Molecular Characterization of *Pasteurella multocida* Vaccine Strains

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A dissertation submitted to the University of Khartoum in partial fulfillment of the requirements for the degree of M. Sc. in Microbiology

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June, 2010
Dedication

To my mother

Father

Brother, sister and friends

With great love
Acknowledgments

First and foremost, I would like to thank my Merciful Allah, the most beneficent for giving me strength and health to accomplish this work. Then I would like to deeply thank my supervisor Dr. Awad A. Ibrahim for his advice, continuous encouragement and patience throughout the period of this work.

My gratitude is also extended to prof. Mawia M. Mukhtar and for Dr. Manal Gamal El-dein, Institute of Endemic Disease.

My thanks extend to members of Department of Microbiology Faculty of Veterinary Medicine for unlimited assistant and for staff of Central Laboratory Soba.

I am grateful to my family for their continuous support and standing beside me all times.

My thanks also extended to all whom I didn’t mention by name and to the forbearance of my friends, and colleagues who helped me.

Finally I am indebted to all those who helped me so much to make this work a success.
Abstract

The present study was carried out to study the national haemorrhagic septicaemia vaccine strains at their molecular level. The vaccine is bivalent contain *Pasteurella multocida* serotype E:2 and B:2. The vaccine strains were obtained from Central Veterinary Research Laboratory, Department of Biological Product, Soba. Culture characteristic and colonial morphology was ascertained in common laboratory media. The bacterial strains were characterized by using sodium-dodecyl sulphate polyacrylamide gel electrophoresis SDS-PAGE, western blotting and by using PCR for capsular serotyping. Firstly, the two strains were characterized by SDS-PAGE technique. The bacteria were cultured in liquid media, and then the bacterial whole cell lysates were prepared. SDS-PAGE was carried out for both strains and the proteins bands were stained with coomassie brilliant blue. Then the molecular weights of the proteins bands were determined; they were 175 kDa, 165 kDa, 150 kDa, 123 kDa, 102kDa, 90 kDa, 85 kDa, 70 kDa, 64 kDa, 60 kDa, 51 kDa, 42 kDa, 37 kDa, 22 kDa and16 kDa. The protein profiles of both vaccine strains were similar.

*P. multocida* strains protein profiles were investigated by immunoblotting using specific hyperimmune sera prepared in rabbits. Immunostaining with enzyme conjugate was done after transfer of proteins in nitrocellulose acetate paper. Eight proteins in whole cell lysate, of approximately 175 kDa, 102 kDa, 90 kDa, 85 kDa, 70 kDa, 42kDa, 37 kDa, and 16 kDa, were recognized by the sera.
The PCR assay was performed for strain B and E of *P. multocida* by using primers that amplify capsule gene. Two extraction methods for DNA were used. There were the Boiling method and the kits method in which a lysis buffer is used. The two methods of DNA extraction gave good DNA yield. However the kit method was better in this respect than the boiling method. Primers of strain E gave an amplification product of 511 bp for vaccine strain E. In contrast primers of strain B did not amplify strain B DNA vaccine strain but amplified strain B field strain which were obtained from Department of Microbiology Faculty of Veterinary Medicine, University of Khartoum stock culture and the amplicon was 760 bp.
لا يمكنني قراءة النص العربي المكتوب بشكل صحيح من الصورة المقدمة. إذا كنت بحاجة إلى مساعدة في شيء آخر，请告诉我！
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<tr>
<td>AGID</td>
<td>Agar gel immuno diffusion test</td>
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<td>APV</td>
<td>Alum-precipitated vaccine</td>
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<td>APS</td>
<td>Ammonium persulphate</td>
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<tr>
<td>BA</td>
<td>Blood agar</td>
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<tr>
<td>BHI</td>
<td>Brain heart infusin</td>
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<tr>
<td>CSY</td>
<td>Casein-sucrose-yeast</td>
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<tr>
<td>CVRL</td>
<td>Central Veterinary Research Laboratories</td>
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<tr>
<td>CIEP</td>
<td>Counter immuno electrophoresis test</td>
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<tr>
<td>dNTP</td>
<td>Deoxynucleotide triphosphate</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribose nucleic acid</td>
</tr>
<tr>
<td>D.W</td>
<td>Distilled water</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immuno sorbent assay</td>
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<tr>
<td>HS</td>
<td>Haemorrahgic septicemia</td>
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<tr>
<td>IHT</td>
<td>Indirect haemagglutination test</td>
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<tr>
<td>IROMPs</td>
<td>Iron-regulated outer membrane proteins</td>
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<tr>
<td>KDa</td>
<td>Kilo dalton</td>
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<td>LPS</td>
<td>Lipopolysaccharide</td>
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<td>MCA</td>
<td>Maconkey agar</td>
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<tr>
<td>MW</td>
<td>Molecular weight</td>
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<tr>
<td>OAV</td>
<td>Oil-adjuvated vaccine</td>
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<tr>
<td>OMPs</td>
<td>Outer membrane proteins</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>RIP</td>
<td>Radioimmunoprecipitation</td>
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<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
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<tr>
<td>SCE</td>
<td>Sonicated cell extract</td>
</tr>
<tr>
<td>S.C</td>
<td>Subcutaneously</td>
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<tr>
<td>TEMED</td>
<td>Tetramethylenediamine</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris boric EDTA</td>
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<tr>
<td>WCL</td>
<td>Whole cell lysate</td>
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Introduction

Haemorrhagic septicaemia (HS) is a major disease of cattle and buffaloes characterized by an acute, highly fatal septicaemia with high morbidity and mortality (Bain et al., 1982; Carter and De Alwis, 1989; Mustafa et al., 1978). The disease has been recorded in wild mammals in several Asian and European countries (Carigan et al., 1991). Outbreaks mostly occur during change in the climatic conditions, as high humidity and high temperatures.

The disease is caused by *P. multocida*, a Gram-negative coccobacillus residing mostly as commensal bacteria in the upper respiratory tract of animals. The Asian serotype B:2 and the African serotype E:2 (Carter and Heddleston system) (Carter, 1955; Heddleston et al., 1972) corresponding to 6:B and 6:E (Namioka-Carter system) (Namioka and Bruner, 1963), are mainly responsible for the disease.

Haemorrhagic septicaemia occurs in Africa, Asia, Central, South America and Europe. The disease was also reported in Sudan and serotypes E and B were isolated (Shigidi and Mustafa, 1979). The clinical manifestations of the typical disease caused by B:2 or E:2 strains include a rise in temperature, respiratory distress with nasal discharge, and frothing from the mouth, followed by recumbency and death. In the recent past, HS has been identified as a secondary complication in cattle and buffalos following outbreaks of foot and mouth disease (FMD) (De Alwis, 1992; Carter and De Alwis, 1989).
Vaccination is considered an effective mean of controlling this disease. Local isolates are usually used for vaccine preparation. In haemorrhagic septicaemia, capsular antigen, LPS or LPS-protein complex, and outer membrane proteins, including the iron-regulated outer membrane proteins are effective immunogens for serogroups B and E (Carter and De Alwis, 1989). However, no published report is written for immunogenic proteins of *P. multocida* that cause haemorrhagic septicaemia in Sudan. Vaccinations with inactivated whole-cell preparations possess the problem that they do not provide long-lasting immunity (Verma and Jaiswal, 1998; De Alwis, 1999). The key antigens of *P. multocida* B:2 that evoke protective immunity to HS in cattle have been defined and *P. multocida* B:2 outer-membrane proteins (OMPs) have been implicated as protective antigens (Vasfri and Mittal, 1997; Srivastava, 1998). For other serotypes of *P. multocida*, OMPs are recognized as important immunogens. Several OMPs are immunogens and the antibodies produced against these OMPs demonstrate a strong protective action, such antigens may be used as a component of subunit vaccines (Rimier, 2001; Gatto *et al.*, 2002; Prado *et al.*, 2005).

*P. multocida* is characterized serologically by identification of capsular antigens by using passive haemagglutination test and by detection somatic antigens using gel diffusion tests. Serotyping of *P. multocida* is currently only undertaken by regional reference laboratories (Carter, 1955). Five serogroups A, B, D, E and F are currently distinguished in the Carter system. A limitation of the capsule typing is the difficulty in inducing antibodies to specific antigens. Most workers found it relatively easy to induce antibodies
against B and E serogroup specific antigens, but not the other serogroup specific antigens. In many instances a non-encapsulated strain is found to be unserotypeable (Rimier and Rhoades, 1989). A multiplex Polymerase Chain Reaction was introduced as a rapid alternative to capsular serotyping system (Townsend et al., 2001). Using this technique, however only the capsular serotyping information could be ascertained; this are helping in serotyping instead of the conventional method of serotyping.

**Objective of the study:**
- To determine the protein profiles of *Pasteurella multocida* by SDS PAGE.
- To detect immunogenic proteins of *Pasteurella multocida* strains used in vaccine production by immunoblotting.
- To determine the efficacy of PCR using capsule gene primers in compared with serotyping in the study of *P. multocida*. 
1.1 Haemorrhagic septicaemia

Haemorrhagic septicemia is an acute, highly fatal septicaemic disease of cattle and buffaloes, characterized by fatal septicaemia with high morbidity and mortality. The symptoms progress rapidly from dullness and fever to death within hours and recovery is rare (Carter, 1967).

1.2 Etiology

Haemorrhagic septicaemia is caused by specific serotypes within the bacterial species of *P. multocida*; more frequently by two specific serotypes of *P. multocida*, serotype B:2 and E:2 in Asia and Africa, respectively (Carter, 1955; Heddleston *et al.*, 1972). These serotypes are corresponding to the newer 6:B and 6:E classification i.e Namioka-Carter system (Namioka and Bruner, 1963). In this respect a few countries such as Egypt and Sudan, have recorded both serotypes (Farid *et al.*, 1980; Shigidi and Mustafà, 1979).

*P. multocida* is a Gram-negative bacterial pathogen which is the causative agent of a range of diseases in animals, including fowl cholera in avian species (Carter, 1972), haemorrhagic septicaemia in ungulates (De Alwis, 1984), atrophic rhinitis in swine (White *et al.*, 1993), and snuffles in Rabbit (Manning, 1984). This bacterium also causes infection in humans; primarily through dog and cat bites (Talan, 1999).
1.3 Taxonomy

The classification of *Pasteurella multocida* is:

- **Kingdom:** Bacteria.
- **Phylum:** Proteobacteria.
- **Class:** GammaProteobacteria.
- **Order:** Pasteurellales.
- **Family:** Pasteurellaceae.

The family Pasteurellaceae contains genera Actinobacillus, Haemophilus, Lonepinella, Mannheimia and Phocoenobacter. This taxonomy is based on outer membrane proteins (OMPs) for iron acquisitions have roles in infection and pathogenesis. Characterization of cell surface proteins of members of the Pasteurellaceae family including Haemophilus, Actinobacillus, Pasteurella, and the Mannheimia genera of organisms has highlighted several redundant iron acquisition receptors for transferrin, siderophores, and heme/heme-containing protein (Chung *et al*., 2008). More taxanomy was done on the basis of the 16r RNA sequencing and phylogentic analysis (Dewhirst *et al*., 1992).

1.4 Morphological, biochemical and cultural characteristic

*P. multocida* is a small, Gram negative coccobacillary rod, with bipolar staining characteristics from tissues, nonmotile, non-spore forming and non-haemolytic, aerobic to facultative anaerobic and produce indole and ferment carbohydrates with slight gas production (Quinn *et al*., 1994).

1.5 Phenotypic and genotypic characterization

Phenotypic characterization is based on the bacterium biochemical reaction where, dulcitol and sorbitol fermentation method is argued. There were significant variations in the phenotypic properties of *P.*
They have been reported (Heddleston, 1976). Mutters, et al. (1985) reclassified genotypically as members of the genus Pasteurella on the basis of DNA-DNA hybridization studies. Three clusters of *P. multocida* showing 84 to 100, 91 to 100, and 89 to 100% DNA reassociation between strains. There were subsequently classified as *P. multocida* subsp. *multocida*, *P. multocida* subsp. *gallicida*, and *P. multocida* subsp. *septica*. Representatives of the existing capsular types were found to be closely related on the basis of DNA-DNA hybridization (Pohl, 1981), despite the diversity of disease manifestations and hosts. Further, the study of Kuhnert, et al (2000) also showed that variant phenotypes of *P. multocida* shared at least 98.5% 16S rRNA sequence similarity with the recognized subspecies of this species.

### 1.6 Epidemiology

Haemorrhagic septicaemia (HS) is an endemic disease in most countries of Asia and sub-Saharan Africa. Within the Asian Region, countries can be classified into three categories, on the basis of incidence and distribution of the disease. These are respectively countries where the disease is endemic or sporadic, clinically suspected but not confirmed, or free. Economic losses due to HS are confined to losses in animal industry. Only a few attempts have been made to estimate economic losses (Dutta et al., 1990).

Organism causing HS does not survive outside the animal body to any significant degree to be a source of infection. Moist conditions prolong its survival. Thus the disease tends to spread more during the wet season. Also movements of animals, work stress in work animals, levels of low nutrition etc. all of which favour the precipitation of outbreaks (Benkirane and De Alwis, 2002).
1.6.1 Transmission
Infection occurs by inhalation or ingestion of *P. multocida* bacteria. Higher incidence of HS is associated with moist, humid conditions, high animals population density, and extensive free grazing system of management, where large herds graze freely in common pastures (Benkirane and De Alwis, 2002).

1.6.2 Mortality and morbidity
In situations where occasional sporadic outbreaks occur in some regions within endemic countries, mortality may be very high unlike in endemic areas where regular, seasonal outbreaks occur, where losses in each outbreak are low and confined to young animals. The phenomenon of naturally acquired immunity resulting from the so-called non-fatal infection largely controls the mortality and morbidity patterns (Benkirane and De Alwis, 2002).

1.6.3 Host range
Cattle and water buffaloes are the principal hosts of hemorrhagic septicemia (De Alwis, 1984) and it is widely considered that buffaloes are the more susceptible. Outbreaks of hemorrhagic septicemia have been reported in sheep and swine where it is not a frequent or significant disease. Cases have been reported in deer, elephants and yaks. There is as yet no evidence of a reservoir of infection outside the principal hosts; there are cattle, water buffaloes, and bison (Heddleston and Gallagher, 1969). The disease was reported in camels in Sudan in Blue Nile Province and the causative agent was identified to be serotype B:6 (Hassan and Mustafa, 1985).
1.6.4 Geographic distribution

Occurrences of the disease in certain parts of the world like Southeast Asia, where favourable conditions often coincide, are the area of highest incidence. The disease occurs in the Middle East and Africa where the environmental circumstances and predisposing conditions are not as clearly defined as in Southeast Asia. In Asia, the disease is frequently associated with the rainy season and poor physical condition (De Alwis, 1984)

Haemorrhagic septicaemia was recognized in Japan as a specific disease of cattle caused by particular strains of pasteurella as early as 1923. Since 1926, the disease has been controlled, and the last recorded case in cattle in Japan occurred in 1952. The B:2 serotype has been recovered from haemorrhagic septicaemia in countries of Southern Europe, the Middle East, and South East Asia, including China (Anonymous, 1991). This same serotype has been reported from Egypt and the Sudan. The E:2 serotype has been recovered from hemorrhagic septicemia occurring in Egypt, Sudan, the Republic of south Africa, and several other African countries. There is no report of either serotype being recovered from Australia, New Zealand, and countries of South and Central America. There is no evidence that the disease has spread from carrier bison in the western United States to neighboring cattle. Given the conditions in which hemorrhagic Septicemia occurs in endemic areas where primitive husbandry practices, low country plains, and well-defined dry and wet seasons, it seems unlikely that the disease will reach epidemic proportions in the United States (De Alwis, 1984).

The disease was reported in Sudan in Blue Nile, Kassala Northern Kordofan, and Upper Nile Province. The serotypes B:6 and E:6 were
isolated and identified from cases of Haemorrahagic septicaemia in cattle by Shigidi and Mustafa, (1979).

1.7 Antigenic structure and serotyping

Early attempts at serological classification of *P. multocida* date back to the 1920. Agglutination absorption test has identified Groups I, II, III and IV (Cornelius, 1929), whereas Yusef, (1935) used precipitation test to identify Groups I, II, III and IV. Rosenbach and Merchant, (1939) used agglutination fermentation identified Groups I, II and III. Little and Lyon, (1943) used slide agglutination and identified Types 1, 2 and 3. However, 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. On the basis of mouse protection, he was able to identify four types, 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's type I, this designation became fairly well established. Lately, Hudson, (1954) added a fifth serotype.

Carter used a precipitation test (Carter, 1952) and subsequently, an indirect haemagglutination test (Carter, 1955) was able to identify four serological types. These were based on agglutination of human type O erythrocytes coated with crude extracts of outer cell components from the bacterial cultures. These crude capsular extracts supernatants were 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 (Carter1952, 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). Later, 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 type O 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 type O 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 a) 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) 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. Another drawback to this system is that some cultures undergo auto agglutination 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 recognized. This test was originally used to type avian strains from fowl cholera but is now extended to strains from all host species.

1.7.1 Designation of serotypes

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 and a non-HS type B strain of Australian origin as B:3,4. This strain was originally isolated from a bovine wound but has subsequently been associated with occasional HS-like septicaemic disease in cattle in North America and deer in the United Kingdom. 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). Under this system, the avirulent Australian strain is designated 11:B. Since both systems are used in the literature.

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. Broadly, two typing systems are adopted. One is the capsular typing by Carter’s IHA test (Carter, 1955) or by AGID tests (Anon, 1981; Wijewardena, 1982). The other is somatic
typing by the method of Namioka and Murata (Namioka, 1978; Namioka and Murata, 1961b) and by the method of (Heddleston, et al. 1972). It is generally agreed that designation of serotypes should be based on a somatic–capsular combination.

1.8 Pathogenesis
Upon entry of the Pasteurella organism into the animal, it is believed that the initial site of multiplication is the tonsillar region. The outcome of this infection depends on an interaction between the virulence of the organism and its rate of multiplication in vivo, and the specific immune mechanisms and nonspecific resistance factors of the host animal. Thus, the dose of infection is a vital factor and if the organism overcomes the host's defence mechanisms, clinical disease will result. If the defence mechanisms dominate over the organism, this is described as an arrested infection and the animal becomes an immune carrier. Such animals possess solid immunity, and the presence of large numbers of such immune animals following an outbreak of disease contributes to 'herd immunity' (De Alwis et al., 1986).

1.8.1 Lipopolysaccaride (LPS)
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 (Rebers et al., 1967). Purified LPS extracts have been shown to have antiphagocytic activity *in vitro* by using phagocytic uptake assays in an ovine mammary neutrophil system and *[^3]H* labeled type B strain of *P. multocida*. Muniahy et al. (1993) found
that capsular polysaccharide extracts known to contain 20% lipopolysaccharides (LPS), potassium thiocyanate extracts and Westphal type LPS extracts inhibited phagocytosis. These workers also found that when encapsulated cells and de-encapsulated cells were used, the percentage of de-encapsulated cells phagocytosed was significantly higher than when encapsulated cells of *P. multocida* were used. These observations indicated that HS-causing strains of *P. multocida* appeared to possess a factor in their capsule that inhibited the ability of phagocytes to engulf and destroy invading bacterial cells. It is well established that the endotoxins of gram negative bacteria consist predominantly of LPS. The toxic effects of the LPS of *P. multocida* associated with HS have been demonstrated, where it produced experimental HS in calves and pigs by different routes using type B strains. Also administered endotoxin prepared from this strain to a calf induced symptoms and lesions resembled those of experimental infection (Rebers *et al.*, 1967).

### 1.8.2 Capsule

Dissociation of colonies is associated with reduction or loss of virulence and also with loss of antigenicity. Well capsulated cultures make good vaccines; for this reason, vaccine seed cultures are passaged in laboratory animals or even in natural host species periodically. However, the relationship between the capsule and virulence is not absolute. There are capsulated variant cultures that are of low virulence or are avirulent, while non capsulated strains may be virulent (Wijewardana *et al.*, 1986).
1.8.2 Enzyme

*P. multocida* has been found to produce a number of enzymes. Neuraminidase is produced by members of serogroups A, B, D and E (Rimier and Rhoades, 1989). Its activity is found to be highest in strains of serogroup A and D. Activity of neuraminidase of type E was inhibited by homologous antiserum only, while those of types B and D were inhibited by antisera against serogroups A, B, D and E. The production of hyaluronidase and chondroitinase by serotype B:2 associated with HS is well documented (Carter and Chengappa, 1980). Hyaluronidases are enzymes that are normally associated with invasive mechanisms in bacteria, helminths and snake venoms. Type B strains, bearing other somatic antigens, such as the B: 3, 4 cattle and deer strains, fail to produce hyaluronidase. Whilst it may be concluded that hyaluronidase production is a character exclusively restricted to serotype B: 2 strains that cause HS. De Alwis *et al.* (1995) described a type B:2 mutant that was of low virulence to mice and rabbits and a virulent to cattle and buffaloes, yet produced hyaluronidase. No clear relationship has been established between the ability to produce hyaluronidase or any other enzyme and virulence.

1.8.3 Toxins

Serogroups A and D have been found to produce protein toxins, more toxigenic strains being present in serogroup D. These toxins are directly involved in the pathogenesis of disease, as in naturally occurring atrophic rhinitis in swine. No correlation has been found between toxin production and somatic types. Toxins of serogroups A and D are similar, if not identical, and antiserum produced against one neutralizes the other (Rimier and Rhoades, 1989). True exotoxins are not produced by strains
of the B group associated with HS. Toxic effect (endotoxic shock) can be produced by injection of culture supernatants (which contain free endotoxins) or endotoxin preparations. With the exception of a few serogroups A and D strains that produce protein toxins, proteins of *P. multocida* are nontoxic.

### 1.8.4 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. Bacteriocins activity has been demonstrated in bovine and avian strains of *P. multocida* (Rimier and Rhoades, 1989). Thirty-three bovine and bison strains belonging to serotypes A, B and D were tested for bacteriocins activity Chengappa and Carter, (1977); 14 were found to produce bacteriocins. Seventeen strains were susceptible to their bacteriocins. The role of bacteriocins in the pathogenesis of disease has not been investigated.

### 1.9 Immunity to *Pasteurella multocida*

A protective immune response comprises humoral immunity or cellular immunity, or both, is effective to eliminate or reduce the load of organism. Humoral immunity or antibody mediated immunity is a main type of immunity against *P. multocida* specially that LPS of *P. multocida* stimulates antibody production (Wijewardana and Sutherland, 1990).

Proteins are believed to be important immunogens and play a vital role in the protective mechanism. The association of outer membrane proteins (OMPs) with protective immunity has been widely investigated.
Muniandy and Mukkur, (1993) observed that the immunogenicity of certain LPS preparations was due to the presence of OMPs.

Serological relationships exist between LPS of serogroups B and E (Mosier, 1993). Electrophoretic analysis of purified LPS preparations has also established relationships between B and E and some type A strains (Rimier, 1990). This is not surprising, since all strains of both Asian and African origin possess the Namioka somatic antigen type 6 and Heddleston type 2, although in the two serotyping procedures the LPS components used are different (De Alwis, 1987). Although crude LPS preparations are associated with immunity, it has been shown that highly purified LPS are nonimmunogenic to mice and rabbits (Muniandy et al., 1993).

The OMPs of gram-negative bacteria such as porin and OmpA have been considered effective vaccine candidates. Two major OMPs of *P. multocida* are related to the families of porin (protein H; OmpH) and heat modifiable (OmpA). Based on the electrophoretic migration of OmpH, different OMP patterns were identified among capsular serotype strains of *P. multocida*, representing various host species and geographic origins, while the electrophoretic mobility of OmpA of *P. multocida* varied slightly among different strains OmpH possessed both specific and cross-reacting epitopes which are abundantly expressed on the bacterial surface. OmpA possessed cross-reacting epitopes which are not exposed on the cell surface, as shown by immunoelectron microscopy cited by Vasfi and Mittal, (1997).

### 1.10 common antigens

*P. multocida* shares common antigens with other closely related gram-negative bacteria. Antigenic relationships with *Yersinia*
**paratuberculosis, Mannheimia haemolytica, Haemophilus canis, Haemophilus influenza, Actinobacillus lignieresi and Escherichia coli** have been reported (Bain, 1963; Prince and Smith, 1966). Cross-protection has been detected in a study of 11 isolates of *P. multocida* from cases of HS, bovine pneumonia and fowl cholera were showed to belong various serotypes (Rimier, 1996). A serotype A:5 strain and a fowl cholera strain were found to protect against a number of other strains, irrespective of the disease caused. This protection was attributed to antigen components of molecular weight 20-120 kDa.

Homogeneity in protein profiles among 14 strains associated with HS was detected. Strains of Asian and North American origin (B:2) displayed a major protein band of molecular mass 32 kDa. On other hand, strains of African origin (E:2), gave a similar band at 37 kDa. Other bands at 27, 45 and 47 kDa were shared by all strains, irrespective of serotype. Using monoclonal antibodies and an immunoblotting technique Ramdani and Adler, (1993) identified protein fractions of 29 and 36 kDa in the cytoplasmic and periplasmic fractions and 42 kDa in the membrane fraction.

**1.11 Diagnosis**

A clinical, provisional diagnosis of HS is based on a combination of clinical signs, gross pathological lesions and a consideration of relevant epidemiological parameters and other similar diseases prevalent in the locality. A variety of diagnostic techniques have been developed over the years for HS. These include Blood smear, culture and biological tests for isolation of the causative agent as using biochemical, serological tests and molecular methods such as PCR, (Benkirane and De Alwis, 2002).
1.11.1 Clinical Signs:
The majority of cases in cattle and buffalo are acute or peracute with death occurring from 6 to 24 hours after the first recognized signs. In a few outbreaks, animals may survive for as long as 72 hours. Dullness, reluctance to move, and elevated temperature are the first signs. Following these signs, salivation and nasal discharge appear, and edematous swellings are seen in the pharyngeal region and then spread to the ventral cervical region and brisket. Visible mucous membranes are congested, and respiratory distress is soon followed by collapse and death. Recovery, particularly in buffaloes, is rare. Chronic manifestations of hemorrhagic septicemia do not appear to occur (De Alwis, 1992).

1.11.2 Gross Lesions
Widely distributed hemorrhages, edema, and general hyperemia are the most obvious tissue changes observed in infected animals. In almost all cases, there is an edematous swelling of the head, neck, and brisket region. Incision of the edematous swellings reveals a coagulated serofibrinous mass with straw colored or blood-stained fluid. This edema distends tissue spaces. There are subserosal petechial hemorrhages throughout the animal, and blood-tinged fluid is frequently found in the thoracic and abdominal cavities. Petechiae may be found scattered throughout some tissues and lymph nodes, particularly the pharyngeal and cervical nodes, which are also swollen and often hemorrhagic (OIE Manual, 2008).
1.11.3 Collection of rewarding specimen
The septicaemia in HS occurs at the terminal stage of the disease. Therefore, blood samples taken from sick animals before death may not always contain *P. multocida* organisms. A blood sample or swab collected from the heart is satisfactory if it is taken within a few hours of death. If there is no facility for postmortem examination, blood can be collected from the jugular vein by incision or aspiration (Wijewardana *et al.*, 1986).

1.11.4 Morphology and staining
This organism is short rod or coccobacillus, 0.2-0.4 by 0.6-2.5 mm in size. Repeated laboratory subcultures of old cultures or cultures have grown under unfavorable 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. It is a gram-negative organism and in fresh cultures and animal tissues, gives typical bipolar staining, particularly with Leishman or methylene blue stain (Wijewardana *et al.*, 1986).

1.11.5 Growth characteristics and colony morphology
*P. multocida* grows in most common laboratory media such as nutrient agar but the growth is very poor. Special media such as dextrose-starch agar and casein-sucrose-yeast (CSY) medium support an abundant growth. Blood agar and CSY agar with 5% blood (bovine, sheep) are convenient media for routine laboratory culture (Wijewardana, *et al* 1986). The optimum growth temperature is 35-37°C. In enriched media at 37°C; colonies 1-3 mm in diameter are produced after 18-24 hours culture. The organism shows different types of colonies, which are
related to the capsular type. Colonies of types B and E 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. Rough colonies may be produced by old cultures. These are the smallest colonies of all forms, and are noniridescent in oblique light. Production of rough colonies is the result of loss of capsular material or loss of LPS. 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. Dissociation also occurs during storage of stock cultures either in stock culture media or in lyophilised form. In such instances, an animal passage should be carried out upon reconstitution of the stock culture (Wijewardana et al., 1986).

1.11.6 Biochemical properties
Many biochemical methods have been used to study *P. multocida*. These include: catalase, indole, oxidase and sugars fermentation tests. Shigidi and Mustafa, (1979) tested 42 strains of *P. multocida* isolated from different outbreaks of HS and from healthy cattle in various parts of Sudan. The isolates did not cause haemolysis on blood agar (BA) and failed to grow on MacConkey agar (MCA). All strains produced indole, catalase, oxidase and reduced nitrate to nitrite. All the strains fermented xylose, glucose, fructose, galactose, mannose, sucrose and sorbitol with acid production. None of the strains fermented rhamnose, lactose, trehalose, raffinose, dulcitol or salicin. The results were variable with some of the carbohydrates; 16 strains fermented arabinose, four fermented maltose and nine fermented mannitol. None of the strains
changed litmus milk, utilized citrate, liquefied nutrient gelatin or produced urease.

1.11.7 Serological test and serotyping methods

Serological tests for detecting antibodies are not normally used for diagnosis. The indirect haemagglutination test (IHA) can be used for this purpose. High titers detected by the IHA test are indicative of recent exposure to HS. As HS is a disease that occurs mainly in animals reared under unsophisticated husbandry conditions, where disease-reporting systems are also poor, there is often considerable delay in notification of outbreaks. When notification is made in such situations, high IHA titers from 1/160 up to 1/1280 or higher among in-contact animals surviving in affected herds, are indicative of recent exposure to HS (OIE Manual, 2008).

Several serotyping tests are used for the identification of the HS-causing serotypes of *P. multocida*. These consist of rapid slide agglutination (Namioka & Murata, 1961 a) indirect haemagglutination (IHA) test for capsular typing (Carter, 1955) and an agglutination test using hydrochloric-acid-treated cells for somatic typing (Namioka and Murata, 1961b). The agar gel immunodiffusion (AGID) test (Heddleston et al, 1972; Wijewardena, 1982; Anon, 1981) and the counter immune electrophoresis test (CIEP) are also used for this purpose (Carter and Chengappa, 1981)
1.11.8 Molecular methods
Molecular methods such as PCR, ribotyping or restriction endonuclease analysis have an epidemiological significance because they enable strain differentiation within serotypes and hence some epidemiological inferences, for investigations extending beyond routine diagnosis. (Benkirane & De Alwis, 2002).

1.11.8.1 Polymerase chain reaction technique
The polymerase chain reaction (PCR) is a technique developed in 1984 by Kary Mullis, (Mullis, 1990). widely used in molecular biology, microbiology, genetics, diagnostics, clinical laboratories, forensic science, environmental science, hereditary studies, paternity testing, and many other applications. The name, polymerase chain reaction, comes from the DNA polymerase used to amplify a piece of DNA by in vitro enzymatic replication. The original molecule or molecules of DNA are replicated by the DNA polymerase enzyme, thus doubling the number of DNA molecules. Then each of these molecules is replicated in a second "cycle" of replication, resulting in four times the number of the original molecules. Again, each of these molecules is replicated in a third cycle of replication. This process is known as a "chain reaction" in which the original DNA template is exponentially amplified. With PCR it is possible to amplify a single piece of DNA, or a very small number of pieces of DNA, over many cycles, generating millions of copies of the original DNA molecule. PCR has been extensively modified to perform a wide array of genetic manipulations, diagnostic tests, and for many other uses (Saiki et al., 1985; Saiki et al., 1988).
PCR technology can be applied for rapid, sensitive and specific detection of *P. multocida*. The rapidity and high specificity of two of the *P. multocida*-specific assays provide optimal efficiency without the need for additional hybridization. Although the use of hybridization can confirm specificity, this approach is usually possible only in specialized laboratories. The *P. multocida*-specific PCR identify all subspecies of *P. multocida* (OIE, 2008).

Nucleic acid based differentiation of closely related *P. multocida* vaccinal strains was performed after morphological and biochemical characterization. HS-specific and species-specific PCR analysis of *P. multocida* vaccinal strains was demonstrated to be useful in distinguishing hemorrhagic septicemia-causing type B strains. The PCR assay performed for species specific *P. multocida* by using primer pair KMT1T7 and KMTISP6 resulted in amplification of all the strains. Another PCR analysis carried out for HS causing strain conformation by using primer pairs KTT72 and KTSP61 showed that only H.S. causing strains were amplified. It was also observed that PCR amplification performed directly on bacterial colonies or cultures was an extremely rapid, sensitive method of *P. multocida* identification (Townsend *et al.*, 1998).

Recently a multiplex PCR was introduced as a rapid alternative to capsular serotyping system. Comparative analysis of the five capsular biosynthetic regions confirmed a genetic basis for the serological differences observed between strains. By using these genetic differences, a rational, DNA-based typing system for *P. multocida* was developed (Townsend *et al.*, 2001). Notably, the PCR-based system was not affected by the geographical distribution of isolates. For example,
isolates classified as serogroup A by conventional serotyping from
Australia, Vietnam, and the United States have all produced the
appropriate amplicon with the serogroup A \textit{cap}-specific primers.
However by this technique only the capsular serotyping information
introduced a PCR technique specific for \textit{P.mutocida} serogroup A.

1.11.8.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SDS-PAGE
SDS PAGE is a technique used in biochemistry, genetics and molecular
biology to separate proteins according to their electrophoretic mobility;
a function of the length of polypeptide chain or molecular weight as
well as degree of protein folding, posttranslational modifications and
other factors. The solution of proteins to be analyzed is first mixed with
sodium dodecyl sulphate (SDS), an anionic detergent which denatures
secondary and non–disulfide–linked tertiary structures, and applies a
negative charge to each protein in proportion to its mass. Without SDS,
different proteins with similar molecular weights would migrate
differently due to differences in folding, as differences in folding
patterns would cause some proteins to better fit through the gel matrix
than others. Adding SDS solves this problem, as it linearizes the proteins
so that they may be separated strictly by molecular weight primary
structure, or number and size of amino acids. The SDS binds to the
protein in a ratio of approximately 1.4 g SDS per 1.0 g protein although
binding ratios can vary from 1.1-2.2 g SDS/g protein, giving an
approximately uniform mass: charge ratio for most proteins, so that the
distance of migration through the gel can be assumed to be directly
related to only the size of the protein. A tracking dye may be added to
the protein solution to allow the experimenter to track the progress of the protein solution through the gel during the electrophoretic run (Laemmli, 1970).

The protein analysis of *P. multocida* organism is usually performed by (SDS-PAGE) technique. It is important that strains of *P. multocida* used for the production of vaccine be antigenically similar and immunologically homologous to the strains of organisms prevalent in the field (Sridevi *et al.*, 1999).

1.11.8.2.1 SDS PAGE of whole cell lysate

Johnson *et al.* (1991) examined a wide range of *P. multocida* strains of different serogroups by electrophoretic techniques. They found a high degree of homogeneity in protein profiles among 14 strains associated with HS. Strains of Asian and North American origin (B:2) displayed a major protein band of molecular mass 32 kDa like strains of African origin (E:2). Further, it also gave a similar band at 37 kDa. Other bands at 27, 45 and 47 kDa were shared by all strains, irrespective of their serotype.

HS related *P. multocida* isolates, collected from different localities of Pakistan, were characterized on the basis of whole cell proteins by (SDS-PAGE) technique. He found no quantitative difference was observed among different isolates (Nawaz, 2006).

1.11.8.2.2 SDS PAGE of Outer Membrane Protein (OMPs)

The analysis of total membrane proteins by (SDS-PAGE), in cells of serotype B:2 strains grown under iron-replete and iron-restricted conditions (Veken *et al.* 1994; Veken *et al.* 1996), revealed different
specific protein components that were expressed by the same strain, depending on the culture conditions. A variety of protein components of various molecular weights have also been isolated from the Indian vaccine strain P52, by various extraction methods including sonication and precipitation with ammonium sulfate gel. Their immunogenic merits have been tested in rabbits and mice (Pati et al. 1996; Srivastava, 1996).

*P. multocida* strains isolated from adult cattle with HS and calves with typical bronchopneumonia, to determine electrophoretic profiles of OMPs and compare reference strains of the serotypes B:2 and A:3, using SDS-PAGE. The electrophoretic profiles of field isolates of *P. multocida* sampled from adult cattle and calves, and the reference strains, serotypes B:2 and A:3, exhibited 9 to 14 bands with different molecular weights, ranging from 18 kDa to 115 kDa (Jablonska & Opacka, 2006).

### 1.11.8.3 Western Blotting

The Western blot, i.e. protein immunoblot is an analytical technique used to detect specific proteins in a given sample of microbial homogenate or extract. It uses gel electrophoresis to separate native or denatured proteins by the length of the polypeptide (denaturing conditions) or by the 3-D structure of the protein (native/ non-denaturing conditions). The proteins are then transferred from the gel to nitrocellulose membrane, where they are probed detected using antibodies specific to the target protein (Towbin et al. 1979; Renart et al. 1979). This method is used in the fields of molecular biology, biochemistry, immunogenetics and other molecular biology disciplines.

The method originated from the laboratory of George Stark at Stanford. The name western blot was given to the technique by W. Neal (Burnette, 1981) and is a play on the name Southern blot, a technique for DNA detection.
developed earlier by Edwin M. Southern. Detection of RNA is termed northern blotting and the detection of post-translational modification of protein is termed Eastern blotting.

1.11.8.3 Transfer method
Transfer can be done in wet or semi-dry conditions. Semi-dry transfer is generally faster but wet transfer is a less prone to failure due to drying of the membrane and is especially recommended for large proteins, >100 kDa (Hames and Rickwood, 1998).

1.11.8.3.1 Wet transfer
The gel and membrane are sandwiched between sponge and paper (sponge/paper/gel/membrane/paper/sponge) and all are clamped tightly together ensuring no air bubbles have formed between the gel and membrane. The sandwich is submerged in transfer buffer to which an electrical field is applied. The negatively charged proteins travel towards the positively-charged electrode, but the membrane stops them, binds them, and prevents them from continuing on. A standard buffer for wet transfer is the same as the 1X Tris-glycine buffer used for the migration/running buffer without SDS but with the addition of methanol to a final concentration of 20%. For proteins larger than 80 kDa, it is recommended that SDS is included at a final concentration of 0.1%.

1.11.8.3.2 Semi-dry transfer
A sandwich of paper/gel/membrane/paper wetted in transfer buffer is placed directly between positive and negative electrodes (cathode and anode respectively). As for wet transfer, it is important that the membrane is closest to the positive electrode and the gel closest to the negative electrode. The proportion of Tris and glycine in the transfer
buffer is not necessarily the same as for wet transfer (Hames and Rickwood, 1998).

1.11.8.4 Characterization of \textit{P. multocida} antigen by immunoblotting

Immunoblotting was done for serotype B:2. As a step for identification of individual antigens that may protect against HS, proteins present in a sonicated cell extract (SCE) and outer-membrane protein (OMP) preparation of a wild-type \textit{P. multocida} serotype B:2 were investigated by immunoblotting with sera from calves that had been protected against challenge with a virulent strain of \textit{P. multocida} B:2 by vaccination with a aroA derivative live-attenuated strain B. Five proteins in SCE, of approximately 50, 37, 30, 26 and 16 kDa, were recognised by the sera. In an OMP preparation, two bands, at 37 and 50 kDa, were recognised as strongly immunogenic (Ataei et al., 2009). Using monoclonal antibodies and an immunoblotting technique Ramdani & Adler, (1993) identified protein fractions of 29 and 36 kDa in the cytoplasmic and periplasmic fractions and 42 kDa in the membrane fraction.

Western blot was used to confirm that immunogens of \textit{P. multocida} in rabbits, compared with the radioimmunoprecipitation procedure (RIP) for identification of outer membrane immunogens and also for identification of antibody-accessible proteins on the cell surface is important in the selection of vaccine candidates, indicating that the two systems were similar in detecting \textit{P. multocida} outer membrane immunogens (Lu et al., 1988).

Immunoblotting and ELISA was used to evaluate antigenic complexes immunogenic properties of outer membrane proteins (OMPs) and iron-
regulated outer membrane proteins (IROMPs) prepared from strain of serotype B:2,5 for the immunization of calves. The occurrence of antibodies against specific outer membrane proteins as detected by immunoblotting and ELISA in the sera of immunized cattle suggest a beneficial immunogenicity of the vaccines (Kedrak & Opacka, 2003).

1.12 Prevention and control

In endemic areas the only practical ways to protect animals are by an organized program of vaccination and maintenance of animals in as good a condition as possible. Avoiding crowding, especially during wet conditions will also reduce the incidence of disease. Animals that are exposed to *P. multocida* serotypes 6:B and 6:E and survived are considered solidly immune (OIE Manual, 2008).

1.12.1 Vaccination

Peracute nature of disease, febrile condition of animals and development of resistance against antibiotics usually result in therapeutic failure. Therefore, an effective control of disease could only be achieved by vaccination. The three types of vaccines used against HS are bacterins, alum-precipitated vaccine (APV) and oil-adjuvanted vaccine (OAV). To provide sufficient immunity with bacterins, repeated vaccination is required. Administration of dense bacterins can give rise to shock reactions, which are less frequent with the APV and almost nonexistent with the OAV.
1.12.1.1 Inactivated vaccines

Vaccination is routinely practiced in endemic areas where three preparations are used; dense bacterins combined with either alum adjuvant or oil adjuvant, and formalin-inactivated bacterins injected subcutaneously (s.c.). There preparations give some protection against HS, but they provide only short-term immunity (Chandrasekaran et al., 1994). The high viscosity of oil-adjuvant vaccines makes them unpopular among field users. The oil adjuvant bacterin is thought to provide protection for up to one year and the alum bacterin for 4–6 months. Nonetheless, there is a disadvantage, that maternal antibody interferes with vaccine efficacy in calves (Shah and De Graaf, 1997; Verma and Jaiswal, 1997, 1998; Sawada et al, 1985)

1.12.1.2 Live attenuated vaccines

A live HS vaccine prepared using an avirulent P. multocida strain B:3,4 deer strain has been used for control of the disease in cattle and buffaloes over 6 months of age. It is administered by intranasal aerosol application; a natural route of entry into the host. This allows targeting the immunostimulatory factors at the same sites of the immune system that occur in the natural infection. For live strains to be used as vaccines, the mode of attenuation should be well defined. The vaccine has been recommended by the Food and Agriculture Organization of the United Nations (FAO) as a safe and potent vaccine for use in Asian countries. However, there is no report of its use in other countries and killed vaccines are the only preparations in use by the countries affected with HS (OIE, 2008; Myint et al. 2005; Myint and Carter, 1989).
CHAPTER TWO
MATERIAL AND METHOD

2.1 Pasteurella multocida
Lyophilized vaccine strains B and E were obtained from Biological Department of the Central Veterinary Research Laboratories (CVRL), Soba, Sudan. Lyophilized field strains B and E were obtained from the Department of Microbiology, Faculty of Veterinary Medicine, University of Khartoum.

2.2 Laboratory animals

2.2.1 Rabbits:
Four healthy local breed of rabbits were purchased from the market. The animals were kept under close observation and were fed well for ten days.

2.2.2 Rats:
Two healthy Rats were obtained from the Department of Microbiology Faculty of Veterinary Medicine.

2.3 Glass wares:
Petri dishes, flasks, beakers, bottles and sonicator tubes were sterilized by oven at 160 °c for 2 hours.

2.4 Plastic wares:
Eppendorf tubes, plain tube and rubbers were sterilized by autoclave at 121°c for 15 min.

2.5 Pasteurella multocida strains:
Each of the lyophilized B and E P. multocida vaccine was reconstituted in nutrient broth (Appendix, I) and was incubated at 37°c for 24 hours.
Point five milliliter of each strain culture was revived in a rat by giving subcutaneous injection. The rats died in less than 24 hours and were dissected aseptically. Heart blood, heart and liver were collected and inoculated into blood agar (Appendix, I) and incubated at 37°C for 24 hours. The growth was observed and colonial morphology was checked. Blood smears stained with methylene blue were prepared to check the bipolarity (Carter, 1985).

2.6 Hyperimmune serum production

Hyperimmune serum was prepared against specific strains in rabbits. Vaccine strains B and E were cultured in brain heart infusion broth (BHI) (Appendix, I) for 24 hours in shaking water bath. About fifty ml of culture for each strain were centrifuged at 4000 rpm at 4°C for 15 min (Megafuge, Germany). The pellet was washed 3 times with phosphate buffer saline (PBS) pH 7.2 (Appendix, II), and the final pellets were suspended in 5 ml of 0.3% formalin saline and were left overnight to kill the bacteria. The suspensions were centrifuged at 4000 rpm at 4°C for 15 min and the pellets were suspended in 10 ml normal saline. The turbidity of the cell suspension was adjusted to that of McFarland's tube No. 3 which corresponds to $10^8$ cfu/ml.

2.6.1 Immunization schedule

Rabbits were inoculated intravenously in ear vein at 3 day intervals with 0.2, 0.5, 1.0, 1.5 and 2.0 ml of the killed bacteria suspension. Proof bleeding was done and the sera were detected for presence of antibody by Agar gel immunodiffusion test. The rabbits were inoculated subcutaneously 1 week after the last injection with 0.5 ml of a similar suspension as a booster dose to raise antibody titer. The animals were
bled 10 days later. The serum was separated and stored at –20°C as described by OIE Manual, (2008).

2.6.2 Detection of antibody

Agar gel immunodiffusion (AGID) test was done to detect the presence of antibodies in serum.

2.6.2.1 Agar gel immunodiffusion test

The gel was prepared by dissolving 1 gram of agarose (SIGMA) powder was dissolved in 100 ml of 0.1M PBS (Appendix, II) and was melted in microwave. A volume of 0.5 ml of phenol was added as bacteriostatic agent. The agar solution was poured in 60 mm petri dishes and left to cool and solidify. Wells were cut with gel puncture. Thirty micro litter of whole cell lysate (WCL) bacterial antigen was placed in one well and in other well 30µl of the serum was dropped. The gel incubated in humidity chamber for 24-48 hours. The test was read against the illuminator chamber.

2.7 Whole cell lysate preparation

Each *P. multocida* vaccine strain was cultured in 250 ml BHI broth and incubated at 37°C overnight in shaking water bath. Purity of each culture was checked. The cultures were centrifuged at 4000 rpm for 15 minutes at 4°C in 15 ml falcon tubes and pellets were collected and washed 3X with PBS. The final pellets were redissolved in distilled water vortexed and sonicated in sonicator (MSE-England) for 30 seconds stroke and 30 seconds cooling upto10 cycles, the amplitude was kept 18 rpm in cycles (Srivastava, 1998). Cell debris and unbroken cells were removed by centrifugation at 3000 rpm for 15 min and the supernatants were kept at -20°C until used.
2.7.1 SDS PAGE

The SDS PAGE of whole cell lysate (WCL) vaccine strains were carried out according to Lammeli method (Lammeli, 1979) using electrophoresis apparatus (Bio Rad, Germany) with 12% separating gel and 5% stacking. Polyacrylamide gel was prepared and polymerized by the addition of tetramethylenediamine (TEMED) (Sigma).

2.7.1.1 Preparation of glass plates

Firstly, the glasses were cleaned up with alcohol and the bottom of the electrophoresis glasses were sealed with 2% agarose and were left to solidify.

2.7.1.2 Separating gel

The seperating gel containing 4.2 ml of 30% acrylamide+bisacrylamide, in 1.5 M Tris-HCl,100 μl of 10 %SDS, 3.2 ml D.W and 100 μl 99% ammonium persulphate (APS) (Sigma). Fourteen microliter of TEMED were added immediately before pouring the separating gel and leveled by methanol. Methanol was discarded and distilled water was added and the glass plates were covered with wet tissue and kept for overnight at 4°C for better resolution.

2.7.1.3 Stacking gel

The stacking gel was prepared by adding 1.3 ml of 30% acrylamide+bisacrylamide, in 0.5M Tris-HCl, 100 μl of 10 %SDS, 2 ml D.W and 100 μl of APS. Ten microliter of TEMED was added and immediately poured off the stacking gel and the comb was placed, the gel was left to solidify, then the comb was removed.
2.7.1.4 Sample loading

Ten microliter of sample were mixed with 10 µl of sample buffer (Appendex, III) containing bromophenol blue and boiled for 5 min. Then 10 µl of heated sample was loaded into each well and also 5 µl of protein marker 10-175kDa (gene direx, USA) was loaded along with the sample. The electrophoresis apparatus was filled with running buffer (Appendix, III). Then the apparatus was connected with constant voltage (100v) for 2 hours or until the bromophenol blue reached the bottom of the slides.

2.7.1.5 Staining and destaining of gel

The gels were placed into container with a staining solution (Appendix, III) containing Coomassie brilliant blue dissolved in methanol. Gels were left in staining solution for overnight under slow shaking. The gels were destained in destaining solution (Appendix, III) containing methanol until the bands become visible.

2.7.2 Western blotting

2.7.2.1 Enzyme antibody conjugate:

The conjugate antibody was antiwhole IgG molecule, goat anti rabbit IgG conjugated with horse radish peroxidase enzyme purchased from Adcam Company, Malaysia

2.7.2.2 Protein transferring

Proteins were electrophoretically transferred to nitrocellulose membrane of average pore size 0.45 Mm (Schleicher and Schueul, Germany). The sponge pads were wetted with transfer buffer and were put in the transfer apparatus, place one piece of wet filter paper, the nitrocellulose membrane, the gel, another piece of wet filter paper were placed in respective order. Bubbles were squeezed out by adding transfer buffer
(Appendix, IV) at every step and by rolling with a pipette after each addition. The apparatus was filled with transfer buffer and the lid was closed and was connected to the power supply and run at 100 volts for 1 hour.

2.7.2.3 Immuno peroxidase staining of nitrocellulose membrane

The nitrocellulose membranes were put on a container and were covered with 2% gelatin as blocking buffer (Appendix, IV). Then the membranes were incubated on the shaker with low speed at room temperature for 1 hour. The blocking buffer was discarded and the membranes were covered with primary antibody diluted at 1:200 in serum diluent (Appendix, IV) and incubated on a shaker at room temperature for 1 hour. The membranes were washed with washing buffer (Appendix, IV) three times (5 minutes for each wash at high speed). Then the membranes were covered with secondary antibody (antirabbit horse, raddish peroxidase- conjugated) (Appendix, IV) diluted 1:1000 and incubated for 2 hours. This was followed by washing the membranes three times like in the first wash. Finally the substrate 4-chloro α naphtol (Appendix, IV) was added and incubated for 15 min. Distilled water was added to stop the reaction. The membranes were air dried and preserved in dark until photographed (Towbin, 1979).

2.8 PCR method

2.8.1 PCR Primers

Primers for each strain were obtained as freeze dried oligonucleotide (Vivantis, Malaysia) with sequences that amplify capsule gene with specific molecular weight (MW) band according to strain.

Strain B
CAPB-FWD 5’-CAT-TTA-TCC-AAG-CTC-CAC-C-3’ MW 5668

CAPB-REV 5’-GCC-CGA-GAG-TTT-CAA-TCC-3’ MW 5460

Strain E

CAPE-FWD 5’TCC-GCA-GAA-AAT-TAT-TGA-CTC-3’ MW 6389

CAPE-REV 5’-GCT-TGC-TGC-TTG-ATT-TTG-TC-3’ MW 6096

2.8.2 DNA extraction

Two methods of DNA extraction were used. The first method of DNA extraction was by boiling method and the second method by the kit (PUREGENE, Gentra System, Minneapolis, USA).

2.8.2.1 Boiling extraction

A pure colony of *P. multocida* was inoculated into 5 ml of BHI broth and incubated at 37°C for 24 h. One point five milliliters of this broth culture was transferred into an eppendorf tube and centrifuged at 3000 x g for 10 min. The pellet was washed twice in PBS and the final pellet was resuspended in 100 µl of sterile deionized distilled water. The mixture was boiled for 30 min and immediately chilled on ice for 30 min. The sample was then thawed and centrifuged at 3000 x g for 5 min. The supernatant was stored at -20°C for further use as DNA template as described by Antony et al. (2007).

2.8.2.2 Kit extraction:

DNA was extracted by using DNA isolation kit. Five hundred microlitre of overnight bacterial BHI culture were centrifuged at 15,000xg in 1.5ml micro tube for 5 second to pellet cells and carefully the supernatant were removed. Then 300µl of cell lysis solution was added to the cells pellet,
and the mixture was inspired and expired by micropipette until cells were suspended and incubated at 80°C for 5 min, followed by the addition of 1.5µl RNase a solution to the cell lysate. The samples were then mixed by inverting the tube 25 times and the tubes were incubated at 37°C for 15 minutes, cooled to room temperature and 100µl protein precipitation solution was added to the cell lysate. The tubes were vortexed at high speed for 20 seconds and then centrifuged at 15.000xg for 3 minutes. Then 300µl of 100% Isopropanol were added to the supernatant fluid into a clean 1.5 microfuge tube, mixed by inverting gently 50 times, and centrifuging at 15.000xg for 1 minute to pellet the DNA. The supernatant was poured off and the tubes were drained briefly, after that DNA was washed with 300µl of 75% ethanol, centrifuged at 15.000xg for 1 minute and the ethanol was poured off. The tubes were then allowed to dry for 10 minutes. 20µl DNA hydration solution was added and DNA was dissolved by incubating for 1 hour at 65°C and then stored at -20°C until used.

2.8.3 DNA amplification:

DNA amplification was done in PCR tube, 50 µl reaction mixture was prepared. One microlitres of template DNA was added to a reaction mixture containing 3.2 µM each of primer, 200 µM of each dNTP, 1 X Taq buffer with 1.5 M MgCl₂ and 2 units of Taq DNA polymerase. The amplification reaction was carried out in an automated thermal cycler (Biometra, Germany) according to the following programme, an initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 second, annealing at 56°C for 30 second, extension at 72°C for 30 second and a final extension at 72°C for 5 min.
2.8.3.1 PCR product detection:
Detection was done by electrophoresis to determine molecular weight of bands.

2.8.3.2 Electrophoresis of PCR product
The product was analyzed by 2% agarose gel with 0.05% ethidium bromide in 1x tris boric EDTA (TBE) running buffer (Appendix, V) prepared by adding 2g agarose to 100 ml of TBE buffer and melted in microwave then 1.5 µl of ethidium bromide were added and poured in electrophoresis apparatus, the comb was placed until solidification of gel. Then comb was removed and samples were loaded. Three microlititre PCR product was mixed with 3µl loading dye (Appendix, V) and was loaded into gel wells. Standard molecular size marker low range DNA ruler with fragments size 100 bp was also loaded and used as DNA molecular size marker to ascertain the size of the amplified PCR product.
CHAPTER THREE
RESULT

3.1 Growth characteristic

*P. multocida*, namely B:6 and E: 6 vaccine strains were showed luxuriant growth on blood agar having translucent grayish colonies varies in size and there was no blood hemolysis (Fig,1). In liquid media showed homogenous growth. The growth was better in BHI broth than nutrient broth.

3.2 Morphological characteristic

*P. multocida* when stained with Gram stain showed Gram negative short rod or coccobacilli. Blood smears from injected rats showed bipolar staining bacteria (Fig,2).

3.3 Agar gel immune diffusion test

Sera were collected from immunized rabbits and tested for presences of antibody. The gel showed presence of continuous precipitation line between strain B and strain E against the sera of strain E or B and other line which might be polysaccharide (Fig,3).

3.4 SDS PAGE

For the analysis of proteins of *P. multocida* organism, SDS-PAGE was done according to Lammeli method (Lammeli, 1979). The gel obtained after analysis was stained with Coomassie brilliant blue dye. By this technique different polypeptides bands of the organism’s proteins were observed. The protein profiles of *P. multocida* whole cell lysate prepared for each strain, there were 15 protein bands of molecular weight 175
kDa, 165 kDa, 150 kDa, 123 kDa, 102 kDa, 90 kDa, 85 kDa, 70 kDa, 64 kDa, 60 kDa, 51 kDa, 42 kDa, 37 kDa, 22 kDa and 16 kDa (Fig.4). Some of these bands are major protein with molecular weight 102 kDa, 85 kDa, 70 kDa, 62 kDa, 42 kDa, and 37 kDa. Polysaccharides were observed as a thin a band near the start of the gel.

3.5 Western blotting

Immunoblotting using antiserum against *P. multocida* strain B prepared in rabbits and using nitrocellulose membrane containing bands transferred from gel according to Towbin (1979) method, revealed eight protein bands of molecular weight 175 kDa, 102 kDa, 90 kDa, 85 kDa, 70 kDa, 42 kDa, 37 kDa, and 16 kDa (Fig.5).

3.6 PCR

The two methods used for extraction of DNA gave good yield for DNA, but the Kits method was the best in yield.

The strains of *P. multocida* E and B when subjected to amplification using the primer pairsgave an amplification of 511 bp and 760 bp for strain E and strain B respectively when subjected to gel electrophoresis. Primers of strain E amplified vaccine strain B DNA. Field strains B and E were specifically amplified. Vaccine strain E gave DNA amplification product (band) but vaccine strain B did not give an amplification product (band) when strain B primers were used as depicted in Fig. 6.
Fig. 1 Growth of *P. multocida* in blood agar showed grayish colonies.
Fig. 2 *Pasteurella multocida* bipolar staining with methylene blue

(Blue dots)
Fig. 3 AGID test, continuous precipitation line between antisera against B and E and strain E of *P. multocida.*
Fig. 4 Whole cell lysate proteins profile of *P. multocida* vaccine strains by SDS-PAGE

Lane 1 protein marker, lane 2-5 strain E, lane 6-10 strain B, lane 11 protein marker.

P = polysaccharide.
Fig. 5 Immunogenic bands of *P. multocida* detected with hyper immune serum and developed by enzyme labeled antibody.

M = protein marker
Fig. 6 PCR of *P. multocida* strain E and B field and vaccine strains

Lane M = protein marker, lanes 1-4 boiling extraction, lanes 5-8 kits extraction

1 and 6 = vaccine strain B, 2 and 5 = field strain B, 3 and 7 = vaccine strain E, 4 and 8 field strain E, 9 = vaccine strain B with primer E, 10 vaccine strain B with primer E
CHAPTER FOUR

DISCUSSION

*P. multocida* is an aerobic, chemo-organotrophic organism that has different serotypes that cause disease in animal and poultry. Serotypes B and E cause hemorrhagic septicaemia in cattle and buffaloes. In all countries where pasteurellosis occurs vaccinations are considered as an effective means of controlling this disease and local isolates are usually used for vaccine preparation. Bacterins were used for immunization, in addition to precipitated alum or aluminium hydroxide gel vaccines and dense bacterin. Further several authors immunized cattle with live vaccines. In order to improve the immunogenicity of vaccines, the causative organism has been fractionated and various cell surface components have been studied (Carter and De Alwis, 1989; Chandrasekaran *et al.*, 1994).

In these study cultural characteristics of two *P. multocida* vaccine strains was studied on blood agar. The optimum growth temperature was 37°C after 18-24 hours incubation culture gave translucent grayish colonies that varies in size 1 to 3 mm were seen and there was no blood hemolysis. In liquid media incubated in shaker there was homogenous growth. *P. multocida* is Gram negative short rod that gave bipolar staining from tissue and these confirmed results had been reported by (Shigidi and Mustafa, 1979; Arawwawela *et al.*, 1981; Wijewardana *et al.*, 1986).

The techniques in molecular biology have significantly increased understanding of the epidemiology of Pasteurella diseases. SDS-PAGE has shown to establish the unique properties of the bacterial
proteins (Johnson et al., 1991). In this study the proteins profile of *P. multocida* organism was studied by SDS-PAGE and clear proteins bands were obtained following staining of the gel with Coomassie brilliant blue dye. By this technique, different polypeptides of the organism’s proteins were observed. The protein profiles of *P. multocida* whole cell lysate revealed 15 bands on SDS-PAGE analysis some of these bands are of high molecular weight include 175 kDa, 165 kdDa, 150 kDa, 123 kDa, 102 kDa protein band. Type of the medium used and the method of protein extraction, as the growth on BHI medium enhanced the production of high molecular weight (Jain et al., 2005) which is substantiated by the correlate observation in this study. Jain et al., (2005) who observed expression of protein of molecular weight 102 kDa along with other major OMP bands in capsular type B buffalo isolates. Further they also examined four reference strains of serotype B: 2 to determine their protein profiles and compared them with field isolates. Their results revealed that fractions of molecular weight of 25 to 55, 75, 80, 86 and 90 kDa were most frequently observed among all isolates and reference strains. In this study bands with molecular weight 90 kDa, 85 kDa, 70 kDa, 64 kDa, 60 kDa, 51 kDa, 42 kDa, 37 kDa, 22 kDa and 16 kDa. Some of these bands were found by other authors. Johnson et al. (1991) determined the proteins profile of the capsular serotype B and E strains isolated from animals with hemorrhagic septicemia and placed the isolates in two distinct groups on the basis of the molecular masses (32 to 37 kDa) of the major proteins. They reported that polypeptide of 37 KDa was the most immunogenic of all the isolates and this band was found in African strain 37 kda. This finding is in agreement with our present result.
Detected band, with hyperimmune serum using immunoblotting were with molecular weights 175 kDa, 102 kDa, 90 kDa, 85 kDa, 70 kDa, 42 kDa, 37 kDa and 16 kDa correspond to those bands obtained by Pati et al. (1996) who reported proteins of 44, 37 and 30 kDa determined that they were the major immunogens when use subunit vaccines comprising OMPs from *P. multocida* serotype B:2 in immunizing buffalo calves. There for the present study provided further wider OMPs are protective and could be used in vaccines against haemorrhagic septicaemia.

The identification and sequence analysis of the biosynthetic locus of the capsule of an organism can lead to a greater understanding of its capsular polysaccharide composition and can provide a genetic basis for the serological differences observed between strains. Genetic analysis of the serogroup B biosynthetic locus revealed only three gene products with similarity to proteins known to be involved in polysaccharide biosynthesis, while six gene products had no similarity to known proteins. However, the structure of the type B capsule remains unknown (Boyce et al., 2000).

Determination of the nucleotide sequence of the serogroup E biosynthesis region provided little information about the capsular polysaccharide composition. Region 2 of serogroup E contains nine genes, two of which showed similarity to genes involved in polysaccharide biosynthesis. These two genes have homologs in the *P. multocida* B:2 cap locus, indicating that N-acetyl-D-mannosaminuronic acid is a component of both the serogroup B and the serogroup E capsules; the remaining seven genes, five have
homologs in the B:2 cap locus but still have no known function, one encodes a putative glycosyltransferase, and the other is unique to serogroup E. In our study DNA bands were obtained by serotype E and B using primer E cap locus and there were no bands using primers strain B cap locus but there was band in field strain B. This finding according to Townsend et al., (2001) is indicative the bivalent vaccine contain only strain E.
Conclusions

- Protein profile of *P. multocida* is similar in two vaccine strains.

- Some *P. multocida* proteins as obtained by ultrasonication followed by SDS PAGE were reported by others.

- The study further provided that whole cell lysate, include OMPs of *P. multocida* were immunogenic and could be used in subunit vaccines against haemorrhagic septicaemia.

- Bivalent haemorrhagic septicaemia vaccine when subjected to PCR gave amplification product for strain E only.

- PCR can be used for capsular serotyping of *P. multocida*. 
Recommendations

- Research must be done to determine if there is a difference between vaccine strains and field strains in protein profile.
- Further studies are required to confirm the immunogenicity of these proteins prior to use them as antigen in subunit vaccine.
- Research should be done in subunit vaccine and how to use them by mucosal routes to induce local immunity.
- Strain B of national vaccine should be recharacterized.
Reference


Appendices
Appendix I

Bacteriological media:

**Nutrient Broth:**
- Meat (beef) extracts: 10g
- Peptone: 10g
- NaCl: 5g
- Distilled water: 1000 ml

**Blood agar:**
- Defibrinated blood: 50ml
- Nutrient agar: 950 ml

Nutrient Broth was gelled by addition of 2% agar. Sterilized by autoclaving at 121°C for 15 min. Then the blood was added at 40°C

**Brain heart infusion broth (BHI):**
- BHI: 37g
- D.W: 1000ml

The media were sterilized by autoclaving at 121°C for 15 min.
Appendix II

Buffers:

Normal saline:

NaCl 8.5g
D.W 1000ml

The buffer was sterilized by autoclaving at 121°C for 15 min.

Phosphate buffer saline:

NaCl 8g
NaH2PO4 1.15g
KH2PO4 0.2g
KCl 0.2g
D.W 1000 ml

The pH was optimized to 7.2.

0.1M phosphate buffer (PB), pH 7.8:-

Sodium phosphate (NaH2PO4.H2O) 52.4g
Deionized water to final volume 3.8L

The pH was adjusted to 7.8
### Appendix III

**SDS PAGE Buffers:**

**Running buffer:**
- Tris-OH: 3.02g
- Glycine: 14.4g
- SDS: 1g

800 ml of D.W was added pH was adjusted to 8.3 then completed to 1000ml.

**Sample buffer:**
- Tris- OH: 1ml
- Glycerol: 0.8ml
- SDS 10%: 1.6ml
- 2-mercapto-ethanol: 0.4ml
- Bromophenol blue 0.05%: 0.2ml
- D.W: 4ml

**Staining buffer:**
- GAA: 5.75ml
- Methanol: 28.5ml
- D.W: 62.5ml
- Coomassie brilliant blue: 0.157-0.2g

**De-staining solution:**
- GAA: 10ml
- Methanol: 50ml
- D.W: 100ml
Appendix IV

Western blotting buffers:

Transfer Buffer:
glycine 14.4g
Tris base 3.02g
Methanol 100ml
D.W 900ml
pH was Adjust to 8.3.

Blocking buffer:
Gelatin 2g
PBS 100ml
Gelatin was added to PBS and heated in water bath until dissolve

Serum diluents:
Gelatin 1g
PBS 100ml
Tween20 50 µl
Gelatin was added to PBS and heated in water bath until dissolved. Then Tween20 was added.

Preparation of primary antibody:
Hyperimmune sera 125 µl
Serum diluent 25ml

Preparation of conjugate:
Anti rabbit conjugate 25 µl
Serum diluents 25ml

Washing buffer:
PBS 250ml
Tween20 125 µl

Preparation of substrate:
4-Chloro α-naphthol 0.002g
Methanol 5ml
PBS 25ml
H₂O₂ 100 µl
Appendix V:

PCR reagent:

Tris boric EDTA Buffer (TBE):

Tris base                          108 g
Boric acid                         55 g
0.5M EDTA (pH 8.0)                40 ml
Stock 10x TBE

Loading dye:

Bromophenol blue (11%)            10 µl
Glycerol                          40 µl
DDW                                50 µl