identified are shown in Table 3.

Carter used a precipitation test (Carter, 1952) and subsequently an indirect haemagglutination test (Carter, 1955) and was able to identify four serological types. These were based on agglutination of human '0' erythrocytes coated with crude extracts of outer cell components from the bacterial cultures. These crude 'capsular' extracts were supernatants prepared by heating suspensions of the bacteria at 56°C for 30 minutes and removing the cells by centrifugation. He designated these four capsular types A, B, C and D (Carter, 1952, 1955). The strains that caused HS were grouped into Carter type B. Subsequently, he found that the strains that caused HS in Africa did not fall strictly into any of these groups, though they were related to type B, and they were included in a separate group designated type E (Carter, 1961). Subsequently, however, he found that type C was not a consistent type and it was deleted (Carter, 1963).

This method of identifying serotypes has become established as the Carter indirect haemagglutination test (IHA). Three decades later, Rimier and Rhoades (1987) isolated a consistent type from turkeys which did not fit into any existing serogroups; this was designated serogroup F. Since fresh human '0' erythrocytes may not always be available in a laboratory, the IHA test has been modified by various workers for practical convenience. Carter and Rappay (1962) used formalinised human '0' cells, which could be stored in a laboratory for long periods. More recently, Sawada *et al.* (1982) used glutaraldehyde-fixed sheep erythrocytes. The test has now been modified for the detection of antibodies as well, using erythrocytes coated with cell extracts from known reference cultures. Wijewardana *et al.* (1986) used fresh sheep erythrocytes and adopted the test both for identification of serotype and for antibody detection.

Namioka and Murata (1961) described a simplified and rapid method of identifying the capsular types using a slide agglutination test in which fresh cultures are agglutinated with hyperimmune rabbit sera. Namioka and Murata (1964) and Namioka and Bruner (1963) later developed what is described as a 'somatic' typing test, based on releasing core ('somatic') bacterial components by agglutinating acid (HCl)-treated cells with rabbit antiserum. Using this method, 11 somatic types were identified. Type-specific antiserum was produced by a complicated system of absorptions, as shown in Table 2.3. Another drawback to this system is that some cultures undergo autoagglutination after the HCI treatment and therefore are rendered untypeable.

Heddleston *et al.* (1972) developed an agar gel precipitation test also for somatic typing. In this test, the antigen used was the supernatant of culture suspensions heated at 100°C for one hour.

The antiserum was prepared in chicken. Using this method, 16 different somatic types were recognised. This test was originally used to type avian strains from fowl cholera but is now extended to strains from all host species.

Currently, the most acceptable and widely used serotype designation system is a combination of Carter capsular typing and Heddleston somatic typing. Using this method, the Asian and African HS serotypes are designated B:2 and E:2, respectively (Alwis, 1999).

Since there are only two of Namioka's types (6 and 11) among the capsular type B strains, and only one (6) among the capsular type E strains, a combination of capsular and Namioka typing is also used occasionally (i.e. 6:B and 6:E for the Asian and African strains, respectively). Under this system, the avirulent Australian strain is designated 11:B. Since both systems are used in the literature, to avoid confusion, it is desirable to adhere to a standard system of expression. In the Carter-Heddleston system, the capsular type is expressed first, followed by the somatic type. In the Namioka-Carter system, expression is made in the reverse order (Sarah *et al*., 2003).

Although the serotyping systems provide a convenient method of grouping isolates from a diagnostic point of view, they are not an absolute method of classification of the species. All of the methods described above have their limitations (De Alwis, 1999). A hyaluronidase production test has been described by Carter and Chengappa (1981) for the identification of type B strains that cause hemorrhagic septicemia. These tests can be used to supplement the serotyping systems rather than as substitutes for them.

2.1.5. Cellular components

Capsule

The outer layer of the cell is believed to be responsible for the serogroup specificity. It is composed of polysaccharides, lipopolysaccharides (LPS) and a variety of proteins. Both LPS and polysaccharides are absorbed onto erythrocytes and are believed to play a role in passive haemagglutination (Sarah *et al*., 2003; Mosier, 1993). LPS, displays a high degree of anti-phagocytic activity in an in vitro phagocyte uptake assays. The capsule consists three antigen complexes - alpha, beta and gamma corresponding to a polysaccharide-protein complex, a serogroup-specific polysaccharide and LPS, respectively. Proteins are believed to be important immunogens and are likely to play a vital role in the protective mechanism (Abba *et al*., 2017). The LPS of *P. multocida* are similar to those of other gram negative bacteria. They constitute the

'endotoxins' of the organism, and are the basis of somatic typing. LPS are largely responsible for the toxicity in the HS causing serogroup B:2 and play an important role in the pathogenesis of the disease. Serological relationships exist between LPS of serogroups B and E (Mosier 1993).

Common antigens

Pasteurella multocida shares common antigens with other closely related gram negative bacteria. Antigenic relationships with Yersinia paratuberculosis, P. haemolytica, Haemophilus canis, H. influenza, Actinobacillus lignieresi, Escherichia coli and Neisseria catarrhalis have been reported (Alwis, 1999). Cross-protection has been detected in a study of 11 isolates belonging to various serotypes (Pandey, 2016).

Enzymes

Pasteurella multocida has been found to produce a number of enzymes. Neuraminidase is produced by members of serogroups A, B, D and E (Quinn *et al.*, 2002). Activity of neuraminidase of type E was inhibited by homologous antiserum only, whilst that of types B and D were inhibited by antisera against serogroups A, B, D and E. Carter and Chengappa (1980) reported that hyaluronidases produced by type B are normally associated with invasive mechanisms in the bacteria. No clear relationship has been established between the ability to produce hyaluronidase or any other enzyme and virulence.

Toxins

No correlation has been found between toxin production and somatic types. True exotoxins are not produced by strains of the B group associated with HS. Toxic effect (endotoxic shock) can however be produced by injection of culture supernatants (which contain free endotoxins) or endotoxin preparations (Quinn *et al.*, 2002).

Bacteriocins

Bacteriocins are bacteriocidal proteins produced by many species of bacteria and which are active against members of their own species or closely related species. Production of bacteriocins is believed to be determined by a genetic element. Bacteriocin activity has been demonstrated in bovine and avian strains of P. multocida (Rimier and Rhoades, 1987). Except in special investigations, their role as a diagnostic tool is of limited value.

2.1.6. Genotype analysis

With the advent of molecular biology, it has been possible to characterise isolates on a genotypic basis, providing a firmer and more stable basis than groupings based on phenotypic characteristics. Molecular techniques have now been developed both to identify bacterial types and for further strain differentiation within serotypes (Ujvari *et al*., 2015). Polymerase chain reaction (PCR) tests have been developed to detect strains of *P. multocida* associated with HS from material having low numbers of the organism. A PCR-based fingerprinting method has also been developed for identification of strains causing HS, irrespective of serotype. Ribotyping and field alternation gel electrophoresis are also techniques developed to characterise strains of *P. multocida* that cause HS. A large number of genetic fingerprint profiles have been identified by restriction endonuclease analysis (REA) among isolates of serotype B:2 that cause HS. These profiles are valuable as markers in epidemiological work (Chitarra *et al*., 2016).

2.2. Epidemiology and economic importance of haemorrhagic septicaemia

2.2.1. Geographic distribution of the disease

The global distribution of HS is related to climatic conditions, husbandry practices and the types of animals reared. Haemorrhagic septicaemia is a major disease of cattle and buffaloes occurring as catastrophic epizootics in many Asian and African countries, resulting in high mortality and morbidity. The geographical distribution of HS includes some areas of Asia, Africa, the Middle East and southern Europe. It has never been confirmed in Mexico, Central or South America (OIE, 2012; Karunasree, 2016). During 2013, haemorhagic septicaemia and other pasteurellosis outbreaks were reported to Inter African Bureau for animal resources (AU-IBAR) by 10 countries, with a total of 1,211 outbreaks, 14,122 cases and 2,530 deaths. Ethiopia recorded the highest number of outbreaks (1,063) followed by Niger (81) and Zambia (22), a pattern similar to that of the previous year. Outbreaks occurred most often in January and February (AU-IBAR, 2013). According to the major animal disease outbreaks reported to the APHRD during 2007 to 2011, HS is the most important animal disease with 3857 and 64011 outbreaks and cases respectively (APHRD, 2012). Recently, Bote *et al*. (2017) reported that the prevalence of *P. multocida* was 13 (3.39%) out of 384 samples tested in Assosa and Bambasi districts of Benishangul Gumuz Regional state of Ethiopia.

2.2.2. Seasonal effects on distribution

Haemorrhagic septicaemia is generally associated with wet, humid weather conditions. Massive outbreaks have been widely associated with seasonality. The correlation of HS with wet weather was further evident from studies on geographical distribution of the disease. Whilst dry season outbreaks appear to be contained, outbreaks occurring during wet seasons tend to spread, presumably due to the longer survival of the causative organism outside the animal. There are other wet season associated factors that promote spread of the disease. With the start of the rainy season, considerable movement of animals occurs for draught power to plough the crop fields. An outbreak of HS was also attributed to movement of new animals into the area (Mousatafa *et al*., 2017).

2.2.3. Host susceptibility

The conventional host species are cattle and buffaloes. There is general agreement that buffaloes are more susceptible than cattle. Haemorrhagic septicaemia has also been reported among camels in Ethiopia and Sudan (Mosier, 2016).

2.2.4. Morbidity, mortality and case fatality

The morbidity rate depends on environmental conditions, herd management, the animals' immunity and other factors. Although HS can be seen at any time of the year, close herding and wet conditions contribute to the spread of the disease; the worst epidemics occur during the rainy season. Stressors such as poor nutrition increase an animal's susceptibility to clinical disease and also stimulate shedding of the organism from carriers. All ages are affected where HS is not endemic and the morbidity rate can be high. In endemic regions, outbreaks often occur when healthy carriers are introduced into a herd. In these areas, most adults have some immunity to the organism and clinical cases tend to occur in young animals between the ages of 6 months and 2 years. However, massive epizootics are sometimes seen. The case fatality rate is nearly 100% unless the animal is treated very early while few animals survive once the clinical signs have become apparent (OIE, 2012). A few spontaneous recoveries may be seen, especially late in an outbreak. Up to 20% of the survivors can become carriers for a short period after an outbreak whereas by 6 months, the carrier rate diminishes to 5% or less. Buffaloes are thought to be more susceptible to illness than cattle, with higher morbidity and mortality rates.

2.2.5. Carrier status

A variable proportion of animals (between 1.9% and 44.4%) in a given population are latent carriers (De Alwis, 1999). These animals intermittently become active and shed virulent pasteurellae. The field observations and experimental findings outlined above led to the conclusion that two types of carriers exist - latent and active. It appears that in endemic areas, where frequent outbreaks occur, and most adult animals harbour Pasteurellae in their tonsils. From time to time, active multiplication takes place in the tonsils and the organism spills over into the nasopharynx and is shed in nasal secretions (Karunasree, 2016).

2.2.6. Presumptive epidemiological cycle

In an endemic area, after one outbreak of disease, a large number of surviving animals become latent carriers. They intermittently shed the organisms, the frequency diminishing with time. Since the herd immunity is also high, there are no fresh clinical cases. The first clinical case occurs when a shedder comes into contact with a susceptible animal, which will invariably be one born after the previous outbreak, or one introduced into the herd from elsewhere.

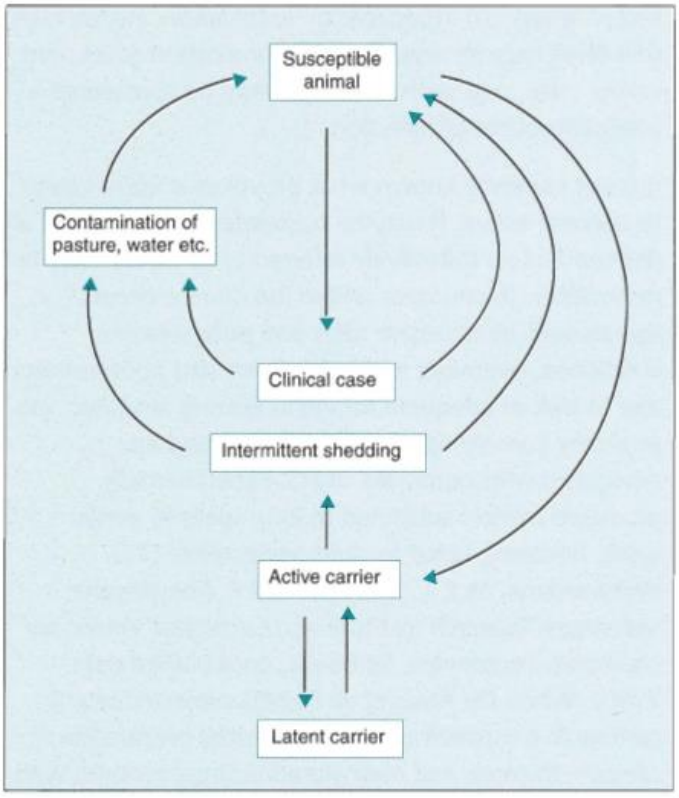

Figure 1. Presumptive epidemiological cycle for hemorrhagic septicaemia (Source:- Karunasree, 2016)

2.2.7. Economic importance

Hemorrhagic septicaemia is a disease of utmost economic importance particularly in Asia and Africa. The contribution of the cattle and buffalo to the milk production as well as draught animals in the farm fields and crop is the staple diet in many countries. Few countries have attempted to quantify the losses due to HS, and there is no uniformity in the methods adopted. The studies are not strictly comparable, but will reflect the trends. Most of the available information is derived from passive reporting systems. Most estimates of losses take into account only direct losses, i.e. value of animals that die of HS. A true estimate of losses should take into account a variety of factors which constitute indirect losses. These include loss of productivity (milk, meat, draught power, and cost of alternate sources of draught power) and impairment of the reproductive potential of the animals. It may therefore be concluded that no accurate estimates are available on the actual deaths due to HS, and the available information on direct and indirect economic losses is incomplete (Zamri-Saad, 2016).

2.2.8. Zoonotic Risk

The *P. multocida* serotypes that cause HS have not been recovered from human infections. However, because many serotypes have the potential to infect people, appropriate precautions should be taken when dealing with suspected cases of HS or HS-like disease (Mosier, 2016).

2.3. Treatment, Prevention and Control

2.3.1. Treatment

Treatment with antibiotics is of limited value unless carried out in the very early stages. Most field cases are not detected in the early stages, making treatment ineffective. In an organised farm incontact animals can be checked regularly and animals showing an increased temperature can be separated and treated with antibiotics.

The oldest therapy recommended is intravenous treatment with sulfadimidine sodium 33.33% at a dosage of 1ml/2.3kg bodyweight has been practised. However, the large volume of drug to be injected, the practical difficulties of intravenous therapy in the type of animal involved and the consequences of leakage of the drug into the surrounding tissues all weigh heavily against this treatment. Resistance to antibiotics in HS strains of *P. multocida* has not been a major problem. Serum therapy is only of theoretical interest (Benkirane and De Alwis, 2002).

2.3.2. Prevention and Control

There are three categories of measures for prevention and control of HS. These include measures to be adopted in endemic countries on a prophylactic basis, measures to be taken in the event of an outbreak and measures necessary for prevention of spread across regional or national borders. Vaccination is best done two to three months before the high-risk season so as to ensure peak immunity during the period of maximum risk. This will enable information on suspected outbreaks to reach animal health authorities as quickly as possible. In most endemic countries HS is listed as a notifiable disease. Farmers are educated to recognise signs of the disease. In endemic areas, a significant proportion of animals are latent carriers and are potential sources of infection. In non-endemic areas, animals are not regularly exposed to infection, lack naturally acquired immunity, are not usually vaccinated, and are highly susceptible. An outbreak originating from an activated carrier in such an instance can be explosive. If contact between such animals is unavoidable, it is of utmost importance that susceptible animals from non-endemic areas are vaccinated at least two weeks before contact with animals from an endemic area. All countries where HS is endemic agree that using vaccination as a control measure has reduced the occurrence of the disease (Zamri-Saad, 2016). Vaccination is recommended even in the face of an outbreak. Animals showing clinical signs are treated with a parenteral broad-spectrum antibiotic. Deep burial or effective incineration of dead animal carcasses is recommended. No country has ever attempted to eradicate HS. This reflects the belief that the existence of carriers makes eradication too hard.

2.3.3. Vaccination Programs

Planning a Vaccination Program

The quality of vaccine is of utmost importance. A highly potent vaccine with reasonable shelf life and duration of immunity should be available. It should be a sufficiently free-flowing liquid for easy injectability. Oil adjuvant vaccine based on the newer formulae satisfies these requirements to a large extent. Proper storage facilities should be available. Vaccines should be stored in a refrigerator at 4-8°C. Before use, bottles of vaccine should be placed at room temperature overnight. Good vaccination coverage is very important. It is generally believed that if a coverage of 70% or higher is achieved, the chances of an outbreak are minimised (Benkirane and De Alwis, 2002).

Post vaccinal adverse reactions

Post-vaccination shock reactions have been reported in a small proportion of animals, particularly with dense broth bacterins or the alum precipitated or aluminium hydroxide gel vaccines. The frequency of such reactions ranges from 0.1% to as high as 10%. The occurrence of reactions is irregular and therefore cannot be reproduced predictably. Shock reactions are virtually absent with the oil adjuvant vaccine. Shock reactions may occur with some batches of vaccine but not with others (Zamri-Saad, 2016).

Measuring immunity following vaccination

A variety of tests have been used in different countries to measure immunity in vaccinated animals. These include the passive mouse protection test (PMPT), indirect haemagglutination (IHA) test and antibody enzyme-linked immunosorbent assay (ELISA). The tests have been used to study the duration of immunity in vaccinated animals in controlled experiments, as well as for evaluating herd immunity under natural field conditions. In some instances, attempts have been made to correlate the results of these tests to the outcome of direct challenge. Furthermore, immunity to HS is predominantly humoral. The contributory role of cell mediated immunity (CMI), however, cannot be discounted (Zamri-Saad, 2016).

Vaccination Failures

Instances of outbreaks of disease despite vaccination are not uncommon. The cause of these failures does not necessarily reflect on the quality of the vaccine. If stringent quality control tests have been carried out and proper methods of storage and handling under tropical conditions have been observed, the 'vaccine quality' factor can be eliminated. Other factors that may cause vaccination failures include vaccination coverage, age at vaccination, health status of vaccinated animals and infection with other diseases (Zamri-Saad, 2016).

Simultaneous and Combined Vaccination

In tropical countries where these diseases exist, it is not possible to round up the animals and restrain them several times during the year for vaccination. Therefore the ability to administer more than one vaccine simultaneously is an advantage (Sarwar *et al*., 2015).

2.4. Vaccine production and quality control

2.4.1. Vaccine Production

Haemorrhagic septicaemia is preventable using vaccines containing the causative bacterial agent. However, Pasteurella is a poor immunogen and a large amount of antigen (usually whole bacteria) therefore has to be injected. This procedure occasionally leads to endotoxic shock. One way of overcoming this problem is to use a suitable adjuvant (Sarwar *et al*., 2015). Adjuvants potentiate the action of the immunogen, and provide a depot effect, delaying absorption by a slow release mechanism that simulates the administration of multiple doses. Vaccine production involves selection and maintenance of a seed culture, preparation of a bulk dense culture, inactivation of the dense culture, and formulation of the vaccine (Gowrakkal *et al*., 2014).

Selection and maintenance of seed culture

Vaccines produced from fresh field isolates are more effective than those produced from seed cultures propagated in vitro in laboratories over long periods. Bacteria grown in vivo gave cross-protection between Asian strains (B:2), African strains (E:2) and the Australian strain 989 (B:3,4), whereas laboratory subcultures gave only homologous protection. There has been some speculation as to whether strains exist in nature that have special immunogenic merit. The Burmese 'Katha' and the Indian ‘P52’ strains have been used even in countries other than that of their origin because they were believed to have special immunogenic properties. De Alwis (1999) reported that the density of growth and therefore the dry weight yield of whole bacteria, varied between strains and that no consistent immunogenic differences were demonstrable when the mice were challenged with a local isolate. It is likely that strains that are well capsulated and therefore contain a full complement of antigens give higher dry matter yields, and may therefore be better immunogenic strains for vaccine production (Gowrakkal *et al*., 2014). Considering the above information, it appears that it is best to select as the vaccine seed an isolate of known serotype designation which has been tested and proven to be immunogenic and which gives high dry weight yields. Once a relationship between dry weight and turbidity of growth is established, turbidimeter readings can be used as an index of dry weight. Once the seed culture has been selected, it is good practice to passage it in a natural host (a calf) at least once a year. (Zamri-Saad, 2016).

Preparation of a bulk dense culture

Various media have been developed in different countries to grow dense cultures. Dense suspensions of bacteria can be obtained by growing the cultures in a solid agar medium in roux flasks and harvesting the bacteria in physiological saline. This is a laborious process and before harvesting and pooling each flask has to be examined, at least visually, for contaminants. Liquid media are the most commonly used, because they are less time consuming to process. Liquid media consist of a protein as a source of amino acids (digest of casein, beef extract etc.), a fermentable sugar and growth promoters such as yeast extract or autodigest of pancreas etc., in a phosphate buffered medium. The protein content of the medium could be reduced to one-tenth of the quantity used before without significantly affecting growth. Yeast extract is highly effective in increasing the yields and the presence of buffer and the addition of sucrose increases yields leading to production of a medium that cost about one-third as much as the conventional medium previously used (Khan *et al*., 2013).

Bulk production of dense cultures using either liquid or solid media requires maintenance of an optimum temperature and pH and a steady supply of air through an aeration process. The main aeration methods used are vortex aeration and sparger aeration. Vortex aeration uses a vortex tank while in sparger aeration systems, a current of sterile (filtered) air is dispersed through the liquid medium using a filter candle (OIE, 2012).

Two common bulk culture methods have been used. One uses a simple culture system with a large vessel and the other makes use of a commercial 'fermentor' as the culture vessel and requires more specialised procedures and trained personnel. In simple culture system, a 40 litre vessel containing the medium is placed in a water bath at 37°C, thereby eliminating the need to use an incubator room. A fermenter provides a bulk culture vessel in which a constant temperature, pH and oxygen tension can be maintained throughout the growth period with churning of the medium and aeration. It is a closed system, where the medium is sterilised and cooled to 37°C in situ and where seeding can be done and samples withdrawn for testing at any stage without opening the vessel (Khan *et al*., 2013).

Inactivation of dense cultures and production of vaccines

Dense cultures produced are inactivated by the addition of 0.5% formalin (36-40% formaldehyde solution). The turbidity is standardized against a reference containing the equivalent dry

weight/volume of 1.5 g/litre. Once a dense suspension of inactivated bacteria is obtained, it is used to prepare one of the four types of vaccine used against HS (Khan *et al*., 2013; OIE 2012).

Types of hemorrhagic septicaemia vaccine

The four different types of vaccine used against HS include broth bacterins; alum precipitated vaccine; aluminium hydroxide gel vaccine; and oil adjuvant vaccines (OIE, 2012).

Bacterins are the simplest form of vaccine and consist of a suspension of whole cells. The common bacterins consist of inactivated broth cultures and are referred to as broth bacterins. Rarely, agar wash bacterins are produced by harvesting the growth from agar plates or from roux flasks. If dense, formalinised suspensions are injected, shock reactions occur in a small percentage of animals, which is presumably due to free endotoxin present in the preparation. The formalinised bacterial suspension (bacterin) should therefore be diluted so as to contain no more than 0.5 mg bacteria (dry weight) per mL and a 3ml dose may be administered. Another disadvantage is that the antibody response to plain bacterin is poor and only provides rapid immunity for about six weeks. Repeated booster doses therefore need to be given (OIE, 2012).

Alum precipitated vaccine is the most widely used vaccine in Asia and Africa. It consists of a bacterin to which potash alum has been added to give a final concentration of 1 %. If derived from aerated culture, the density of the bacterin should be adjusted to approximately 0.75mg dry weight per ml, so that a 3ml dose will contain a minimum of 2.0 mg bacteria. The disadvantages of this type of vaccine are that it only provides reliable immunity for three to four months and shock reactions can also occur. It is important to thoroughly mix the contents of the bottle before use because precipitation with alum causes the cells to settle at the bottom. If this precaution is not taken, animals receiving vaccine from the bottom of the bottle will receive a large dose of bacteria, and the chances of shock will be increased (De Alwis, 1999).

Aluminium hydroxide gel vaccine shares common properties with the alum precipitated vaccine. Aerated cultures are blended with aluminium hydroxide gel at a final concentration of 3%. Standardisation is required to ensure that a 3-mL dose contains approximately 2mg of dry bacterial substance. Although six months immunity is sometimes claimed (and twice yearly vaccination is therefore practised), reliable immunity following a single dose at primary vaccination may be similar to the protection obtained (OIE, 2012).

An oil adjuvant vaccine for HS was first developed in the 1950s (De Alwis, 1999). It consists of a water in oil emulsion, where the aqueous phase consists of a dense broth culture and the oil phase a light mineral oil. Various mineral oils have been used. The aim of oil adjuvant vaccine was initially to reduce the toxicity of the vaccine, but there is also delayed absorption and enhanced immune response. In preparing oil adjuvant vaccines, equal or nearly equal volumes of the bacterial suspension (aqueous phase) and oil need to be used. Thus, the bacterial content (i.e. antigen content) of the bacterin is halved in the emulsified vaccine. If a conveniently administered 2ml dose is to be used, the minimum requirement of 2 mg dry bacteria should be present in 1.5ml of bacterin. A bacterial density of 1.5mg/ml is therefore a reasonable target for dense culture production using aerated cultures. In order to increase the antigen content per unit volume of vaccine, it is logical to increase the proportion of the aqueous phase, but this result in a marked increase in viscosity of the emulsion. For the preparation of the emulsion, a turboemulsifier is generally used. The oil with the melted lanoline is sterilised in a sealed vessel (Afroz *et al*., 2016). The propeller of the emulsifier is inserted into the vessel and, while this mixture is thoroughly churned, the bacterin is added gradually over a few minutes. The resulting emulsion should be pure white in colour, and stick evenly to the walls of a glass vessel (Sivakumar, 2011).

Product packaging

Once the vaccine is put in recipient vial, it is sealed with sterile stoppers. All the processes described will have to comply with the standards defined for GMP that will involve several quality control tests and an adequate infrastructure and separation of activities to avoid cross-contamination. Finally, the vaccine is labelled and distributed (Lee and Nguyen, 2015; OIE, 2012).

2.4.2. Quality control tests

In process control

Proper concentration of bacterial growth, the capsulation of the bacteria, purity of culture and efficient inactivation all need to be checked during the manufacture process.

Final vaccine batch test

Test for freedom from contamination

Test for freedom from aerobic and anaerobic bacterial contamination of the final vaccine is conducted by inoculating 0.5ml of the vaccine in liquid tryptose soy broth and fluid thioglycollate medium respectively. Freedom from fungal contamination is conducted by inoculating 0.5ml of the vaccine in liquid tryptose soy broth. The inoculated media are incubated for a week at 37^0C and 25^0C for bacterial and fungal contamination test respectively, 1ml of the media sub-cultured into corresponding media and final reading is made after a week (AU-PANVAC, 2016).

Safety test

Two seronegative cattle are vaccinated with twice the recommended dose and observed for 10–14 days for adverse effects. Five mice are inoculated intramuscularly with 0.2 ml each of the vaccine, and observed for 5 days. The blood of any mouse that dies is cultured for *P. Multocida* (AU-PANVAC, 2016).

Potency test

A potency test in mice is the most feasible method. Each of 25 mice is vaccinated intramuscularly with 0.2 ml of vaccine, and again 14 days later. On day 21, the mice are divided into five groups of five, each group being challenged with respective dilutions of a 6–8-hour broth culture of a field strain in the range 10^{-4}–10^{-8}; 25 unvaccinated controls are similarly challenged, and all mice are observed for 5 days. The median lethal dose (LD_{50}) can then be calculated in order to obtain an indication of the dose that is sufficient to protect cattle. Vaccines prepared in the manner described give at least 10^4 units protection in the vaccinated mice (AU-PANVAC, 2016; OIE, 2012).

2.4.3. Vaccine shelf life

OIE (2012) reported that when stored at 4-8°C in a refrigerator, loss of potency is minimal for six months for oil adjuvanted HS vaccine. It was also indicated that increasing in the content of lanolin improves stability, but also increases the viscosity, a distinct disadvantage. Use of other emulsifying agents such as ‘Arlacel’ helps to produce thinner, stable emulsions. Sheikh *et al*. (1995) reported the significantly drastic effects of storage on the potency of the alum precipitated HS vaccine (APV). The APV vaccine, studied through challenge infection of vaccinated rabbits (post 60 days of vaccination), showed 100% potency when stored at 4 °C for 30 days. The potency dropped to 20% when storage period was extended to 60 or more days. At 30 °C, the

potency reduced by 40, 40 and 60%, respectively, after 30, 60 and 90 days of storage, while, at 37 °C, the decrease was 60, 60 and 100% after 30, 60 and 90 days of storage, respectively. On the other hand, the oil adjuvanted HS vaccine (OAV) carrying water in oil emulsion remained stable at room temperature for 6 months (OIE, 2012).

2.5. Vaccine Research and Development

2.5.1. The ideal vaccine

A considerable amount of research has focused on a search for a better vaccine. It is widely recognised that an ideal vaccine should be easy and economical to produce, stable for use, easy to administer, have no adverse reactions and have high level of immunity with a minimum delay after vaccination and lasting for at least a year. None of the vaccines developed so far satisfy all of these requirements. Attempts to find an ideal vaccine have met with varying degrees of success, but none has replaced the existing vaccines for routine prophylactic use (Jorge and Dellagostin, 2017).

2.5.2. Improved vaccines

Attempts to produce better HS vaccines can be classified into the use of other adjuvants, production of oil adjuvant vaccines of low viscosity, production of vaccines based on purified extracts of the organism and on the identification of the protective antigens, and use of live vaccines.

Adjuvants other than oil

In Africa, a type E:2 vaccine was produced using 2% sodium alginate as adjuvant (De Alwis, 1999). Comparative trials showed that this and the oil adjuvant vaccine both provided one year of immunity as indicated by survival on challenge. In Iran, a saponin-lysed vaccine was introduced in the 1930s but was subsequently replaced by a saponin-formalised vaccine. None of these vaccines have gained widespread acceptance (Lee and Nguyen, 2015).

Improved oil adjuvant vaccines

There have been several attempts to improve oil adjuvant vaccines by using new formulations to reduce viscosity to improve injectability, concentrating the antigen to permit small doses to be given, and by using double emulsification to produce a thinner, more easily injectable vaccine (Mudassar *et al*., 2014).

Vaccines incorporating purified extracts

Studies on the various cell components of *Pasteurella multocida* serotypes that cause HS have indicated that the cell capsule is associated with serogroup specificity, virulence and immunogenicity. This led to the belief that capsular extracts may be superior to whole cells as antigen in vaccines. It was believed that the protein components of the capsule were immunogenic and were responsible for protection. Two major antigenic components were recognized, the capsular antigen and the endotoxin. Rabbits immunised with purified crude capsular extract failed to produce adequate passive protective antibodies, and none of the rabbits survived subcutaneous challenge with viable organisms. However, calves immunised with this vaccine developed passive mouse protective antibodies (Joshi *et al*., 2013).

Attempts were made to separate in pure form polysaccharides, proteins and lipopolysaccharides of serotype B:2 in order to determine the most immunogenic components. It was found that capsular polysaccharide in a highly purified state was non immunogenic while lipopolysaccharide (LPS) extracts prepared by the Westphal method were protective in mice. The protectivity of LPS was abolished by treatment with phenol or digestion with proteinase. Thus, it was concluded that the protectivity of these preparations was actually due to the contaminating outer membrance proteins (OMPs). Several attempts have also been made to identify and isolate the immunogenic proteins. All of these attempts demonstrate the role of the protein fractions of the cells in providing some degree of protection (Joshi *et al*., 2013).

Studies with type B:2 strains of *P. multocida* showed that bacteria grown in iron-restricted media gave better protection than those grown in iron-replete medium. This superior protection was attributed to a high molecular weight protein fraction. The research on vaccines based on identification of immunogenic components of the cell, it can be concluded that no single component is totally responsible for immunity. All fractions including LPS, proteins and polysaccharides evidently contribute towards immunity (Zamri-Saad, 2016).

DIVA vaccines

Qureshi and Saxena (2016) reported the development of a Pasteurella bacteriophage based marker vaccine (containing an iron restricted protein and an adjuvant) and an immunoblot based DIVA assay for HS in bovines.

Live vaccines

Natural exposure to live organisms produces greater immunity than immunisation through inactivated vaccines. This has been specifically shown in HS, where the naturally acquired immunity in exposed animals that results from arrested infection has been found to be superior to vaccinial immunity (Tabatabaei *et al*., 2007). If we can find an organism that has all the properties of the field strains, including all the antigen components, but has lost its virulence, it will constitute the basis for an ideal live vaccine. Tabatabaei *et al*., 2007 reported a live-attenuated vaccine, which would mimic the early stages of the natural infection, might be expected to confer more solid and long-term protective immunity.

2.5.3. Protection between serotypes

Cross-protection between Asian and African strains was reported when bacteria grown in vivo were used to immunise mice and that this cross-protection disappeared, whilst homologous protection remained with successive subculture (De Alwis, 1999). It is unlikely that a single antigenic component, linked with a specifically identified gene is responsible for total protection. It is therefore unlikely that a DNA recombinant vaccine will appear within the foreseeable future (Shah *et al*., 2016).

2.5.4. Future prospects for vaccine development

Three interesting broad areas appear to have emerged for future vaccine research. First, newer adjuvants that have been developed for low-viscosity emulsions, including the production of double emulsions, appear to be promising (Afroz *et al*., 2016; Abdelaziz *et al*., 2015). It is important that new emulsions should be tested not only for low viscosity and stability of emulsion, but also for retention of potency under different conditions of storage. Secondly, development of better media for bulk culture production to provide culture conditions that would cause the fullest expression of antigenic components also appears to be a promising area (Khan *et al*., 2013). The third interesting area is that of live vaccine development. Studies on virulence factors and their genomic basis may help to produce suitable avirulent mutants by genetic manipulation (OIE, 2012).

3. MATERIAL AND METHODS

3.1. Study area

Production of HS vaccine candidates and stability study was conducted in the Pan African Veterinary Vaccine Centre (AU-PANVAC) laboratories while immunogenicity study was conducted in Addis Ababa University Business Enterprise Bishoftu dairy, poultry and cattle fattening farms located at Bishoftu town. AU-PANVC is the only mandated organization in Africa to provide independent quality control of veterinary vaccines. Bishoftu town is situated 47km south east of the capital city, Addis Ababa. It is found at 9°N latitude and 4°E longitudes at an altitude of 1850m above sea level in central highlands of Ethiopia (CSA, 2012).

3.2. Materials

Bacterial strain and adjuvants

Pasteurella multocida B:2 strain (Ethiopian isolate) available at AU-PANVAC was used both as a vaccine seed and challenge strain. Montanide™ ISA 61 VG readymade (Seppic, France) was used as an oil adjuvant while aluminium potassium sulphate (10%) obtained from the National Veterinary Institute (NVI), Ethiopia was used as an alum adjuvant.

Polymerase chain reaction reagents

A pair of *P. multocida* serogroup B:2 specific primers KTT72 (5'-AGG-CTC-GTT-TGG-ATT-ATG-AAG-3') and KTSP61 (5'-ATC-CGC-TAA-CAC-ACT-CTC-3') specific to *Pasteurella multocida* B:2 purchased from Eurofin and Qiagen DNeasy blood & tissue extraction kit purchased from Eurodyne were used for identity test.

Study animals

White albino mice weighing 18 to 22g were used for potency test while cross breed (Local-Holstein) calves aged between 12 and 18 months were used for immunogenicity study (appendix V).

3.3. Methodology

3.3.1. Study design

Experimental study was conducted from November, 2017 to May, 2018 to assess the stability and immunogenicity of inactivated bacterin, alum precipitated and oil adjuvanted vaccine

candidates. The study had two phases. Phase 1 focused on the production of the vaccine candidates for the study and the second phase dealt with stability and immunogenicity studies. The three vaccine candidates were produced, stored in a refrigerator (4ºC) and three vials of each candidate were randomly selected once a month to conduct stability study (potency test on mice and identity test with polymerase chain reaction) for a period of 5 months. For potency test, 100 mice were randomly divided into four experimental groups. Group 1 was assigned for inactivated bacterin, group 2 for alum precipitated vaccine, group 3 for oil adjuvanted vaccine and group 4 was left as unvaccinated controls. The median lethal dose (LD_{50}) was calculated by the Spearman-Karber formula. Shelf life of the vaccine formulations was designated as the last time at which the potency and/or identity test results remain in an acceptable threshold as described by WHO (2006). For immunogenicity study, nine calves were randomly divided into three groups, each having three calves. Group 1 was assigned for inactivated bacterin, group 2 for alum precipitated vaccine and group 3 for oil adjuvanted vaccine. All the calves were bled prior to vaccination and blood samples were collected at 28, 42 and 56 days post primary immunization. Sera were subjected to analysis by indirect hemmaglutination test to measure antibody titres in response to vaccinations. The values obtained were converted to $\log_{10}$ before analysis. Mean antibody titres were then calculated as stated by Villegas and Purchase (1989) and Burgh (1998).

3.3.2. Production of vaccine candidates

Culture preparation and inactivation

Tryptose broth and agar were prepared, supplemented with 10% horse serum and pre-incubated overnight at 37^0C to check for sterility (Appendix IV). In a class II microbiological safety cabinet, the freeze-dried *P. multocida* B: 2 vaccine seed was reconstituted with 2ml tryptose broth and streaked on tryptose agar plate using sterile disposable inoculation loop. The plate was incubated overnight at 37^0C. On the next day, a colony was then picked with a sterile loop, inoculated into three bottles containing 25ml tryptose broth and incubated at 37^0C overnight. Twenty five millilitres of the inoculum culture was inoculated into each of three bottles containing 500ml tryptose broth. The bottles were incubated at 37^0C with vortex aeration until maximum turbidity was obtained within 18 hours. A 100µl aliquot of the culture was taken from each of the bottles for optical density (OD) measurement using spectrophotometer at 600nm. Then, the growth was terminated by the addition of formalin (37%) to a final concentration of 0.5% (OIE, 2012). Sterile saline solution was then added into the inactivated mass culture to

standardize the turbidity against a reference containing the equivalent dry weight/volume of 1.5 mg/ml.

Inactivated bacterin vaccine

One litre of standardized bacterin containing the equivalent dry weight/volume of 1.5 mg/ml was used as an inactivated bacterin vaccine.

Alum precipitated vaccine

Nine hundred millilitres of diluted culture was admixed with 100ml aluminium potassium sulphate (10% stock solution) to achieve its 1% concentration. The pH was adjusted to 7.2 by adding sodium bicarbonate and the aluminised culture was incubated at 25^0C for 24 hrs.

Oil-based vaccine

Five hundred millilitres of the formalin inactivated dense culture (1.5 mg/ml) was admixed with equal volume of Montanide™ ISA 61 VG as an oil emulsion adjuvant (50/50 v/v). The mixture was blended at 3000xg at 31°C for 30 minutes.

Vaccine filling and packaging

Forty millilitres of each of the three candidates were distributed into 50ml sterile polypropylene vial. The vials were aseptically stoppered and sealed with aluminium caps. Fifty vials of each candidate were produced for the study.

Quality control

All the quality control tests including freedom from contamination in tryptose soy broth and thioglycollate medium, safety and potency in mice, identity by PCR and residual formalin content were conducted for each of the three candidates according to procedures stated in AU-PANVAC (2016) and OIE (2012). Procedures for HS vaccine quality control are detailed in appendix 1.

3.3.3. Comparative stability study

Potency test

Potency test was conducted on mice according to procedures stated in AU-PANVAC (2016) and OIE (2012). One hundred mice were randomly divided into four experimental groups. Group 1 were assigned for inactivated bacterin, group 2 for alum precipitated vaccine, group 3 for oil adjuvanted vaccine and group 4 for unvaccinated controls. Each of 25 mice in group 1, 2 and 3 were inoculated intramuscularly with 0.2ml of inactivated bacterin, alum precipitated vaccine and oil adjuvanted vaccine respectively. Group 4 was left as unvaccinated control. After 14 days, the mice were boosted with 0.2ml of each of the same vaccine formulations. Challenge strain was prepared according to procedures described in AU-PANVAC (2016) (appendix IV). On day 21, the mice of each vaccine formulation were randomly divided into five groups. Each group were challenged with respective dilutions of the challenge strain in the range of 10^{-4} - 10^{-8}. These dilutions were purposively selected to obtain 100% dying and 100% surviving mice in unvaccinated controls after challenge experiment. Twenty five unvaccinated controls were similarly challenged. The mice were observed for mortality daily for 5 days. Finally, the median lethal dose (LD_{50}) was calculated by the Spearman-Karber formula. The vaccine passed the test if the difference in protection between the vaccinated and unvaccinated controls was at least 1 fold dilution (1 Log_{10}).

Identity test

The identity of each of the vaccine candidate was tested by PCR (AU-PANVAC, 2016; OIE, 2012). A pair of *P. multocida* serogroup B:2 specific primers KTT72 and KTSP61 targeting somatic antigen were used. Extraction of total DNA from the vaccine sample was conducted by Qiagen DNeasy Mini kit according to procedures stated on DNeasy® Blood & Tissue Handbook (appendix I). Then, PCR master mix was prepared, tubes were properly closed and placed into the thermocycler and run according the AU-PANVAC (2016) program (appendix II). At the end of the PCR program, 2µl of loading buffer was loaded for 5µl of amplified DNA on 1% agarose gel. Four microlitre of DNA molecular weight marker (DNA ladder) was loaded into the first wells. The electrophoresis was then run at 100 volts for one hour. Finally, the gel documentation system was used to snap the gel. The expected amplified fragment size for HS serogroup B was 620bp. Vaccine samples showing amplified band at 620bp were considered as positive while absence of band was regarded as negative.

3.3.4. Immunogenicity study

Immunization of calves

Nine calves were randomly divided into three groups, each having three calves. Each calf of group 1, 2 and 3 was injected subcutaneously with 2ml of each of inactivated bacterin, alum precipitated and oil adjuvanted vaccines respectively. Each calf of all the three groups was boosted on day 28 post-priming with the same vaccine using the same dose and route of vaccination. Primary and booster vaccinations were conducted 2 and 30 days after production of the vaccine formulations. Blood sample (5ml) was collected from each calf of each group on 0, 28, 42 and 56 days post priming. Each of the blood samples was transported to the laboratory in ice box and was incubated at 37^{o}C for 2hr. The serum from each blood sample was separated and stored at -20^{o}C in properly labelled vials till evaluation of immune response by indirect hemmaglutination test.

Indirect hemmaglutination test

Indirect hemmaglutination test (IHA) was conducted according to procedures stated in OIE (2012) and Qureshi and Saxena (2014). A 6 to 8hr broth culture of *P. multocida* serogroup B:2 was seeded on to casein sucrose yeast (CSY) agar containing 5% blood and incubated overnight at 37°C. On the following day, the growth was harvested in 3ml physiological saline containing 0.3% formalin and incubated overnight at room temperature. The cell suspension was centrifuged at 1000xg for 15 minutes and subjected to three washings with normal saline solution (NSS). The bacterial sediment was suspended in NSS to match the opacity with tube number 3 of MacFarland opacity scale. This suspension was then heated at 56°C for 30 minutes in a water bath with frequent shaking, centrifuged at 6000xg for 15 minutes at 4°C, and the clear supernatant fluid was stored at –20°C.

Sheep blood was collected aseptically into an anticoagulant and centrifuged at 500xg for 10 minutes. The packed RBCs were washed three times in sterile physiological saline (NSS). A 10% (v/v) suspension of sheep RBCs was prepared in NSS and stored at 4°C. Equal volumes of glutaraldehyde solution (1% v/v in NSS) and 10% washed RBC suspension were mixed and allowed to stand at 4^{o}C for 30 minutes with intermittent gentle stirring. The sensitized sheep RBCs (SRBCs) were given three washes in NSS to remove free gultaraldehyde. A 10% suspension of SRBCs in NSS containing 0.1% sodium azide was prepared. The gluteraldehyde fixed SRBCs (G-SRBCs) were stored at 4^{o}C. A 10% suspension of G-SRBCs was mixed with

an equal volume of PBS containing 0.005% tannic acid (w/v), and incubated at 37ºC for 30 minutes with occasional shaking. The tanned G-SRBCs (TG-SRBCs) were pelleted and washed three times with PBS to yield a 10% suspension. One volume of packed TG-SRBCs and 15 volumes of the antigen extract, approximately were mixed and incubated for 1-2hr at 37ºC in a water bath with frequent shaking. The sensitized cells thus prepared were washed 3 times with NSS and resuspended in chilled NSS to obtain a 1% suspension. To remove the heterophile antibodies, all the test serum samples (3 volumes) were adsorbed with packed SRBCs (1 volume) for 2hr at 37ºC with periodic shaking before the test proper. The RBCs were removed by centrifugation. The supernatant was collected and used for the test. Normal saline (160µl) and adsorbed serum (40µl) were added to the first well (1:5 dilution), and 100µl of NSS was added to all the other wells of a U bottom 96 well microtitre plate (Tarson). Two fold serial dilutions of serum were made. An equal volume of the 0.5% sensitized RBC suspension was added to all the wells. The plates were agitated briefly and left at room temperature for 2hr. Positive, negative and saline controls were included in each test run. The first reading was taken after 2hr and a final reading after 18hr. A course agglutination of the RBCs along the sides of the concave wells was taken as a positive reading, and the formation of a button at the centre of the wells as negative. Titre of each test sera was considered as the reciprocal of the highest dilution giving positive result. Mean titre values of IHA antibody titres were calculated to compare the immunogenicity of the vaccine formulations.

3.3.5. Ethical clearance

The animal experimentation was approved by the Pan African Veterinary Vaccine Centre (AU-PANVAC) animal welfare and ethics committee (Appendix VI). A total of 600 mice were used for potency test while 9 calves were used for immunogenicity study. Animal experiments were carried out according to the animal ethics principles and guidelines employed elsewhere.

3.3.6. Data management and analysis

Data entry and management from both the stability study (identity and potency tests) as well immunogenicity study was conducted using Microsoft office Excel 2013. Data analysis was undertaken using STATA version 13. Descriptive statistics were utilized according to their needs. ANOVA was used to determine statistical significance between the vaccine candidates with respect to their immunogenicity. Student's *t* test was used for pair wise comparison of means. Effects were reported as statistically significant if p-value is less than 5% ($P<0.05$).

4. RESULTS

4.1. Stability results

4.1.1. Identity result

Identity test of inactivated bacterin, alum precipitated and oil adjuvanted vaccine was conducted on days 0, 30, 60, 90, 120 and 150 post production by using PCR. Hemorrhagic septicemia causing type B:2 isolates of *Pasteurella multocida* when amplified with the primer pair (KTT72/KTSP61) produced a product of approximately 620 bp (Appendix III). The *Pasteurella multocida* B:2 specific nucleic acid for inactivated bacterin, alum precipitated and oil adjuvanted vaccine was detectable until days 120, 90 and the last day of testing (day 150) respectively. However, DNA amplification was not obtained on days 120 and 150 in the alum precipitated vaccine while it was not obtained on day 150 in the inactivated bacterin group (Table 4).

Table 4. Summary of identity results for inactivated bacterin, alum precipitated and oil-based *Pasteurella multocida* vaccine

Vaccine formulation	Identity result over storage time					
	Day 0	Day 30	Day 60	Day 90	Day 120	Day 150
Inactivated bacterin	+	+	+	+	+	-
Alum precipitated vaccine	+	+	+	+	-	-
Oil adjuvanted vaccine	+	+	+	+	+	+

4.1.2. Potency result

Potency test was conducted on mice at 0, 30, 60, 90 and 150 days of storage after production of the vaccine formulations. In general, these results revealed that both the inactivated bacterin and alum precipitated vaccine had an acceptable potency result (i.e. $\geq$ 1.0 $\log_{10}$ difference with unvaccinated controls) until day 30 while that of the oil adjuvanted vaccine was acceptable until day 90 (Table 5).

Table 5. Potency results for inactivated bacterin, alum precipitated and oil based *Pasteurella multocida* vaccine

Vaccine Type	Potency result (Log_{10} difference with unvaccinated controls)					
	Day 0	Day 30	Day 60	Day 90	Day 120	Day 150
Inactivated bacterin	1.2	1.0	0.2	0	0	0
Alum precipitated vaccine	1.2	1.0	0.8	0	0	0
Oil adjuvanted vaccine	1.2	1.3	1.4	1.0	0.6	0.2

All the three vaccine formulations had 1.2 log_{10} on initial time of the experiment. The potency of alum precipitated vaccine decreased by 0.2 log_{10} every month until day 60 and dramatically dropped by 0.8 log_{10} in the third month. Similarly, the potency of inactivated bacterin dropped by 0.2 log_{10} in the first 30 days and reduced by 0.8 log_{10} in the second 30 days. However, the oil adjuvanted vaccine showed relatively stable potency until day 60 as well as 0.4 log_{10} consistent decrease in potency on days 90, 120 and 150 (Figure 2).

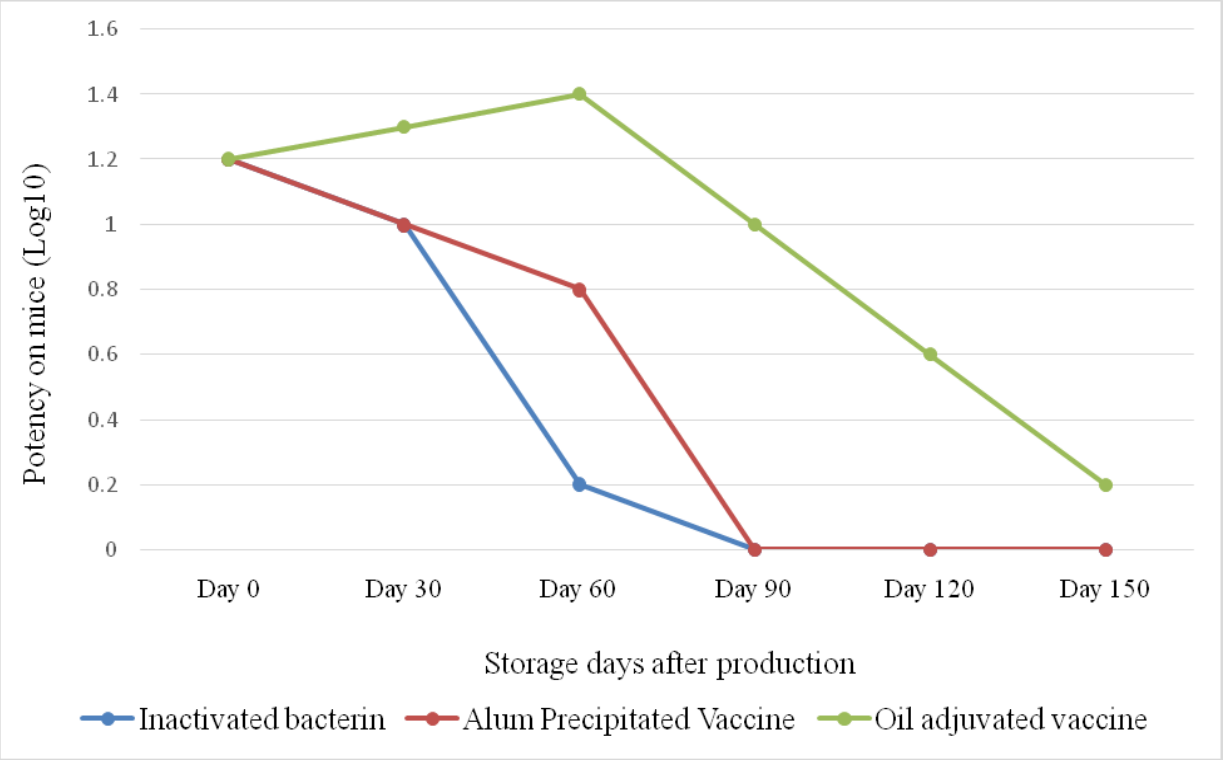

Figure 2. Potency of inactivated bacterin, alum precipitated vaccine and oil adjuvanted vaccine on mice at 0, 30, 60, 90, 120 and 150 days of storage after production

4.2. Immunogenicity result

The mean IHA titres recorded for calves in the three vaccination groups during immunogenicity study are depicted in Table 6 and Figure 3. The post vaccination antibody titres in cattle vaccinated with all HS vaccine formulations revealed an increase over the initial prevaccination stage.

In the inactivated bacterin group, there was a significant antibody level increment ($P<0.05$) from day 28 to day 42, with mean $\log_{10}$ antibody titres of 1.0 and 1.3 respectively, whereas the antibody increment was not significant from day 0 to day 28 and from day 42 to day 56. In the alum precipitated vaccine group, there was a significant increase ($P<0.05$) in titres from day 0 to days 28 and 42, with $\log_{10}$ antibody titres of 0.6, 1.9 and 2.2 respectively. Whereas the antibody response increased non-significantly from day 42 to day 56 post immunization with mean antibody titres ($\log_{10}$) of 2.4 at day 56. In the oil adjuvanted group, the antibody titres significantly increased ($P<0.05$) from day 0 to days 28, 42 and 56.

On day 0, mean antibody $\log_{10}$ titres of 0.80, 0.57 and 0.23 were recorded for calves in the inactivated bacterin, alum precipitated and oil adjuvanted groups respectively. However, the antibody level difference in the three vaccine groups on day 0 was not statistically significant ($P>0.05$). On day 28, the antibody response of 1.0 $\log_{10}$ in the inactivated bacterin was significantly lower than 1.9 $\log_{10}$ of alum precipitated vaccine and 1.6 $\log_{10}$ of oil adjuvanted vaccine ($P<0.05$). Although the antibody response of calves vaccinated with alum precipitated vaccine was higher than the oil adjuvanted vaccine on day 28, the difference was not statistically significant ($P>0.05$). On day 42, mean antibody response of 2.2 $\log_{10}$ for alum precipitated vaccine and 2.3 $\log_{10}$ for oil adjuvanted vaccine were significantly higher than 1.3 $\log_{10}$ mean antibody titre of inactivated bacterin. On day 56 post primary vaccinations, the highest mean antibody titre of 3.4 $\log_{10}$ was recorded for oil adjuvanted vaccine group followed by 2.4 $\log_{10}$ for alum precipitated and 1.6 $\log_{10}$ for inactivated bacterin. Comparison of mean antibody titres between the three vaccine formulation groups by ANOVA revealed that the antibody titres were significantly higher in oil adjuvanted group at day 56 ($P<0.05$) than the alum precipitated group, meanwhile the alum precipitated group was also significantly higher than the inactivated bacterin.

Table 6. Antibody response of calves to inactivated bacterin, alum precipitated and oil adjuvanted *Pasteurella multocida* vaccine formulations (n=9)

Vaccine formulation	Days post vaccination	Mean antibody IHA titre (Log_{10})	Standard Error	Confidence Interval (95%)
Inactivated bacterin	0	0.80^{a}	0.10	0.60 - 1.00
	28	1.00^{a}	0.17	0.65 - 1.35
	42	1.30^{b}	0.17	0.95 – 1.65
	56	1.60^{b}	0.17	1.25 – 1.95
Alum precipitated vaccine	0	0.57^{a}	0.30	-0.03 - 1.17
	28	1.90^{b}	0.17	1.55 - 2.25
	42	2.20^{c}	0.17	1.85 - 2.55
	56	2.40^{c}	0.20	1.99 – 2.81
Oil adjuvanted vaccine	0	0.23^{a}	0.23	-0.24 - 0.71
	28	1.60^{b}	0.17	1.24 – 1.95
	42	2.30^{c}	0.10	2.10 - 2.50
	56	3.40^{d}	0.17	3.05 – 3.75

Means with no common superscript differ significantly (P<0.05).

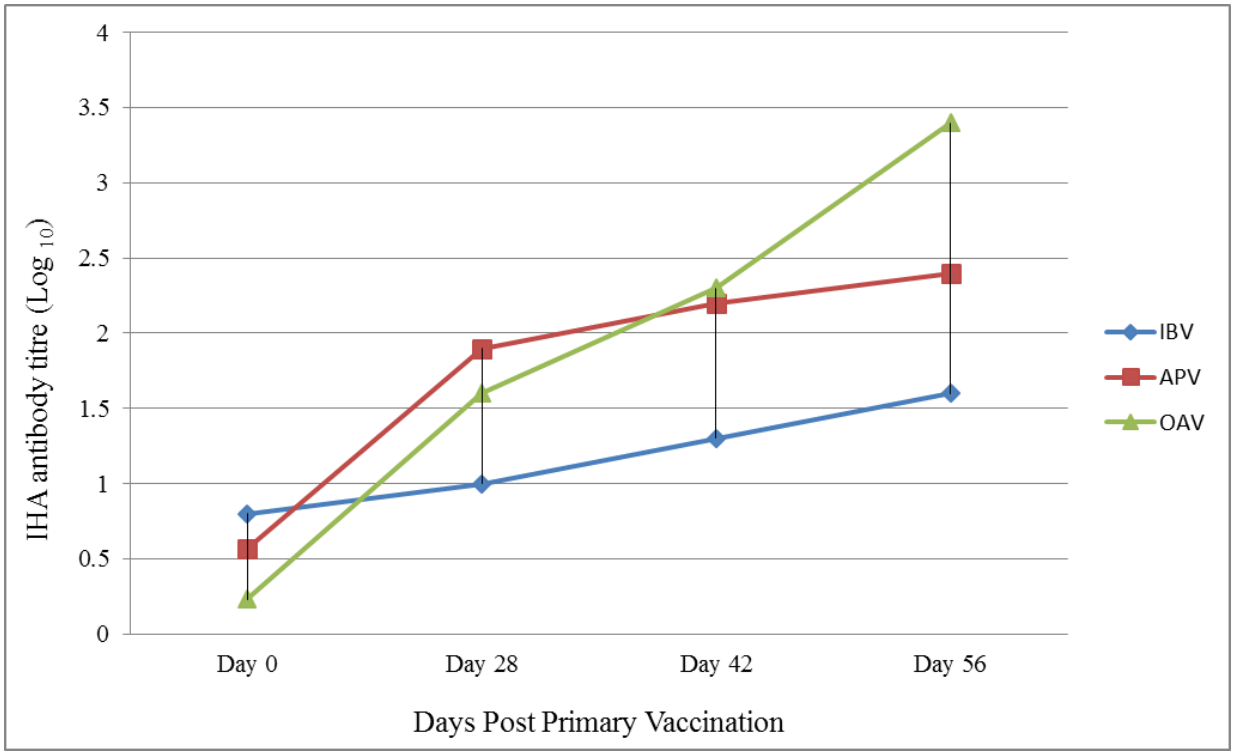

Figure 3. Mean antibody titres (Log_{10}) of calves in inactivated bacterin (IBV), alum precipitated (APV) and oil adjuvanted (OAV) groups at 0, 28, 42 and 56 days post vaccination

5. DISCUSSION

Haemorrhagic Septicaemia (HS) caused by *Pasteurella multocida* is an important disease of cattle and buffalo which causes heavy economic losses (Bote *et al*., 2017; Ujvári, *et al*., 2015). Due to sudden onset, brief duration and fatal nature of the disease, treatment in most cases is not possible and the only practical method of control and prevention is carried out by vaccination. A highly potent vaccine with reasonable shelf life and duration of immunity should be available. AbdelAziz *et al*. (2015) reported that montanide is a ready-to-use oily vaccine adjuvant based on high-grade injectable mineral oil. It produces double emulsion vaccine very fluid, stable, well tolerated and induces short and long term immune response (Seppic, 2013). In the present study, this adjuvant was compared with the conventionally used aluminium potassium sulphate adjuvant and the inactivated bacterin without adjuvant. So the findings are important to contribute valuable information in the understanding of the different formulations of hemorrhagic septicemia vaccines to be used in the control of hemorrhagic septicemia.

In this study, both identity and potency were tested as indicators of vaccine stability at day 0 and monthly for 5 months. Potency test was conducted by vaccination and challenge experiment on mice. All vaccine formulations had the same potency at day 0. The effect of duration of storage on the potency of inactivated bacterin and alum precipitated vaccine formulations in terms of mice protection indicated that the refrigeration temperature of 4°C for 30 days storage gave an acceptable protection $\log_{10}$ (i.e. 1.0 $\log_{10}$ difference with unvaccinated controls for both IBV and APV) and a decline thereafter. On day 60, a 0.2 $\log_{10}$ drop of potency was observed for alum precipitated vaccine while the drop was as high as 0.8 $\log_{10}$ for inactivated bacterin. On day 90 and thereafter, the potency dropped to 0 $\log_{10}$ for both inactivated bacterin and alum precipitated vaccine showing there was no difference between vaccinated and unvaccinated groups. This is in agreement with the study of sheikh *et al*. (1995) who reported the drop of potency of the alum precipitated vaccine from 100% on day 30 post production to 20% after challenge infection of vaccinated rabbits when stored at 4ºC for a further 30 days. The potency test of oil adjuvanted vaccine gave an acceptable result with 1.0 $\log_{10}$ difference with unvaccinated controls until 90 days of storage at 4ºC. Later, the potency of oil adjuvanted vaccine consistently dropped by 0.4 $\log_{10}$ to 0.6 $\log_{10}$ and 0.2 $\log_{10}$ on days 120 and 150 respectively. The drop in potency of vaccine formulations might be attributed to the loss in its stability and denaturation of the antigen as described by Chandrasekaran *et al*. (1987) and Vipulasiri *et al*. (1982).

In addition, identity test was conducted by using PCR and the results indicated that *Pasteurella multocida* serogroup B:2 specific nucleic acid was detected until day 90 for alum precipitated vaccine and day 120 for inactivated bacterin. Thereafter, it was not possible to detect the nucleic for both formulaions. However, the DNA was detected until the end of this study (i.e. day 150) for oil adjuvanted vaccine. The PCR results showed that both the inactivant (formalin) and the adjuvant (aluminium potassium sulphate) have an effect on the stability of the nucleic acid. This might be due to the inhibition of DNA amplification and deterioration of the nucleic acid. To the best of my knowledge, the present study for the first time revealed the effect of HS vaccine storage on the stability of *Pasteurella Multocida* serogroup B:2 nucleic acid by PCR.

The stability results showed the significantly drastic effects of storage on the potency of alum precipitated vaccine and inactivated bacterin. Shelf life of the vaccine formulations was designated as the last time at which the potency and/or identity test results remain in an acceptable threshold as stated by WHO (2006). In this study, the shelf life of both the inactivated bacterin and alum precipitated HS vaccines was found to be only 1 month. This is in agreement with the study of sheikh *et al*. (1995). On the other hand, the shelf life of oil adjuvanted formulation was revealed to be 3 months. This is not in agreement with OIE (2012) who reported that oil adjuvanted HS vaccine can be stored at 4–8°C for 6 months without any significant loss of potency. This might be due to the difference in the type of oil adjuvant used.

This study was undertaken to investigate factors possibly influencing the efficacy of the prevalent alum precipitated HS vaccine and to develop a more efficient and stable vaccine. The conventionally used alum precipitated vaccine is accepted to be stable for one year at a refrigeration temperature of 4°C (Sori *et al*., 2018), a fact which is unfortunately overlooked and is generally responsible for the low efficacy of the vaccine. This study showed that the shelf life of alum precipitated vaccine is equivalent to the time required to conduct complete quality control test. Considering the transportation and storage conditions, shelf life might even be worse at field level. This might be the cause of vaccine failure in the field.

For immunogenicity study, each group containing three calves were vaccinated and booster dose was given with the three HS vaccine formulations. Sera were collected on days 0, 28, 42 and 56 post primary vaccinations to assess the immune response. In the inactivated bacterin group, there was no strong immune response of calves to confer protection, although there was a significant antibody titre increase 2 weeks (day 42) after boosting. The peak mean antibody titre in the inactivated bacterin group was 1.6 $\log_{10}$ on day 56, which is lower than that of the international

standard requirement of 2.0 $\log_{10}$ to confer protection (Sheikh *et al*., 1995). This is in agreement with AbdelAziz *et al*. (2015), Mudasar *et al*. (2014) and Tariq *et al*. (1997), who suggested the poor immunogenic nature of Pasteurella and the need to potentiate the immune response by adding suitable adjuvant. The post vaccination antibody titres in calves vaccinated with alum precipitated HS vaccine revealed an increase over the initial prevaccination stage, with protective mean antibody response observed at 42 days post vaccination(2.2 $\log_{10}$), plateauing up to day 56 post primary vaccination (2.4 $\log_{10}$). This is in agreement with the reports of Qureshi and Saxena (2014). On the other hand, the antibody titres for oil adjuvanted vaccine significantly increased at days 28, 42 and 56 following vaccination, with peak antibody response observed at day 56 (3.4 $\log_{10}$). This is in agreement with the works of Seppic (2013).

The antibody response in alum precipitated vaccine was similar to that of oil based vaccine until day 42 post vaccination. However, a more pronounced immune response was observed on day 56 in the oil adjuvanted vaccine group. It seemed to be due to the reason that Montanide ISA VG 61 makes a water in oil emulsion, the antigen in between the oil phase is slowly released to the immune system inducing short and long term immune response as described by Mudasar *et al*. (2014). Increase in the immune response in case of oil based vaccine was gradually increasing until the end of this experiment. It seemed to be due to the absence of external aqueous phase and this would help to use oil based vaccine to induce a sustained immune response in animals. These results corresponded to the works of Sarwar *et al*. (2015) and Muneer *et al*. (2005). It was reported by OIE (2012) that the antibody response of animals to bacterin containing alum is relatively short lived providing protection for 3-4 months while oil based vaccine can provide protective immunity up to 1 year after vaccination. Since this study was only conducted until day 56 post primary post vaccination, it was not possible to assess the longer periods of immune response to the vaccine formulations.

6. CONCLUSSION AND RECOMMENDATIONS

The use of therapeutics against hemorrhagic septicaemia is not efficient due to acute nature and short duration of the disease. As a result, vaccination remains the only feasible way of controlling the disease. To obtain an effective hemorrhagic septicemia vaccine, the vaccine needs not only good antigen but also preferable adjuvant to enhance the antigen immunogenicity while retaining stability. In Ethiopia, alum precipitated hemorrhagic septicemia vaccine is the widely used and is discredited for having limited value in terms of both degree and duration of immunity as well as poor stability. The present work included comparative studies of stability and immunogenicity of inactivated bacterin, alum precipitated and oil adjuvanted hemorrhagic septicemia vaccine. This study revealed that inactivated bacterin without adjuvant was poorly stable and weakly immunogenic while alum precipitated vaccine was similarly poorly stable inducing protective immune response, but, plateauing after 42 days of vaccination. Oil adjuvanted vaccine showed better stability with gradual increase and more pronounced immune response. So it could be concluded that oil adjuvant (Mantonide ISA 61 VG, water in oil adjuvant) is a preferable adjuvant to hemorrhagic septicemia vaccine and could be used as a best alternative to the conventionally used aluminium potassium sulphate providing better stability and higher immune response.

Therefore, based on the above conclusion the following recommendations are forwarded:

- Since the inactivated bacterin is poorly immunogenic indicating the need for incorporation of suitable adjuvant and the conventionally used alum precipitated vaccine is poorly stable with less pronounced immune response, oil adjuvanted HS vaccine should be produced for the control of the disease,
- Considering the low stability of the vaccine, vaccination of HS vaccine should be carried out immediately after production,
- Further stability and immunogenicity studies should be conducted incorporating a range of different oil adjuvant preparations,
- Further experimental and field trials of the oil adjuvant vaccine should be conducted to evaluate the efficacy and safety at field level, and
- A live attenuated vaccine should be developed to elicit stronger protective immunity with better stability of the vaccine.

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8. APPENDICES

Appendix 1. Hemorrhagic septicemia vaccine quality control procedure (AU-PANVAC, 2016; OIE, 2012)

i. Sterility test

- *Material and equipment*
  - Sterile pipette
  - Sterile syringe
  - Laminar Flow
  - Incubator
  - Anaerobic jar
- *Media and reagent*
  - Fluid Thioglycollate Medium
    - L-Cystine 0.5 g
    - Sodium chloride 2.5 g
    - D-Glucose monohydrate ($C_6H_{12}0_6.H_2O$) 5.5 g
    - Agar, granulated (< 15% moisture) 0.75 g
    - Yeast extract (water-soluble) 5.0 g
    - Pancreatic digest of casein 15.0 g
    - Sodium mercaptoacetate 0.5 g
      or Mercaptoacetic acid 0.3 ml
    - Resazurin sodium solution 1.0 ml
      (1 in 1000, freshly prepared)
    - Distilled water to 1000 ml
    - Mix the L-cystine, sodium chloride, D-glucose, agar, yeast extract and pancreatic digest of casein with the water and heat until complete dissolution.
    - Add the sodium mercapto-acetate or mercapto-acetic acid and, if necessary adjust the solution with 1M sodium hydroxide to give a final pH of 7.1 ± 0.2 after sterilization.
    - Pass through a course filter while the solution is still hot.
    - Add the resazurin solution and distribute 9.5 ml per tube, which provide a ratio of surface to depth of medium is such that during subsequent incubation not more

than the upper third of the medium has undergone a colour change indicative of oxygen uptake.

- Sterilize in an autoclave at 121° C for 20 minutes.
- If more than the upper one-third has acquired a pink colour, the medium may be regenerated just before use by heating in a steam water bath until the pink colour disappears and then cooling rapidly.

- Soyabean Casein Digest Medium
  - Pancreatic digest of casein 17.0 g
  - Papain digest of soyabean 3.0 g
  - Sodium chloride 5.0 g
  - Di-potassium hydrogen phosphate 2.5 g
  - D-Glucose monohydrate ($C_6H_{12}0_6.H_2O$) 2.5 g
  - Distilled water 1000 ml
  - Dissolve the solids in the water, warming slightly to effect solution.
  - Cool the solution to room temperature, and, if necessary, adjust with 1M sodium hydroxide in order to obtain a pH of 7.3 ± 0.2 after sterilization.
  - Filter, if necessary, to clarify and dispense into suitable vessels.
  - Sterilize by heating in an autoclave at 121°C for 15 to 20 minutes.

- *Procedure*
  - Clean the exterior of ampoules and closures of vials and bottles with a suitable decontaminating agent, and gain access to the contents in an aseptic manner.
  - Reconstitute the vaccine with 2 ml sterile distilled water per vial.
  - Inoculate 0.5 ml of reconstituted vaccine into each tube of a series of six tubes of SBCDM and three tubes of thioglycollate medium each containing 9.5 ml of medium (i.e the whole content of reconstituted vaccine vial must be tested so as to give a ratio of vaccine suspension to test medium of between 1:10 and 1:20).
  - Inoculate each of 2 tubes of SBCDM and 1 tube of thioglycollate medium with 1 ml of the fluid used for reconstitution (these are the diluent controls).
  - For each test two tubes of SBCDM and 1 tube Thioglycollate medium are included as uninoculated medium controls.
  - Incubate all the thioglycollate and one half of the SBCDM test media tubes for 7 days at 35°C to 37°C in the test intended to detect bacteria and the other half of SBCDM media tubes at 20 to 25°C (room temperature) in the test intended to detect fungi.

- After 7 days following incubation subinoculate 1 ml from each tube of the previously inoculated media into a corresponding series of fresh media. These are then incubated as earlier described for a further minimum period of 7 days.

➢ *Interpretation of Results*

- The vaccine passes the test for sterility if no growth of microorganisms is observed in any of the broths/media during the incubation period and at its conclusion.
- The test is valid if no growth is observed in any of the controls, i.e. un-inoculated media (medium control), medium inoculated with the fluid used for reconstituting the vaccine. If growth occurs in either control, the test should be repeated.
- The vaccine fails the test for sterility if the test is valid and growth is observed in the media inoculated with the vaccine under test.

ii. Safety test

a. Safety test on laboratory animals

➢ Two seronegative cattle are vaccinated with twice the recommended dose and observed for 10–14 days for adverse effects.

➢ Five mice are inoculated intramuscularly with 0.2 ml each of the vaccine, and observed for 5 days. The blood of any mouse that dies is cultured for P. multocida.

b. Residual formalin content

➢ *Material& Reagents*

- 15 ml Tubes
- 10 ml graduatedpipettes
- 1000 µl Tips
- -Test tube rack
- Reagent A: 2.5% HCl (v/v)
- Reagent B: 0.2% Formalin solution,(v/v)
- Reagent C: Indicator: To 0.1 g basic fuchsine add 90 ml of distilled water and 1g of Na3SO3 and 1 ml of HCl
- Vaccine: filtered to remove eventual colorants

➢ *Procedure:*

- For each sample:
  - Add 10 ml of reagent A to tube labeled a.
  - Add 11 ml of reagent B to another tube labeled b.

- 2. Add 0.05 ml of well homogenized vaccine sample to each of the tube «**a**»and«**b**».
- 3. Prepare a set of reference for formalin concentration as follows:

Tube No	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Reagent A	10	10	10	10	10	10	10
Reagent B	0.15	0.20	0.225	0.250	0.275	0.3	0.35

- If the vaccine contains a colorant i.e. gentian violet it is necessary to filter it before the test. Then wait 1 hour to allow discoloration by HCl before proceeding with the next step
- Add 1 ml of reagent C to all tubes labeled a. The tube labeled b will be used as opacity control.
- Mix the content of tubes and leave them until a good color is developed: 15 to 30 minutes.
- Shake the tubes to have a good bacterial suspension. Compare each group of tube with that of the formalin reference.

➢ *Calculation of the formalin content*

- The calculation of formalin content is done by comparing the colors in tubes «**a**» with those of tubes containing the measured quantity of formalin using the table below.

Tube No	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Formalin % per 0.05 ml	0.6	0.8	0.9	1.0	1.1	1.2	1.4
Formalin % per 0.1 ml	0.3	0.4	0.45	0.5	0.55	0.6	0.7

- E.g. If the color in tube «**a**»corresponds to that of the reference 4, the vaccine sample has a formalin content of:
  - 1% if there was 0.05 ml per tube
  - 0.5 % if there was 0.1 ml per tube.

iii. Potency test

➢ Each of 25 mice is vaccinated intramuscularly with 0.2 ml of vaccine, and again 14 days later.

➢ On day 21, the mice are divided into five groups of five, each group being challenged with respective dilutions of a 6–8-hour broth culture of a field strain in the range of 10^{-4} to 10^{-8}; 25 unvaccinated controls are similarly challenged, and all mice are observed for 5 days.

- The median lethal dose (LD_{50}) can then be calculated in order to obtain an indication of the dose that is sufficient to protect cattle: vaccines prepared in the manner described give at least 10^4 units protection in the vaccinated mice.

iv. Identity test

- Extraction of DNA is conducted according to Appendix 2.
- Classical PCR and gel electrophoresis is conducted according to Appendix 3.
- The presence of amplicon on the position of 620bp indicates positive result while absence of amplicon is interpreted as a negative result.

v. Stability test (Emulsion stability by centrifugation)

- The vaccine is vigorously mixed
- 40mls is poured into a 50ml centrifuge tubes
- Centrifugation is done at 4000 RPM for 1hr (1000g for 30 minutes)
- Separation should be less than 5%, ideally 1% for good stability

Appendix 2. Extraction/purification of total DNA from vaccine sample Qiagen DNeasy Mini Kit

MATERIALS REQUIRED			
Biosafety cabinet		Micro-Pipettes: 10 - 100 µl; 100 - 300µl; 100 - 1000µl	
DNA Extraction Kit (RNeasy)		Filter Tips: 10 - 100 µl; 100 - 300µl; 100 - 1000µl	
Centrifuge		Vaccine Sample	
Vortex		Normal saline	
Micro-centrifuge tube 1.5ml or 2 ml			
	PROCEDURE		
S/N	**STEPS**	**PROCEDURE**	**ENSURE TO**
1	**Preparation of sample**	Reconstitute freeze dried vaccine with 2ml of Normal saline	Or collect liquid inactivated vaccine ready to use for extraction
2	**Lysis step**	To 180µl **Buffer ATL** in 1.5/2 ml micro-centrifuge tube, Add 20µl **proteinase K**, and 100µl of vaccine sample.	Mix thoroughly by vortexing, and incubate at **56°C for 1 hour** with occasional sample agitation, in a thermomixer, shaking water bath, or on a rocking platform
		Add 200µl **Buffer AL** to the mixture,	Mix thoroughly by vortexing
3	**Binding Preparation**	Then add 200µl ethanol (96–100%), and mix again thoroughly by vortexing.	It is essential that the sample, Buffer AL, and ethanol are mixed immediately and thoroughly by vortexing or pipetting to yield a homogeneous solution.
4	**Transfer to the columns and binding to the membrane**	Pipette 700µl of that mixture (including any precipitate) into the DNeasy Mini spin column placed in a 2 ml collection tube (provided).	Centrifuge at 6000 x g (**8000 rpm**) **for 1 min**. Discard flow-through and the collection tube
5	**AW1 washing**	Place the DNeasy Mini spin	Centrifuge for **1 min** at 6000 x g

		column in a new 2 ml collection tube (provided), add 500 µl of **Buffer AW1**	(**8000 rpm**). Discard flow-through and the collection tube
6	**AW2 washing**	Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), add 500 µl**Buffer AW2**,	Centrifuge for **3 min** at 20,000 x g (**14,000 rpm**) to dry the DNeasy membrane. Discard flow-through and the collection tube
7	**Elution**	Place the DNeasy Mini spin column in a clean 1.5 ml or 2 ml microcentrifuge tube, and pipet 100 µl**Buffer AE** directly into the DNeasy membrane.	Incubate at room temperature for 1 min, then centrifuge for **1 min** at _6000 x g (**8000 rpm**) to elute DNA.
8	**Storage**	Close the tubes, identify and store DNA on ice if to be used immediately, or at -20°C +/-5°C or -70°C +/-10°C.	

Appendix 3. Hemerrhagic septicemia (Sero group B) identity by classical PCR

1. **Vaccine Type: Haemorrhagic Septicaemia (*P. multocida*sero group E)**
   - Type of test
     - Conventional PCR
   - Primers
     - **KTT72:** 5'-AGG-CTC-GTT-TGG-ATT-ATG-AAG-3'
     - **KTSP61**: 5'-ATC-CGC-TAA-CAC-ACT-CTC-3'
   - Template
     - DNA
2. **PCR Master Mix preparation for 25µl reactions**

   - In 1.5 ml micro centrifuge tube prepare the master mix according to the table below

Reagents	**25 µl mix for 1 reaction**	**25 µl mix for n reactions**	**Final Concentration**
Water	10 µl		-
Taq PCR buffer 10X	2,5 µl		1 X
dNTPs mix 2mM each	2,5 µl		200 µM
KTT72 5µM	2,5 µl		0,5 µM
KTSP61 5µM	2,5 µl		0,5 µM
Taq polymerase 5U/µl	0,125 µl		0.625U/25 µl
Volume without DNA	20 µl		
Template DNA	5 µl		
Volume final	25 µl		

NB: Mix thoroughly by vortexing and distribute 20µL of mix into each PCR tube. Add 5µl of extract template DNA to the corresponding tube, Close properly the tubes and place into the thermocycler and run according the following program.

3. **PCR Thermal Cycle**
   a. Initial denaturing step 95^{o}C – 5 min
   b. followed by 40 cycles (Denaturation step 95^{o}C – 1min, Primers annealing step 55^{o}C – 1min , Extension step 72^{o}C – 1min)
   c. Final extension step 72^{o}C – 5 min

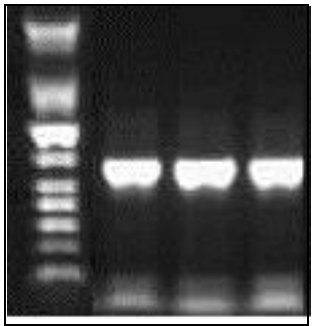
4. Electrophoresis

- At the end of the PCR program, load2µl of loading buffer for 5µl of amplified DNA on 1% agarose gel (1g of agarose in 100 ml of TBE 1X + 4 µl of Gel red)
- Load 4 µl of DNA molecular weight marker (DNA ladder) in to the first and the last wells
- Run the electrophoresis at 100 volts for 1hour
- Amplified fragment size – **620bp**

5. Gel Capture

- Use the Gel documentation system to snap the gel.

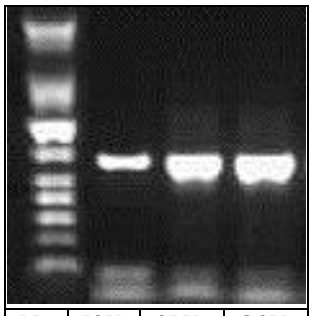
Appendix 4. Results of identity test by classical PCR



M W	IAV	APV	OAV

Conventional PCR for HS (Sero group B sp)

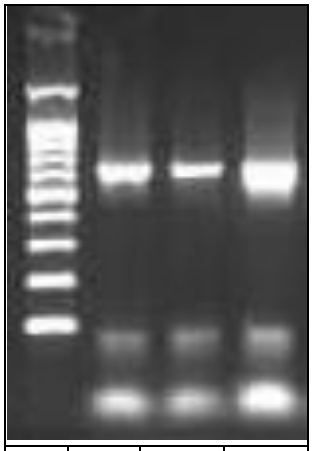
Gel picture showing the bands for inactivated bacterin (IBV), alum precipitated vaccine (APV) and oil adjuvanted vaccine (OAV) at day 0 post production with 100bp marker on the left



M W	IAV	APV	OAV

Conventional PCR for HS (Sero group B sp)

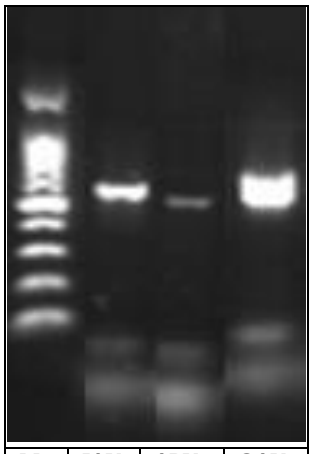
Gel picture showing the bands for inactivated bacterin (IBV), alum precipitated vaccine (APV) and oil adjuvanted vaccine (OAV) at day 30 post production with 100bp marker on the left



M W	IAV	APV	OAV

Conventional PCR for HS (Sero group B sp)

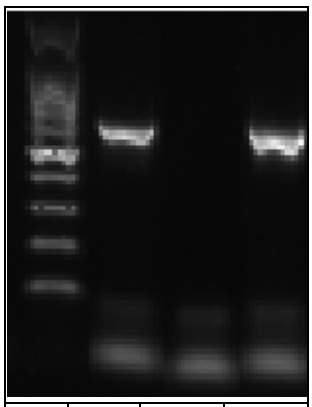
Gel picture showing the bands for inactivated bacterin (IBV), alum precipitated vaccine (APV) and oil adjuvanted vaccine (OAV) at day 60 post production with 100bp marker on the left



M W	IAV	APV	OAV

Conventional PCR for HS (Sero group B sp)

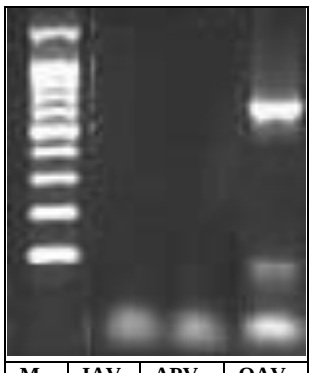
Gel picture showing the bands for inactivated bacterin (IBV), alum precipitated vaccine (APV) and oil adjuvanted vaccine (OAV) at day 90 post production with 100bp marker on the left



M W	IAV	APV	OAV

Conventional PCR for HS (Sero group B sp)

Gel picture showing the bands for inactivated bacterin (IBV), alum precipitated vaccine (APV) and oil adjuvanted vaccine (OAV) at day 120 post production with 100bp marker on



M W	IAV	APV	OAV

Conventional PCR for HS (Sero group B sp)

Gel picture showing the bands for inactivated bacterin (IBV), alum precipitated vaccine (APV) and oil adjuvanted vaccine (OAV) at day 150 post production with 100bp marker on

Appendix 5. Preparation of media and challenge strain for potency test

Day 1

1 Prepare 2L of tryptose broth
2 Dispensed into 6 x 270ml, 1 x 235ml and 2 x 90ml
3 Add 1.3g bacteriological agar to each 90ml bottle
4 Autoclave all bottles 121^{o}C for 15 minutes
5 Add complement inactivated thawed horse serum to the solid and liquid media, final concentration of 10%
6 Dispense 9ml into 22 tubes and 3ml into 6 tubes
7 Pour the agar into 5 petri-dishes
8 Inoculate Multocida B into 2 agar plates and 2 tubes (3mL). Incubate at 37^{o}C overnight.
9 Put the rest of media at 37^{o}C for sterility

Day 2

Pick a colony and inoculate into 3ml media.

Leave at room temperature overnight

Day 3

1. Take un-inoculated media samples for zeroing spectrophotometer (from 300ml broth)
2. Pipette 2mls of inoculum from overnight culture into 300ml broth
3. Incubate at 37^{0}C with shaking/stirring for 6-8hrs
4. Take an aliquot for OD measurement. Use when culture OD is 0.35 at 600nm (1 cm light path) or use microplate OD= 0.15 at 620nm (100µl per well)
5. Make serial dilution 10^{-1} – 10^{-11} on ice. These to be made in duplicate
6. Immediately take one set of tubes for inoculation, and incubate the other set at 37^{o}C overnight.

NB: The second set of tubes incubated overnight is to assess the end point for the titration.

Appendix 6. Animals used for the study

Mice used for potency test

Calves used for immunogenicity study

Appendix 7. Animal Use Ethical Clearance Certificate

Union Africaine
Centre Pan Africain des Vaccins Veterinaires
OIE Collaborating Center for Quality Control of Veterinary Vaccines
OIE AU-PANVAC
FAO Reference Centre for Technical Assistance in Quality Control of Veterinary Vaccines
African Union
Pan African Veterinary Vaccine Centre
P.O Box. 1746 Debre-Zeit, Ethiopia
Email: aupanvac@africa-union.org

Pan African Veterinary Vaccine Centre (AU-PANVAC) Animal Welfare and Ethics Committee

ANIMAL USE ETHICAL CLEARANCE

Chairman
Dr Nick Nwankpa
Director, AU-PANVAC

Secretary
Dr Charles Bodjo
Senior animal disease diagnostic reagents officer, AU-PANVAC

Scientific members
Dr Gelagay Ayelet,
Ethel Chitsungo,
Rahamatou Boukuri Cisse,
Buziki Jean De Dieu,
Hassen Belay,
Yehchaye Degefa,
Adama Diakite, and
Dr Paul Ankeli
AU-PANVAC

This is to certify that the under mentioned project proposal has been cleared and approved by AU-PANVAC Animal Welfare and Ethics Committee, at its meeting held on October 10, 2017 at AU-PANVAC, Debre Zeit, Ethiopia.

Project Title: A Comparative study of the stability and immunogenicity of inactivated bacterin, alum precipitated and oil adjuvated hemorrhagic septicemia vaccine.

Name of the investigator: Hassen Belay

Project Supervisor: Dr Nick Nwankpa

Species and No. of animals used: 600 Mice and 9 Calves

Name of the institution: AU-PANVAC

Dr Nick Nwankpa
Director, AU-PANVAC