

Thesis Ref .No-----

ISOLATION, IDENTIFICATION AND ANTIMICROBIAL SUSCEPTIBILITY OF
PASTEURELLA MULTOCIDA FROM CATTLE WITH HEMORRHAGIC
SEPTICEMIA IN ASSOSA AND BAMBASI DISTRICTS, BENISHANGUL GUMUZ
REGIONAL STATE, ETHIOPIA

MSc Thesis



BY

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JUNE, 2017

BISHUFTU, ETHIOPIA

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A thesis submitted to the School of Graduate Studies of Addis Ababa University in
partial fulfillment of the requirements for the degree of Master of Science in Veterinary
Microbiology,

By

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June, 2017
Bishoftu, Ethiopia

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Date of Submission: 16/06/2017

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ACKNOWLEDGMENTS

I feel I do have a lot to say but as it is always said, words cannot fully express feelings. What I can say is only thanks to my advisor, Dr. Asmelash Tassew for his unreserved advice and devotion in correcting this paper. My gratitude also goes to all staff members of Assosa Regional Veterinary Diagnostic Laboratory especially those in the microbiology Department staff members”w/o” Yeshareg Abebe and also for dr Bosona Fantahun for their limitless laboratory diagnostic technique support. My thanks go to BGRS Agricultural and Natural Resource Bureau for moral and budget support especially to Ato Mulleta Womber the head of bureau with great appreciation. Also my especial thanks go to my wife, Kuribachew Desta for all her share which cannot be listed out due to limitation of space and time. Finally I would like to thank my family, and all my relatives and friends. Above all, Thanks a lot to God.

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LIST OF ABBREVIATION AND ACRONYMS

AST	Antimicrobial susceptibility testing
Bp	Base pair
BGRS	Benshangul Gumuz Regional State
BHI	Brain heart infusion
CB	Cross breed
CSY	Casein sucrose yeast
EA	Environmental Agency
ETB	Ethiopian Birr
HS	Hemorrhagic septicemia
H	Hour
HPA	Health protection agency
OMPA	Outer membrane protein A
pH	Power Hydrogen
INR	Indian Rupees
LPS	Lipopolysaccharide
MR	Methylene red
MDR	Multi drug resistant
NCCLS	National Committee for Clinical Laboratory Standards
NVI	National Veterinary Institute
OIE	Office International des Epizooties
TP	Test procedure

ABSTRACT

A cross sectional study was conducted from November 2016 to May 2017 in Bambasi and Assosa districts in Benshangul Guzum Regional State with the objectives of isolation, identification and determination antimicrobial susceptibility profile of *Pasteurella multocida* isolated from cattle infected with Hemorrhagic Septicemia. The study was also aimed to detect the agent using classical PCR. Specimens were collected purposively from cattle in areas with outbreak of Hemorrhagic septicemia and a total of 384 swab samples were collected aseptically from cattle and transported to Assosa Regional Veterinary Laboratory using a cold chain system. Specimens were cultured on MacConkey agar and blood agar. Different biochemical tests were also used in order to identify and characterize the agent of the suspected disease. The prevalence of *P. multocida* was 13 (3.39%) out of 384 samples tested). All the isolates were subjected to the in vitro antimicrobial sensitivity tests and the result indicated the presence of different degrees of susceptibility and resistance to most of the antibacterial agents. The degree of susceptibility ranges from 15.4% for Tetracycline upto 61.5% for Sterptomycin, and resistance is between 15.4% for Sterptomycin, Clindamycin and Chloramphicol and 69.2% for Tetracycline. Similarly, (60 %) of this discs showed the presence of multidrug resistance. The result of this test clearly indicates that, Sterptomycin, Clindamycin and Chloramphicol could be prospective drugs of choice. Analysis of PCR assay revealed the presence of *P. multocida* serotype B2. In conclusion, the presence of *P. multocida* serotype B2 in the selected areas can be considered as potential pathogens in causing Hemorrhagic septicemia in cattle especially where there is no any practice of vaccination. Therefore, strict measures like proper vaccination and antibiogram test to select effective drugs should be implemented.

Key words: *Antimicrobial sensitivity test, Benishangul Gumuz Regional State, Cattle Haemorrhagic Septicemia, Isolation, Pasteurella multocida, PCR*

1. INTRODUCTION

Haemorrhagic septicaemia (HS) is an acute, fatal, septicemic disease of cattle and buffaloes caused by specific serotypes of the bacterium family *Pasteurellaceae* and genus *Pasteurella* which recently classified in the genera *Pasteurella*, *Mannheimia*, and *Bibersteinia* (Blackall *et al.*, 2007). Bacteria of the family *Pasteurellaceae* are involved in a variety of economically important diseases in food-producing animals. Pasteurellosis is a multi-factorial respiratory disorder (Catry, 2005).

Among these genera *Pasteurella* is the causative agent of HS specifically species *P. multocida*. *P. multocida* was first found in 1878 in fowl cholera-infected birds. However, it was not isolated until 1880, by Louis Pasteur the man whom *Pasteurella* is named in his honor (Katherine, 2008). Now strains of *P. multocida* are grouped serologically into 5 capsular types (A, B, D, E and F) and 16 somatic lipopolysaccharide-types (1–16). *P. multocida* strains have also been characterized by outer membrane protein (OMP)-type and 16S rRNA-type. 16S rRNA-typing revealed that the majority of clinical isolates belong to a single lineage containing seven 16S-types. However, a range of capsular types, OMP-types and host species were represented, indicating significant heterogeneity between closely related strains (Richard, 2009). From these serotypes B2 and E2 are only the causative agents of HS in cattle mainly in Asia and Africa (Garner, 2003).

P. multocida is isolated from upper respiratory tract and blood sample that result in high rates of morbidity and mortality in cattle. *P. multocida* is causative agents of several economically significant veterinary diseases. Serious infectious diseases as fowl cholera, bovine hemorrhagic septicemia, and porcine atrophic rhinitis are caused by *P. multocida* (Michael, 2008).

P. multocida is common inhabitants of the tonsils and nasopharynx of a variety of healthy cattle. In cattle, *P. multocida* are believed to be opportunistic bacteria that colonize the lung and other organ after some predisposing risk factors. The initiating risk factors can

be from stresses by mildly pathogenic agents such as FMD, parasitic infections, as well as from mechanical dust, heavy rain, and transportation.

In most instances, these insults alone do not result in significant epidemics with high morbidity or mortality; however, when these and other stressors are compounded by infection with *Pasteurellaceae*, the result can be increased morbidity and death (Michael, 2008). Generally infection results when an animal is compromised by any of the variety of stress factors such as inclement weather, transportation, malnutrition, bacterial invasion of host defense, viral infections, nasopharyngeal colonization and dehydration (Hawari *et al.*, 2008).

The diagnosis of the disease is based on the clinical signs, gross pathological lesions, morbidity and mortality patterns, and confirmation by isolation of the pathogens and their conventional and molecular characterization (Ragy, 2005).

In the Benshanigul Gumuz Reginal State, there is high cattle movement for cattle market, grazing and water. This is because of the large cattle market place in Assosa and Bambasi towns in the first place. Secondly the cattle come from Oromia Regional State and even cattle always come into the region form border of Sudan. So there is high risk of unvaccinated cattle coming in contact with cattle from Sudan and other in Ethiopia boarder the regions because Sudan and Egypt are the endemic countries in Africa with both *P. multocida* serotype B2 and E2 which are the cause of HS (De Alwis, 1982). On the other hand, base line data about the status of HS in cattle is not available in Ethiopia so far except in (Ethiopia animal health yearbook, 2011).

Therefore, this study was conducted with the following objectives:

- ❖ Isolation and Identification of *P. multocida* from cattle in the study area
- ❖ Molecular detection of *P. multocida*
- ❖ Assessment of the potential risk factors associated with HS
- ❖ Determination of the antimicrobial susptibility pattern of *P. multocida*

2. LITERATURE REVIEW

2.1. Biology of Pasteurellaceae

Pasteurellaceae is a small, Gram-negative coccobacilli or rods which may show bipolar staining. It may grow on most laboratory media with the exception of bile containing media such as MacConkey agar for *P. multocida* (Hawari *et al.*, 2008). It is non motile and non spore forming, fermentative, with few exceptions; ferment sugars like glucose, sucrose, and sorbitol and most of them produce acid from common sugar but not H₂S gas. *P. multocida* is aerobic or facultative anaerobic with fastidious growth requirements. *Pasteurellaceae* is oxidase, catalase and Indole positive, and urease negative. Its growth on artificial media was enhanced by the addition of serum or blood, on which they appear after 24 hours of incubation as round, smooth, greyish colonies of moderate size (1-2 mm in diameter) (Catry, 2005).

2.2. Epidemiology

Hemorrhagic septicemia (HS) is an acute, fatal, septicemic disease of cattle and buffaloes caused by specific serotypes of the bacterium *Pasteurella multocida* which is based on capsular Sero-types currently have serotype A, B, D, E, F and 1-16 somatic type. From these serotypes B2 and E2 are only the causative agents of HS in cattle. Host range with susceptibility include cattle, buffaloes, pigs, domestic and wild birds, rabbits and wound infections in dogs, cats and humans (Garner, 2003). The tonsils of up to 5% of healthy water buffalo and cattle are colonized by small numbers of *P. multocida* serotype B: 2 or E: 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, or work stress.

Although outbreaks can occur at any time, disease is most prevalent during the rainy season. Increased outbreaks associated with high rainfall are most likely due to the multiple stressors present during this time and the moist conditions, which prolong the survival time of the organism in the environment. Infection occurs by contact with infected oral or nasal secretions from either healthy carrier animals or animals with clinical disease, or by ingestion of contaminated feed or water.

Infection begins in the tonsil and adjacent nasopharyngeal tissues. Subsequently, *P. multocida* strains have also been characterized by outer membrane protein (OMP)-type and 16S rRNA-type. 16S rRNA-typing revealed that the majority of clinical isolates belong to a single lineage containing seven 16S-types. However, a range of capsular types, OMP-types and host species were represented, indicating significant heterogeneity between closely related strains. Bacteraemia leads to dissemination and rapid growth of bacteria in various locations, tissue injury, a host cytokine response, and release of lipopolysaccharides that results in a rapidly progressing endotoxemia. Clinical signs can appear 1–3 days after infection, and death can occur within 8–24 hr after the first signs develop (Richard, 2009).

In endemic areas, HS affects older calves and young adults, and morbidity and mortality are variable. In non endemic areas, epizootics can occur with high morbidity and mortality that can reach 100%. Water buffalo tend to be more susceptible and have more severe clinical disease than cattle. Recovery can stimulate acquired immunity to homologous and often heterologous strains of *P. multocida*, and some of these animals become healthy carriers that can provide a source of infection for future outbreaks (Michael, 2008).

The two common serotypes of *P. multocida* associated with disease in these species are types B:2 (in Asia) and E:2 (in Africa). All age groups are affected with *P. multocida*, but in cattle the most susceptible age group is between 6 months and 2 years. Both morbidity and mortality varies between 50 and 100%, and animals that recover require a long convalescence. Morbidity depends upon the immune status of the herd, either by acquired

immunity by natural infection or by vaccination. Incidence of disease is also reduced significantly by vaccination (Benkirane and Alwis, 2002).

The phenomenon of naturally acquired immunity resulting from non-fatal infection largely controls the mortality and morbidity patterns. It is, on the other hand the mechanism through which animals that have recovered from hemorrhagic septicemia acquire a long lasting carrier status, which render the prevention of new outbreaks difficult (Alwis, 1990b). Infection occurs by inhalation or ingestion of *P. multocida* bacteria. The outbreaks are more often associated with wet humid weather during the rainy season. During the intervening period, the causative agent persists on the tensile and nasopharyngeal mucosa of carrier animals (Alwis, 1990a).

In Ethiopia, in years 2008 the morbidity rate of the cattle were 0.9%, mortality rate 0.2%, the number of dead cattle due to HS were 7376, and number of cases record in cattle were 31,277. In year 2009 the morbidity rate was 0.9%, mortality rate 0.1%, the number of dead cattle due to HS were 2976 and number of cases record in cattle were 20,412 again reported. In year 2010 morbidity rate was 0.6%, mortality 0.1%, the number of dead cattle due to HS were 3381 and number of cases record in cattle were 17096. In 2011 morbidity 0.426%, mortality 0.1%, the number of dead cattle due to HS was 2517 and number of cases record in cattle 10613 (Ethiopia animal health yearbook, 2011).

The total cattle death from 2008-2011 for four consecutive years were 16250 from total 79398 number of cases the case fatality of these four years 20.4 % consumption .Alum potassium sulphate adjuvant bovine pasteurellosis/HS/ vaccine production and distribution in Ethiopia in 2010/11 fiscal year was 5,008,700 doses for prevention and control of HS outbreak that is for domestic use. In year 2010/11 In Ethiopia HS outbreak report were shown that unconfirmed 600 diseases outbreak of HS were reported from all regions of Ethiopia including Benshanigul Gumuz Regional State (Ethiopia animal health yearbook, 2011).

2.2.1. Global distribution of *P. multocida* (HS)

Haemorrhagic septicaemia occurs in Southern and Southeast Asia including Indonesia, Philippines, Thailand and Malaysia, in the Near and Middle East, Southern Europe (including the U.S. S.R.) and in North, Central and East Africa. It has also been reported in the Republic of South Africa (Carter G.R. (1982). Ethiopia, Gambia, Ghana, Kenya, Liberia, Libya, Uganda, Malawi, Mauritania, Niger, Tanzania, Uganda and Zaire are Countries where the hemorrhagic septicemia is reported to occur on clinical or bacteriological grounds listed as category A. On the other hands Countries like Tongo, Guinea-Bissau, Mozambique, and Rwanda are countries where HS is reported as an exceptional occurrence, or is suspected to occur but not confirmed listed as category B (Bain, 1982).

2.2.2 Pathology

In cattle, postmortem examination reveals pronounced hyperemia of the internal organs and multiple hemorrhages in serous membranes, mucous membranes and different organs, especially the lungs and muscles. The kidney and liver show cloudy swellings. In sub-acute cases, the subcutaneous tissue of head, neck and throat is infiltrated with gelatinous material and is studded with hemorrhages with serous infiltration between deeper layers of muscle. The unconsolidated part is hyperemic and edematous (Mathy *et al.*, 2002).

2.2.3 Pathogenicity

Pathogenicity is generally related to serogroup of the organism. The pathogenesis is similar in all species. Acute cases of hemorrhagic septicemia are clinically characterized by sudden onset of fever and death in about 24 h. On rangeland, animals may be found dead without any clinical signs. Affected animals have painful swellings about throat, dewlap and brisket, and have severe dyspnea. Haemorrhagic septicaemia is the same in

both cattle and other species. Death in hemorrhagic septicemia is due to respiratory failure and toxemia. (Al-Haddawi *et al*, 2001).

The pathogenesis of hemorrhagic septicemia in buffaloes infected with *P. multocida* is poorly understood. However, the characteristic of sudden onset leading to the rapid death of infected animals is due to endotoxic shock (Horadagoda *et al.*, 2001).

2.3. Diagnostic Techniques

The diagnosis of HS depends on the isolation of the causative organism, *P. multocida*, from the blood or bone marrow of a dead animal by culture and biological methods, and the identification of the organism by biochemical, serological and molecular techniques (DeAlwis, 1999). Clinical diagnosis is usually done by characteristic signs, gross pathological lesions, herd history, morbidity and mortality pattern, species susceptibility and age group affected (OIE, 2009). Laboratory confirmation is based on demonstration of Gram-negative, bipolar, pleomorphic bacterial cells in fresh blood smears following staining with Gram's, Leishman's or Methylene Blue. Bacterial isolation is based on standard techniques using BHI broth and sheep blood agar culture; subsequent tests may include mice inoculation, identification by colony morphology, pathogenicity testing, biotyping and anti- biogram determination (Holt *et al.*, 1994; OIE, 2009).

2.3.1 Bacteriological culture media

Nutrient agar, Blood agar (nutrient agar with 5% ox or sheep blood), Casein-sucrose-yeast (CSY) broth, CSY agar CSY-blood agar, Dextrose-starch agar, Enriched tryptophan agar as shown in (annex II) (with 5% sheep blood) and MacConkey agar is used as selective medium because *P. multocida* cannot grow on MacConkey. The colony morphology of *P. multocida* like shape, color, consistency, size and a gram staining of culture media is considered. Growth inhibition, motility, hemolytic property of growing bacteria was also seen (Wijewardana *et al.*, 1986). Microscopic examination of cultured

colony for gram negative, small size rod shape of coccobacilli was being appreciated. For mixed bacteria growth sub culturing was done to get pure colony.

2.3.2 Biochemical tests

Pasteurella species have both an oxidative and fermentative metabolism. The optimum growth temperatures can 37°C. Glucose and other carbohydrates are catabolised with the production of acid but no gas production (Songer and Post, 2005). Oxidase, Catalase and Indole test are the biochemical test used for *P. multocida* isolation (Janda and Mutters, 2010).

2.3.3. Molecular techniques

PCR assay for HS-associated type B serotypes of *P. multocida*, Townsend *et al.* (1998) described the development of PCR assays for species and type-specific identification of *P. multocida* isolates. Oligonucleotide primers designed during the sequencing of an HS associated type B specific clone isolated by genomic subtractive hybridization formed the basis of a type specific PCR assay for the detection of HS-associated type B serotypes of *P. multocida*. Polymerase chain reaction (PCR) assays for the specific identification of *P. multocida* and serotypes implicated in HS have been developed for identification within 3–4 h (Biswas *et al.*, 2004). The complexities associated with presumptive diagnosis by conventional methods have been overcome by more rapid PCR assays that can be applied directly on bacterial colonies, bacterial culture lysates, mixed bacterial cultures and infected clinical materials to permit rapid disease diagnosis (Choi and Chae, 2001; Shivachandra *et al.*, 2004,).

In most countries with endemic disease, molecular assays for primary screening of *P. multocida* isolates for rapid and specific diagnosis of HS have been introduced in parallel to existing conventional methods (Benkirane and De Alwis, 2002; Biswas *et al.*, 2004). A *P. multocida*-specific PCR assay developed by Townsend *et al.* (1998) has been widely

employed worldwide for initial species identification; this test gives a positive signal by an amplicon of 460bp with field isolates irrespective of

capsular type (A, B, D, E and F). Two independently isolated gene sequences unique to HS-causing serogroup-B *P. multocida* have been utilized in the development of serogroup B-specific PCR assays. The Haemorrhagic septicaemia serogroup-B developed by Townsend *et al.* (1998) remains highly specific for HS causing serogroup B serotype 2 or 5, and identifies target strains by the amplification of an approximately 620bp fragment. The primer pair KTSP61-KTI72 specifically amplifies a product of approximately 560bp in all *P. multocida* isolates possessing the type B capsular antigen and either type 2 or 5 as the dominant somatic antigen (Townsend *et al.*, 1998).

Table 1: Oligonucleotides used for the amplification of species-specific and virulence associated genes of *Pasteurella multocida*.

Oligonucleotides	Size of product (bp)	Gene	Gene function (Description)
KMT1/SP67	460	KMT-sequence	Capsule serotypes
ToxA fwd/rv	866	toxA	Toxins (Dermo necrotic toxin)
Synth ewd/rv	1264	pmHAS	Hyaluronidase (Hyaluronan synthase)
PMOut/ rv	219	Omp87	Protectins(Outer membrane protein 87)

Source: (Deressa *et al.*, 2004 ; Hawari *et al.*, 2008 ; Xibiao *et al.*, 2009)

2.4 Treatment and prevention

2.4.1 Chemotherapy

Treatment is of little use once signs of HS have appeared, but could be effective in the early stages (OIE, 2009). Practically, the procedure employed is to check the rectal temperature of all in-contact animals after the first case has been reported, and to start antibiotic therapy immediately (Benkirane and De Alwis, 2002).

Generally, clinical cases of HS are extensively treated with Oxytetracycline, Co-trimoxazole, a combination of Penicillin and Streptomycin or Sulphaquinoxaline. However, in the recent times, shifts in antibiotic sensitivity spectrum of *P. multocida* are evident and there have been increases in incidences of high morbidity and mortality (Biswas *et al.*, 2004; Kumar *et al.*, 2004).

Compromised therapy may be due to emergence of multi-drug-resistant strains as a result of widespread use of antibiotics as a supplement in feed, for prophylaxis and growth promotion, for subtherapeutic use in the feed, and for metaphylactic antibiotic therapy. The studies identified a rise in multiple drug-resistant strains of *P. multocida* (Biswas *et al.*, 2004), especially for Sulphadiazine, the drug of choice in the field for control of HS, as well as many other commonly used antibiotics such as Amikacin, Carbencillin, Erythromycin and Penicillin, where there is gradual development of resistance (Kumar *et al.*, 2004).

Strains of *P. multocida* vary in susceptibility to chemotherapeutic agents. Most *P. multocida* isolates were resistant to sulphadiazine with varied sensitivity to Chloramphenicol, Gentamicin, Tetracycline, Kanamycin, Penicillin-G, Streptomycin Sulphonamides and Trimethoprim as assessed by agar dilution and determination of the susceptibility of Transconjugants and transformants by disc diffusion assay (De Alwis, 1999; Shivachandra *et al.*, 2004). However, *P. multocida* is known to develop resistance against some antibacterial agents. In a study carried out in India, resistance was reported

specifically to amoxicillin (100%), metronidazole (67%), sulphadiazine (100%) and trimethoprim(67%) (Prabhakar *et al.*, 2010). Similarly, resistance against arbenicillin (100%), cephalexin (80), doxycycline (80%) and nalidixic acid (67%) was record in Iran (Zuber, 2009).

2.4.2 Vaccination

Vaccination of susceptible animals in endemic areas is the only practical approach to prevent HS (Benkirane and De Alwis, 2002; OIE, 2009). In most countries, various formulations of HS vaccines are available for vaccination of animals (Verma and Jaiswal, 1998). The Previous investigations on isolation and serotyping of *P. multocida* from cases of HS reveal the presence of other serotypes (A: 1) as well (Kumar *et al.*, 2004). Moreover, it has been observed at field level that despite proper vaccination, HS does occur among cattle and buffaloes indicating the imminent need to revamp/change existing vaccines as well as vaccination strategy against HS (Biswas *et al.*, 2004).

In control programmers, during an outbreak, one should resort to immediate whole herd vaccination, irrespective of previous vaccination history. The use of either broth bacterin or oil adjuvant vaccine is recommended. Sanitary measures include early detection and isolation of new cases and their immediate treatment with antibiotics, deep burial of carcasses or incineration, and the prevention of movements of animals to disease-free areas. In endemic areas the prevention measures include, vaccination on a routine prophylactic basis, preferably two to three months before the high-risk season (monsoon), awareness of the disease among farmers backed up by a good disease reporting/disease information system, segregation of animals from endemic and non-endemic areas to avoid contact with carriers (Benkirane and De Alwis, 2002). In Ethiopia there are 600 HS disease outbreak reports in 2010/2011 fiscal years. To overcome these outbreaks Ethiopia have produced and distributes 5,008,700 doses attenuated Bovine pasteurellosis vaccine to all the regions in the country for prevention and control HS of (Ethiopia animal health yearbook, 2011). Number of vaccinated animals is not included in the yearbook report.

3. MATERIALS AND METHODS

3.1. Study area

Assosa town is the administrative centre of the Benishanigul Gumuz Regional State, and located 676 km West of Addis Ababa. Assosa zone is one of the 3 administrative zones of Benshangul Guzum Regional State. It is found in the Southwest part of the region. The study was conducted from November 2016 to May 2017 at two selected districts in Assosa Zone of Benshangul Gumuz Regional State, namely Bambasi and Assosa. Assosa district is composed of 70 administrative peasant associations and A assosa town “kebeles” (Assosa District livestock and fishery development Office), which is located at 9.60° and 10.45° N and 34.20° and 34.58° E longitude. The altitude of Assosa ranges from 580 to over 1544 meter above sea level.

The total area of the district is 2317 Km^2 of which area is characterized by low land plane agro-ecology according to National Meteorology Service Agency (NMSA, 2007) with average annual rainfall of 1316 mm with uni-modal type of rainfall that occurs between April and October. Its mean annual temperature ranges between 16.75°C and 37.9°C . The total human population of the district is 104,147. Assosa district has the livestock population 36,916 cattle, 23,500 goats, 14,325 sheep, and 5,890 donkeys, 35125 poultry (Assosa District Office of Livestock and fishery resource development, 2017 and personal communication).

Bambasi district is located in Benschignigul Regional State Southernwest of the Assosa zone and 616 km West of Addis Ababa at 9.45° - 9.75° N and 34.35° - 34.88° E with a minimum and maximum altitude of 1350m and 1770 m above sea level. The district is composed of 42 administrative peasant associations and 2 Bambasi town “kebeles.” The total area of the district is 2100 Km^2 of which the average minimum and maximum annual rainfall are 900mm and 1200mm; while the average minimum temperature is 23°C and maximum

temperature is 32⁰C. The total human population of the district is 62693. Bamibasi Woreda has the livestock population 38964 cattle, 11,990 goats, 3452 sheep, 1995 donkeys, and 38442 poultry (CLSI, 2012).

To represent the study area 14 “kebeles” from Assosa and 13 “kebeles” of Bambasi districts were selected. These districts were selected on the basis of history for presence of cattle pasteurellosis/HS/, cattle movement to market, grazing land, watering points and size of cattle population by the district office of livestock and fishery development. The study areas of both districts were similar and which is low land plane agro- ecology. The map below shows both Assosa and Bambasi districts (Assosa Zone Office of Livestock and fishery resource development and personal communication)

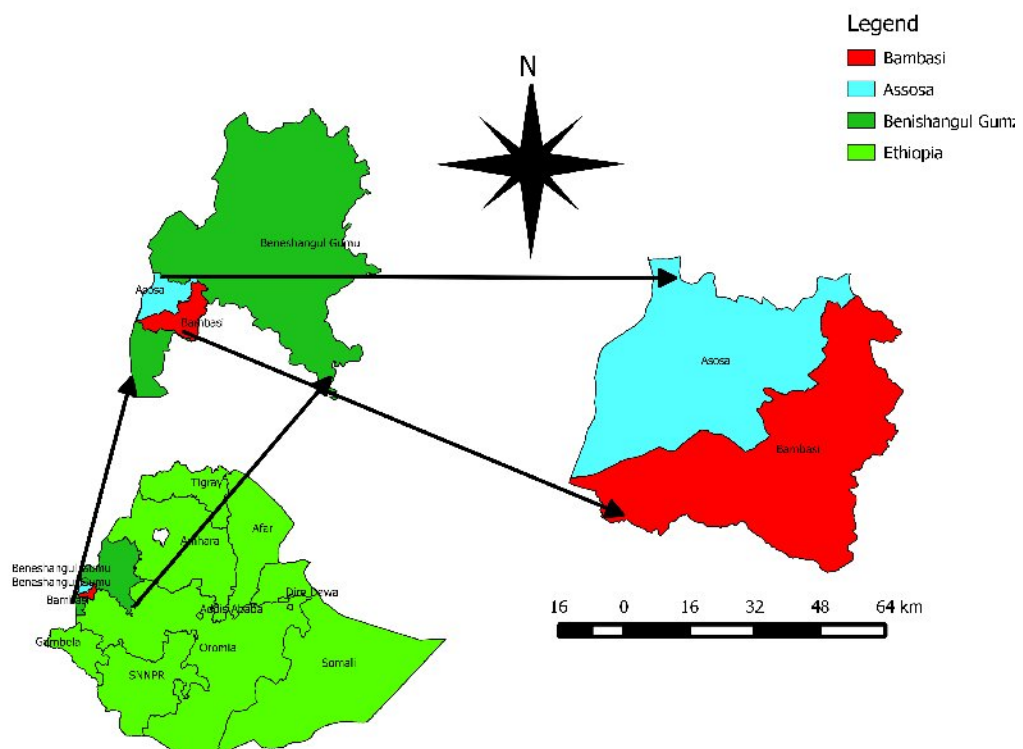


Figure1: Map of the study area showing relative location of Assosa and Bambasi districts in Benshangul Gumuz Regional State

Source: (Mulaw *et al.*, 2011)

3.2 Study population

The study population was HS infected cattle which are indigenous breed found in selected districts. For the Isolation and identification of *P. multocida* with the emphasis of Serotype B2 from indigenous cattle breeds, different risk factors were considered. These factors include sex, age, body condition, herd management system, herd size and vaccination history.

3.3 Study design

The study design was a cross-sectional study and it was designed to determine prevalence of HS and to isolate *Pasteurella multocida*.

3.4. Sample size determination

Since purposive sampling technique was used, samples were collected from areas with outbreaks of HS. Hence, the number of cattle sampled from Bambasi and Assosa district was 197 and 187 respectively based on number of sick animals that were considered as outbreak.

3.5. Sampling methods

The districts were selected purposively since each district has high cattle population, large market where cattle come from different Oromia districts and Assosa zone make close contacts to each other. Individual animals were selected purposively based on the presence of clinical sign of HS and those animals which were contact with sick once also sampled this was either non vaccinated or vaccinated.

3.6 Study methods

3.6.1 Sample Collection

Each and every sampled cattle was examined clinically for symptoms of HS and any other disease conditions and the findings were recorded. Sex, age, breed, body condition, management system, vaccination history of the sampled animals were recorded. Sample collections procedure involved blood collection from jugular vein or nasal swab after disinfecting the skin around sampling site or around the noses by 70% ethyl alcohol. The sample was taken deep in to nasal cavity by 5cm long swab after disinfecting. Samples were collected from clinical sick cattle which were selected purposely from the two study districts in a separate heparinized vacutainer tube for blood sample and in test tube with 6ml saline for swab which were labeled and coded immediately after sample collection.

The vacuteiner containing blood sample were preserved under cold chain of ice pack and swab sample containing Transport enrichment medium (TEM) or Modified Stuart's or Amie's transport medium in nutrient broth. The samples were transport to Assosa Regional Veterinary Laboratory for culturing and then isolation and identification was conducted. In the laboratory nasal swabs were incubated immediately at 37⁰C for 24 hours in incubator. From febrile individual whole blood was collected also for bacteria culture and for appreciation of bipolar by methylene blue staining (Ashraf *et al.*, 2011).

3.6.2 Bacterial isolation

After 24 hours of incubation of nasal swabs in nutrient broth were streaked onto sheep blood agar plates and again was incubate at 37⁰C for 24 hrs. The blood sample was streaked on blood agar media immediately after arrival to laboratory. After 24 hour incubation the colony color, shape, size, consistency, odder, smoothness, and roughness was characterized. Gram stain was used to identify gram negative, short rod, cocobacilli. Then for pure colony growth onto sheep blood agar plates was subcultured at 37⁰C for 24

hour. Presence and absence of haemolysis was visualized and those isolates which were identified as gram negative was further identified by molecular detection method. After CSY media preparation, the pH of the media was adjusted to 7.3-7.4 and autoclaved at 121°C and 15 bar pressure for fifteen minutes. After cooling of the media, a colony of the *P. multocida* was inoculated and all the flasks were incubated at 37°C in incubator shaker. Dry cell mass were checked after 24 hours of the bacterial culture. MacConkey agar was used as a selective medium because *P. multocida* as do not grow on it (EA, 2002).

After 24 hours incubation of a whole blood or nasal swab sample on blood agar or nutrient agar mixed bacterial colony was observed in almost all of the samples. The three morphologically similar pasteurilloceae genera and species were expected to grow upon sub culturing of colonies on to blood agar and MacConkey agar at the same time. Cultures with colony characteristics of round (smooth) edge, greyish color, small to moderate size and mucoid consistency which were either hemolytic or non haemolytic, and not grow on MacConkey agar or grow on Mac Conkey agar were observed.

3.6.3 Biochemical tests

For further identification of the culture and Gram stain bacteria which were suggestive of *pasteurella/Mannheimia/Bibersteinia*, different biochemical tests were conducted by selecting those which aid in differentiation among *pasteurella/ Mannheimia/ Bibersteinia* genera and species. These included Indole and Catalase test fermentation of maltose, lactose, glucose, sucrose, trehalose and Oxidase, Sorbitol, and motility tests. The data obtain were recorded and compared for confirmation of the isolates to which species they were belong.

The oxidase test was used to determine those organisms which possessed the cytochrome oxidase enzyme. The test was used as an aid for the differentiation of Neisseria, Moraxella, Campylobacter and Pasteurella species (oxidase positive).

Wet filter paper was also used. A strip of filter paper was soaked with a little freshly made 1% solution of the reagent. A speck of culture was rubbed on it with a platinum loop. A positive reaction was indicated by an intense deep-purple color, which appeared within 5-10 seconds, a “delayed positive” reaction by coloration in 10-60 seconds, and a negative reaction by absence of coloration or by coloration later than 60 seconds.

Direct Plate Method was used by adding 2 -3 drop of reagent directly to suspect colonies on an agar plate. The Kovac’s oxidase reagent was used and the result was seen with in 5 to 10 minutes dark purple color change as positive reaction. The Swab method was also done by dipping swab into reagent and then touches an isolated suspect colony and was observed for color change within 5-10 seconds and it’s result was similar with wet filter paper.

Catalase test was used by placing a small amount of growth from culture onto a clean microscope slide. To avoid a false positive result it was not used metal loop. A few drops of H₂O₂ were added onto the smear. It was mixed with a tooth pick. Since the metal loop gives false positive result when we use with H₂O₂, it was avoided metal loop or needle use with H₂O₂ in this test. A positive result was shown the rapid evolution of O₂ as evidenced by bubbling. A negative result was no bubbled or only a few scattered bubbles. Biohazard glasses were disposed in disposal container.

Indole test was used on a sterilized test tube containing 4 ml of SIM broth culture. Inoculate the tube aseptically by taking the growth from 18 to 24 hrs culture. Incubate the tube at 37°C for 24-28 hours. 0.5 ml of Kovac’s reagent was added to the SIM broth culture. The result was read for the presence or absence of red ring at the top of broth. 384 So among 384 sample tested only 13 were indole positive. *P. multocida* was Indole positive and *Actinobacillus* spp and *Manhemia haemolytica* were Indole negative (HPA, 2010).

3.6.4. *In vitro* Antimicrobial Sensitivity Testing

For antimicrobial susceptibility evaluation study, bacterial cultures were identified by their species were used to check species which are resistant or susceptible to commonly used antibiotics in the selected districts and to identify a drug which is efficient for *P. multocida*/HS/. In the laboratory, isolate colonies were suspended in nutrient broth (approximately 0.5 McFarland turbidity) and were spread/ swabbed onto a media (Mueller-Hinton agar) which was dispensed on a Petri dish. Then antimicrobial discs were fixed individually at different sites on the surface of inoculate agar plate evenly. Finally it was allowed growing at 37⁰C overnight and its zone of complete inhibition was measured to nearest millimeter using a ruler by holding on the back of inverted Petri dish. The result was compared to the standards (Zone Diameter Interpretive Standards and equivalent Minimum Inhibitory Concentration Break Points of the NCCLS Performance Standards for Antimicrobial Susceptibility Testing) and the species of the isolates were related with a drug effective against them or with a drug to which they are resistant (Kehrenberg, 2001).

3.6.5 Molecular detection of *P. multocida*

After bacteria culture and biochemical tests were done. Those gram negative and morphologically identified *P. multocida* specifically Oxidase, Catalase, Indole and Sorbitol positive but negative for lactose and maltose, non motile and non hemolytic, isolates were selected for Molecular detection. Conventional PCR for *P. multocida* type B6/2/ isolation and identification test were done by taking the bacteria sample from nutrient broth which were inoculated from study area isolates culture media kept at +4⁰C freezer and transported under cold chain to National Veterinary Insitute (NVI) for molecular detection.

At NVI, the isolates DNA were extracted by heating the isolates in the broth agar media of 200µl aliquots each for 15 minutes at 100⁰C. Then salt and detergent solution containing compound sodiumdodecyl sulfate (SDS) were added to break down and

emulsify the fat and protein of cell membrane of bacteria. Finally alcohol was added to precipitate the DNA from the solution by floating the DNA on the top of solution since the alcohol is lighter than water. The separated DNA were spooled on stirring rod or filter sieve and pulled from the solution and it was washed by buffer solution to remove the added alcohol and SDS which might interfere the result during PCR reaction in agarose gel diffusion. After washing DNA the extract was centrifuged at 12,000 rpm for 3 minutes and dry and kept under +4°C freezer.

Next to DNA extraction, master mix preparation which was done by adding RNase free water 3µl x 8 = 24µl for total reaction, Primer-KTT 72-Fow-5pm/µl 5'-AGGCTCGTTT-GGATTATGAAG-3' 2µl x 8 = 16µl, Primer KTSP 61-REV-5pm/µl 5'-ATCCGCTAACACACTCTC-3' 2µl x 8 = 16µl, IQ Super mix 10µl x 8 = 80µl and Add Template (DNA 3µl for each isolate, mixed by vortex and centrifuge mixture 12000 rpm for 30 seconds.

After master mix preparation was finished the PCR reaction was ran using thermo cycler machine. It was done for denaturation (at 95°C for 5 minutes), annealing (at 55°C for 1 minute and extension/elongation (at 72°C for 1 minute) as shown in (annex X) which require 40 cycles to complete the reaction and 122 minutes were required to finish the reaction. Finally the agarose gel was prepared by adding 1.5% agarose gel for 20 minutes to form viscous then it was added 4µl Gel red with Loading dye, 10 µl PCR product and 10 µl markers (Ladder). It was ran Electrophoresis for 1 hour using 120 voltage. Stained with Ethidium bromides and visualized by UV illumination. It was around 620bp positive result as shown in figure 9.

3.7 Data management and Analysis

For interpretation of the results, the whole data was entered into the Microsoft Excel sheet Data management and Analysis t, and then it was analysed using SPSS version 20. The association between the dependent variables and independent variables (age, sex, body condition, vaccination status and management practices was analyzed. Chi-square (χ^2)

tests for repeat measure was used to test relationship between dependent variable (*P. multocida* distribution) and different independent host and environmental factors. For the antimicrobial susceptibility evaluation test, the diameters of zone of inhibitions were assumed as the dependent variables. Antibiotic susceptibility was determined by comparing zone of inhibition of each drug with 0.5 Mcfarland turbidities. SPSS version 20 software was used. For all analysis, 95% CI and $P < 0.05$ was set for statistical significance of an estimate. Regarding the molecular detection, the banding patterns of individuals' strains were scored based on the presence or absence of the bands with the appropriate base pairs.

4. RESULTS

In the present study, a total of 384 clinically sick cattle belonging to small households were sampled for whole blood and/or nasal swab at Bambasi and Assosa Districts, and whole blood or swab samples were processed microbiologically for isolation and identification of *P. multocida*. Variations in sex, age, management, animals' body condition (availability of improved feed and water supply) and vaccination status were used as risk factors for assessing the occurrence of hemorrhagic septicemia in cattle. Based on the bacterial identification and biochemical test, 13 isolates (3.38%) that were found non-hemolytic (Figure 4) on blood agar and didn't grow on MacConkey agar were grouped as *P. multocida*.

4.1. Culture Characteristics

Out of 384 sample collected out break HS, 13 isolates were grown on blood agar nutrient agar and the grown isolate were subjected to sub culturing for pure colony appreciation and bacterial colonies were characterization as rod coccobacilli, smooth and rough colony, gram stain was shown gram negative, the 13 sample isolates were not grown on MacConkey, on blood agar hemolysis of 13 isolate were not seen (Figure 4). After each bacterial colony was characterized, identification of bacteria through primary and secondary biochemical tests was conducted. Moreover, *P. multocida* was identified based on methylene blue staining showing bipolar staining. So based on above activities *P. multocida* was identified from 13 isolates (Figure 2,3,5,6 and7).

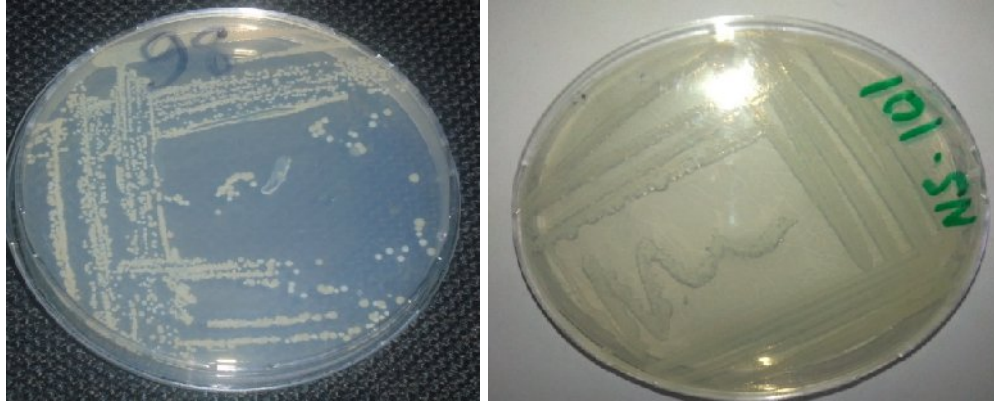


Figure 2: Colony of *P. multocida* grown on nutrient agar

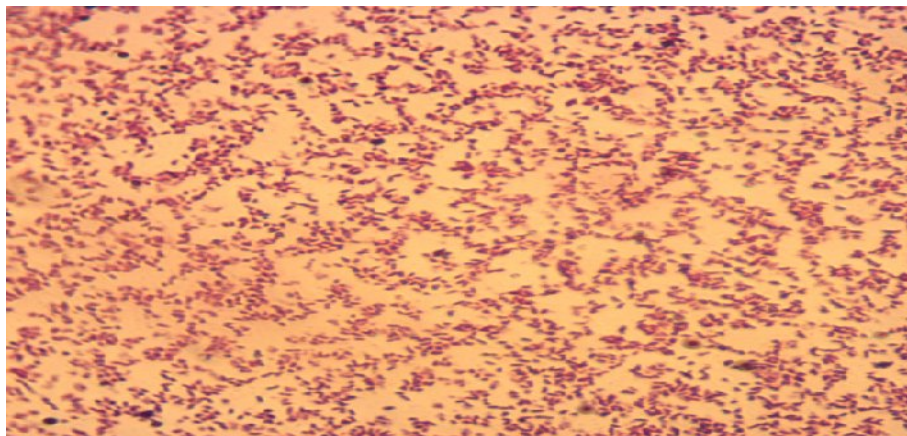


Figure 3: Gram stained isolate of *P. multocida*



Figure 4: Growth of *P. multocida* on blood agar without hemolysis

4.2. Biochemical activities of the isolates

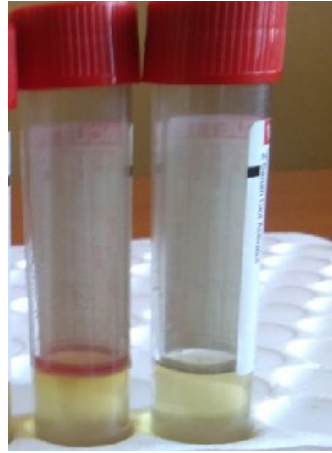
The results from the multiple biochemical tests are illustrated in the following figures.



Figure 5: Oxidase test positive of *P. multocida*



Figure 6: positive catalase test of *P. multocida*



A B

Figure 7: Indole positive test shows red ring formation at the top of SIM broth (A) where as Indole negative is indicated in B.

Table 2: Distribution of total *P.moltocida* in cattle populationin Assosa and bambasi “woreda” association with different epidemiological risk factors

Factor	Level	No of examined	Prevalence (%)	X ²	P-value
Districts	Assosa	187	9 (4.81%)	12.39	0.0983
	Bambasi	197	4 (2.30%)		
Age	Young age <= 2year	113	9 (7.96%)	5.33	0.070
	Adult age > 2<=5 years	179	4 (2.23%)		
	Old > 5 years	92	0 (0.0%)		
Sex	Male	178	4 (2.25%)	2.077	0.357
	Female	206	9 (4.67%)		
Management	extensive	211	10 (4.74%)	58.92	0.000
	intensive	173	3 (1.73 %)		
Body condition	Poor	126	7 (5.56%)	87.16	0.000
	Medium	114	3 (2.63%)		
	Good	144	3 (2.08%)		
Vaccination	Nonvaccinated	126	8 (6.35%)	160.2	0.000
	Vaccinated	258	5(1.94%)		

4.3 Antimicrobial susceptibility testing

Table 3: Antibiotic susceptibility profiles of *Pasteurella multocida* isolate
From nasal or whole blood of cattle Assosa Bambasi districts

Antimicrobial agent	Content	Zone of inhibition in mm		
		Resistant	Intermediate	Susceptible
Tetracycline	10µg	9 (69.2%)	2 (15.4%)	2 (15.4%)
Chloramphenicol	30µg	2 (15.4%)	8 (61.5%)	3 (23.1%)
Kanamycin	30µg	8 (61.5%)	2 (15.4%)	3 (23.1%)
Clindamycin	10µg	2 (15.4%)	4 (30.8%)	7 (53.8%)
Gentamycin	10 µg	7 (53.8%)	3 (23.1%)	3 (23.1%)
Sterptomycin	30µg	2 (15.4%)	3 (23.1%)	8 (61.5%)

After overnight incubation of representative isolates on Mueller Hinton agar plates, it was used according to NCCLS's Standard on zone size interpretation chart (Appendix-IX). Almost all *P. multocida* isolates displayed high resistance to various discs used during the test. When drug sensitivity were concerned the antibiotics available in study area used. Attached antibiotic impregnated discs (Tetracycline, Sterptomycin, Gentamycin, Clindamycin and Chloramphenicol) fixed at different sites on Muller Hinton agar, a zone of bacterial growth inhibition was measured to the nearest millimeter and the result was as presented in the (table 3).

The drug sensitivity tests from isolates in selected study districts were resistant to Tetracycline (69.2%), kanamycin (61.5%) and Gentamycin (53.8%). Whereas susceptible to Sterptomycin (61.5%) and clindamycin (53.8%) and chloramphenicol (61.5%) intermediate susceptible.



Figure 8: Antimicrobial drug sensitivity test on sample from Assosa and Bambasi districts. 1) TTC, 2) Sterptomycin, 3) Clindamycin, 4) Gentamycin, 5) Kanamycin

Table 4: Multi drug resistance of isolates (MDR) of *P. multocida*

pattern	Multi drug resistance Pattern	Total NO
For three	TTC,S,GEN (1), TTC,KA,GEN (6)	7 (53.8%)
For four	TTC,KA,GEN,CLI (2)	2 (15.38%)

The overall MDR of isolates of *P.multocida* was 9(69.2%) (Table: 6) and the remaining 4 (30.77%) isolates were non multi drug resistant. were non MDR.

Out of 13 isolates 9 were MDR to different common in vitro used discs in study areas. Out of these isolates 6 (46.15%) were resistant to 3 drugs which were Tetracycline, Kanamycin and Gentamycine and 1 (7.7%) isolate was resistant to 3 drugs such as Tetracycline, Streptomycin and Gentamycine. On the same ways other 2 isolatse were again resistant to 4 drugs like Tetracycline, Kanamycin, Gentamycine and Clindamycin. Generally 9 (69.23%) isolates shown multi drug resistant to in vitro antimicrobial discs tested in study area(Table 4).

4.4. Molecular detection of *P. multocida* isolates

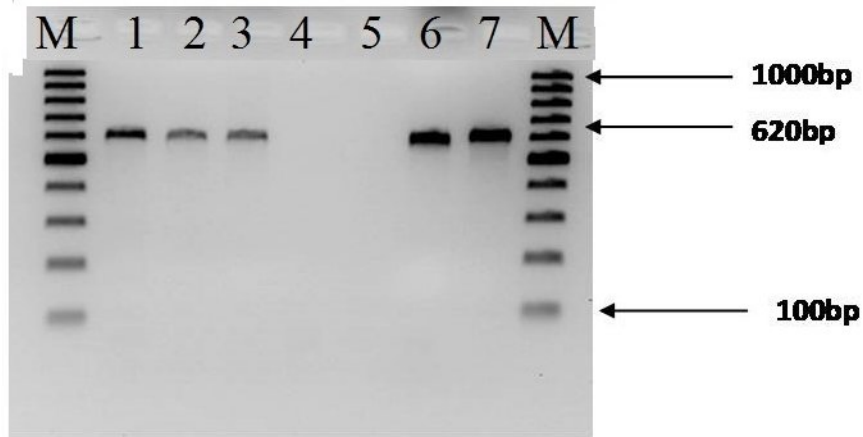


Figure 9: Conventional PCR showing the amplification of 620 bp fragment of *p. multocida* type B 6/B2 from the samples taken from HS infected cattle

Key: Lane 1: DNA marker, lanes 1-3: samples (extracts from the isolates), lane 4 -5: negative controls and lane 6 and 7: known positive controls.

The PCR result indicated that the isolates from Assosa and Bambasi districts were positive 620 bp fragments of *P. multocida* type B6/B2 which were the causative agents of HS (Figure 9).

5. DISCUSSION

In this study, *P. multocida* was isolated and identified from suspected cases or out breaks of HS of cattle in Assosa and Bambasi ditrictes by conventional bacteriological method. On blood agar media, the isolated bacteria produced small, round, grayish colonies with no hemolysis. Gram's staining revealed presence of Gram negative small rod shaped bacteria. The isolated organisms fermented gulcose, sucrose and but not maltose and lactose. These fermented sugars produced acid without gas. The organisms also gave positive indole test and negative methyl red (MR). All these findings are similar to those reported by Cheesbrough (2006) as specific for *Pasteurella* spp. To the best of our knowledge, this is probably the first study in Assosa and Bambasi districts describing the PCR based detection of *P. multocida* from suspected clinical cases or disease out breaks of HS. The isolated organisms were also found Gram negative and morphologically they were coccobacillary in shape. On Blood agar the isolated organisms produced grayish, opaque, circular, translucent colonies and with no hemolysis that resembles the characteristics colonies of *P. multocida*, as described by Choudhury *et al.* (1985) and Rahman *et al.* (2016). Biochemically the isolated organisms were found positive for oxidate, catalase, indole tests, negative from MR.

The organisms were found positive for sucrose, dextrose, mannitol and negative for lactose and maltose. Results of these biochemical tests suggested that the isolated organisms could be considered as *P. multocida*. Shivachandra *et al.* (2011) also reported similar biochemical characteristics for *P. multocida* type B. characteristics colonies of *P. multocida*, as described by Choudhury *et al.* (1985).

The overall prevalence of *Pasteurella multocida* in nasal swab samples collected from 384 clinically sick cattle 13 (3.39%) were isolated. Assosa and Bambasi districts were in the same low land plane agro-ecology even though the prevalence of HS was greater 9 (4.81%) in Assosa district than Bambasi district 4 (2.30%). The finding of this is in agreement with Prevalence of 3 (30. 00%) study of *pasteurella* spp. conducted by

Ahmed, *et al.*, (2015) in Baghdad, Iraq. But this study percentage is very less when compared with the previous ones.

Age distribution of the total positivity shows that the isolation rate of the agents were not statistically significant among different age groups ($P=0.070$). The status of the isolation rate /prevalence /of the agent was decreased when age of cattle increase so older animals are relatively resistant to HS than younger.

On the same ways age distribution of the total positivity shows that the isolation rate of the agent is not statistically significant among different age groups ($P=0.070$.) But the status of the isolation rate /prevalence /of the agent decreased when age of cattle increase so older animals are relatively resistant to HS than younger. Younger cattle less than or equals 2 year old were 9 (7.96%) and adult cattle greater 2 year and less than or equal to 5 years old were 4(2.23%) positive for isolated agents older cattle greater than 5 years were 0 (0.00%). On this study anybody can conclude that young cattle were twice more susceptible to the isolates than the adult cattle. This study shows agreement with young age susceptibility report by (De Alwis, *et al.*, 1976)

Sex distributions of the agent show no statistically significant variation even if there were variations observed. Females are 9 (4.69%) positive while male are 4 (2.25%). It is possible to say in study area females was more susceptible than male cattle. Even though there were not previous result discussed sex risk factors for occurrence of hemorrhagic septicemia, the sex difference might have seen in female during lactation, pregnancy and heat period/on estrus/ due to compromised natural immunity leads to stress on female cattle than male. This is similar with the study conducted to isolate pneumonic *M. hemolytica* and *P.multocida* by Dereje *et al.*, (2013) in Badele district from apparently health cattle.

Vaccination practices have shown statistically significant which were similar $P=0.000$ for HS occurrence. 8 (6.35%) of non-vaccinated and 5 (1.94%) of the vaccinated animals was susceptible to isolates. This study result was related with reported of veterinary

immunology (Dowling, *et al.*, 2004). It is concluded that HS can be prevented/minimized its occurrence by proper and seasonal vaccination program.

Cattle with good management practices were less susceptible to the isolated agent of HS than the poorly managed cattle. Cattle with poor body condition 7(5.56%) were also affected by the isolates *P. multocida*. Whereas cattle with medium body condition were 3(2.63%) and cattle with good body condition 3 (2.08%) were infected by isolated agents. This study is related to the positivity report of disposing factor of Sheikh *et al.* (1996) in India.

Out of 13 positive animals to HS those with poor body condition, poor management practices and non vaccinated were about 1/3 (30.77%). This study is in agreement with the report of De Alwis and Sumanadasa (1982).

Antibiotics are important remedies in modern farm animal production. The use of these chemical agents should be based on an accurate diagnosis since there is an increasing incidence of bacterial resistance to antibiotics in humans. This phenomenon was attributed to the use of anti-microbial drugs in food-producing animals. Also, there is a concern about possible residues in animal products.

Out of 5 commercially available discs used for drug sensitivity in vitro test 60% was shown resistance to the *P. multocida* isolates identified by biochemical test and conventional PCR detected. The drug sensitivity test discs diffusion for isolates in selected study districts were resistant to Tetracycline (69.2%) and kanamycin (61.5%) and Gentamycin (53.8%).which were agreed with study conducted by Zuber (2009) in Iraq. Whereas susceptible to Sterptomycin (61.5%) and clindamycin (53.8%). This study also agreed with De Alwis, (1999); Shivachandra *et al.*, (2004) in Sri-lanka, Refsdal, (2000) in Siwa Oasis, Egypt. General Tetracycline, Kanamycin and Gentamycine were less effective to the isolates in study area where as Serptomycin, and Clindamycin were the drug of choice for study areas.

In general one can see that there are multiple drug resistance developed by *P. multocida*/HS/ due to long time use of same antibiotics for given animal population as food staff, therapeutics and the nature of bacteria that can form mutation through time under harsh environmental conditions this was agreed with Biswas *et.al* 2004 gradual development of multi-drug resistance in Asia. Generally, Out of 13 isolates 7(53.8%) isolates resist for 3 drugs and 2 (15.38%) isolate resist for 4 drugs. This study has shown that multi drug resistance which was similar with kumer (2004) study reports. In this study only 30.77% of the isolates were shown non multi drug resistance.

Conformation of the isolated organisms as *P. multocida* type B, the causal agent of haemorrhagic septicemia in cattle were done based on PCR as described by Townsend *et al.* (1998) PCR was carried out to confirm the isolate as *P. multocida* type B using the specific primers pairs KTT72 5'-AGG-CTC-GTT-TGG-ATT-ATG-AAG-3' and KTSP61 5'-ATC-CGC-TAA-CAC-ACTCTC-3'. Briefly, for PCR bacterial DNA was first extracted using Wizard genomic DNA Purification kit. Extraction of DNA and its quality was checked by running 5 µL suspension of the extracted DNA in a 1% (w/v) agarose gel. All the PCR was done in a final 25 µL volume containing 12.5 µL PCR mastermix (Promega, USA) 1 µL of each primer (10 pmol), PCR grade water 8.5 µL and DNA template 2 µL. The thermal profile used for the *Pasteurella* genus specific PCR was performed as Townsend *et al.* (2001).

HS causing type-B-specific PCR remains 100% specific for isolate of *P. multocida*. Type B cultures with the predominant somatic antigen being either type 2 or 5 are identified by the amplification of a ~620bp fragment with the KTSP61 and KTT72 primers. The isolates in study area was detected by conventional PCR reaction which result 620bp as *P. multocida* serotype B6/ B2 positive which was similar with Haemorrhagic septicemia serogroup-B developed by Townsend *et al.* (1998) remains highly specific for HS causing serogroup B serotype 2 and identifies target strains by the amplification of an approximately 620bp fragments with the KTSP61 and KTT72 primers.

6. CONCLUSION AND RECOMMENDATIONS

It is concluded that both districts of study area was identified as hemorrhagic septicemia positive which was the major disease of cattle in the area and *P. multocida* is the most common in young as age of animals were the risk factor of the disease. It also demonstrated that hemorrhagic septicemia is a highly complex multi factorial disease particularly in cattle which could be associated with stress, compromised immunity, adverse environmental condition previous illness (co-infection). The isolates in study area were shown susceptibility to some of the drugs on the bases of in vitro test. However, the isolates were shown resistance to some of the antibiotic dics. Moreover, There must be an integrate animals management system, vaccination, controlling of the predisposing factor and use of broad spectrum antimicrobials as a prophylactic and early treatment of sick animal is suggested. There were developments of MDR with in isolates in study area which this pathogen is becoming resistant against the commonly used antimicrobial drugs.

Based on results of this study the following points are recommended:

- ❖ Public awareness must be created among the public about the major preventive measures against HS
- ❖ It should be known to the public that Hemorrhagic septicemia is a disease which can be prevented by implementing a management strategy which can avoid stress.
- ❖ Further investigation using modern techniques like DNA sequencing should be carried out as a confirmation of *P. multocida* strains that will ultimately helpful in designing an effective and efficient prevention and control options
- ❖ A continuous monitoring and evaluation of drug should be implemented.

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8. ANNEXES

Annex I: Field Data Collection Format

Date _____

District _____ Agroecology _____ Season _____

C o d e	O w n er	Sp eci es/ bre ed	A g e	S e x	Rx/Va ccine/ Other	Body condi tion	Managemet

Annex II: Laboratory Test Results Recording Format

Blood agar	Mac Conkey	Gram stain	Oxida se test	Tentat ive Dx	SIM media	Indol test	Catala se test	Sugar ferme ntatio n	Dx

Annex III: Summary of Culture characteristic and Biochemical tests used as reference at identification

Features	<i>P. multocida</i>
Haemolysis	-
Mac Conkey	-
Lactose (TSI)	-
Catalase	+
maltose	-
Indole (SIM)	+
urea	-
Xylose	-
Oxidase	+
Glucose	+
Sucrose	+
H ₂ S Production	-
sorbitol	+
Motility	-

Annex IV: Transporting media

A. Modified Stuart's transport medium

Sodium glycerophosphate	10.0 g
Sodium thioglycollate	0.5 g
Cysteine hydrochloride	0.5 g
Calcium chloride	0.1 g
Methylene blue	0.001 g
Agar	5.0 g
Distilled water	1 litre
pH	7.4 ± 0.

The above ingredients (or 16 g of the commercial dehydrated medium) are dissolved in 1 litre of distilled water. The mixture is then dispensed into 7.0-mL screw-capped bottles and sterilised at 121°C for 15 minutes

C. Transport enrichment medium (TEM)

Bacto agar	3.0 g
BHI broth base	37.0 g
Distilled water	1 litre

Mix well, autoclave to sterilise, cool to approximately 56°C and add the following antibiotics

per litre of BHI solution:

Annex V: Bacteria growth media

A. Nutrient agar (Oxoid, England)

Typical formula (g/l):

'Lab-Lemco' powder	1.0
Yeast extract	2.0
Peptone	5.0
Sodium chloride	5.0
Agar	15.0
Final pH	7.4 ± 0.2 at 25°C

Instruction for use:

Suspend 28g in 1 litre of distilled water. Bring to the boil to dissolve completely.

Sterilize

by autoclaving at 121°C for 15 minutes and display on sterile petridish.

B. Blood agar (nutrient agar with 5% ox or sheep blood)

Beef extract	5.0 g
Peptone	5.0 g
Sodium chloride	5.0 g

Agar	15.0 g
Distilled water	1 litre

C. CSY agar (Wijwardana et al. 1986)

Acid hydrolysate of casein	3.0g
Sucrose	3.0g
Yeast extract	5.0g
Sodium chloride, anhydrous	5.0g
Dipotassium hydrogen orthophosphate, anhydrous (K ₂ HPO ₄)	2.0g
Agar	15.0g
Distilled water	1 litre
pH	7.4+/-0.2

A greater benefit is derived if the first three ingredients are filter sterilised and added later to

the heat-sterilised medium.

CSY media: extract (0.5 g), casein hydrolysate (0.3 g), sucrose (3 g), anhydrous potassium dihydrogen phosphate (0.3 g) and sodium chloride (0.5 g), by dissolving the ingredients in 100 ml distilled water. While to check the effect of different concentrations of yeast extract, casein hydrolysate and sucrose, one lower and one higher concentration were prepared along with the control CSY media

D. MacConkey Agar

Ingredients Amount

Peptone (Pancreatic digest of gelatin)	17 gm
Proteose peptone (meat and casein)	3 gm
Lactose monohydrate	10 gm
Bile salts	1.5 gm
Sodium chloride	5 gm
Neutral red	0.03 gm
Crystal Violet	0.001 g
Agar	13.5 gm

Distilled Water Add to make 1 Liter

E. MacConkey Agar

1. Suspend 49.53 grams of dehydrated medium in 1000 ml purified/distilled water.
2. Heat to boiling to dissolve the medium completely.
3. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.
4. Cool to 45-50°C.
5. Mix well before pouring into sterile Petri plates

MacConkey Agar: Suspend 49.53 grams of dehydrated medium in 1000 ml purified/distilled water. Heat it to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 45-50°C. Mix well before pouring into sterile Petri plates (annex VI). Sodium chloride maintains the osmotic balance in the medium. Neutral red is a pH indicator that turns red at a pH below 6.8 and is colorless at any pH greater than 6.8. Agar is the solidifying agent (EA, 2002). This is used for *P. multocida* as selective medium that is *P. multocida* do not grow on this medium.

AnnexVI: Staining Procedures

A. Gram Stain

1. Place slide with heat fixed smear on staining tray.
2. Gently flood smear with crystal violet and let stand for 1 minute.
3. Tilt the slide slightly and gently rinse with tap water or distilled water using a wash bottle.
4. Gently flood the smear with Gram's iodine and let stand for 1 minute.
5. Tilt the slide slightly and gently rinse with tap water or distilled water using a wash bottle. The smear will appear as a purple circle on the slide.
6. Decolorize using 95% ethyl alcohol or acetone. Tilt the slide slightly and apply the alcohol drop by drop for 5 to 10 seconds until the alcohol runs almost clear be careful not to over- decolorize.
10. Immediately rinse with water.
11. Gently flood with safranin to counter-stain and let stand for 45 seconds.

12. Tilt the slide slightly and gently rinse with tap water or distilled water using awash bottle
13. Blot dries the slide with bibulous paper.
14. View the smear using a light-microscope under oil-immersion.

Annex VII: Biochemical test reagents procedures

Oxidase test: Share open dropper bottle. Open a new reagent droper only if there are none yet

a. To open an new reagent dispenser: Hold reagent dropper upright and point tip away from yourself.

Grasp the middle with thumb and forefinger and squeeze gently to crush the glass ampule inside the dropper. Tap the bottom on the tabletop a few times. Invert the ampule and squeeze gently for drop-by-drop dispensing.

- b. With a sterile swab, obtain a small amount of organism from an agar slant or plate.
- c. Place one drop of reagent onto the culture on the swab.
- d. Positive reactions turn the bacteria violet to purple immediately or within 10 to 30 seconds.

Delayed reactions should be ignored.

Catalase test:

- a. Place a small amount of growth from your culture onto a clean microscope slide. If using colonies from a blood agar plate, be very careful not to scrape up any of the blood agar blood cells are catalase positive and any contaminating agar could give a false positive.
- b. Add a few drops of H_2O_2 onto the smear. If needed, mix with a toothpick. Do not use a metal loop or needle with H_2O_2 ; it will give a false positive and degrade the metal.
- c. A positive result is the rapid evolution of O_2 as evidenced by bubbling.
- d. A negative result is no bubbles or only a few scattered bubbles. Dispose of your slide in the biohazard glass disposal container. Dispose of any toothpicks in the Pipet Keeper

Annex:VIII Phenotypic culture and biochemical test characteristics *P. multocida*

Phenotypic Characteristics	<i>P. multocida</i>
Blood agar (hemolysis)	Negative (-)
MacConkey (lactose fermenter)	No growth
Gram stain	Negative (-)
Colony colour	gray
Colony size	Small to medium
Oxidase	Positive (+)
TSI (lactose fermenter)	Negative (-)
Catalase	Positive (+)
Indole test (SIM)	Positive (+)
H ₂ S Production	Negative (-)
Motility	Negative (-)
maltose	Negative (-)
Trehalose	Negative (-)
Glucose	Positive (+)
Sucrose	Positive (+)

Annex IX: Standards for drug resistance interpretation.

A. Zone Size Interpretive Chart for Antimicrobials

Antimicrobial agents	disc potency	resistance	intermediate	susceptible
Clindaycin	10µg	< 15	16-18	>19
Tetracycline	10 µg	< 14	15-18	>19
Chloramphenicol	30 µg	<12	13-17	>18
Gentamycin	10µg	<12	12 -14	>15
Kanamycin	30 µg	<13	14-17	>18
Sterptomycin	30 µg	<11	12-14	>15