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Isolation, identification and antimicrobial susceptibility of *Pasteurella multocida* from cattle with hemorrhagic septicemia in Assosa and Bambasi districts, Benishangul Gumuz Regional state, Ethiopia

Yami Bote, Kibeb Legesse, Getahun Shawul and Asmelash Tassew

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 Polymerase chain reaction. 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 ssensitivity 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 regularly implemented.

Keywords: Antimicrobial sensitivity test, Benishangul Gumuz Regional State, Cattle Haemorrhagic Septicemia, Isolation, Pasteurella multocida, Polymerase chain reaction

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* [4]. Bacteriaof the family *Pasteurellaceae* are involved in a variety of economically important diseases in food-producing animals. Pasteurellosis is a multi-factorial respiratory disorder [6]. 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 [20]. 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 [29]. From these serotypes B2 and E2 are only the causative agents of HS in cattle mainly in Asia and Africa [17].
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* [23].

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 Foot and mouth disease, 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 [23]. 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 [18].

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 [26].

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 [11]. On the other hand, base line data about the status of HS in cattle is not available in Ethiopia so far except in [16].

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 susceptibility pattern of *P. multocida*

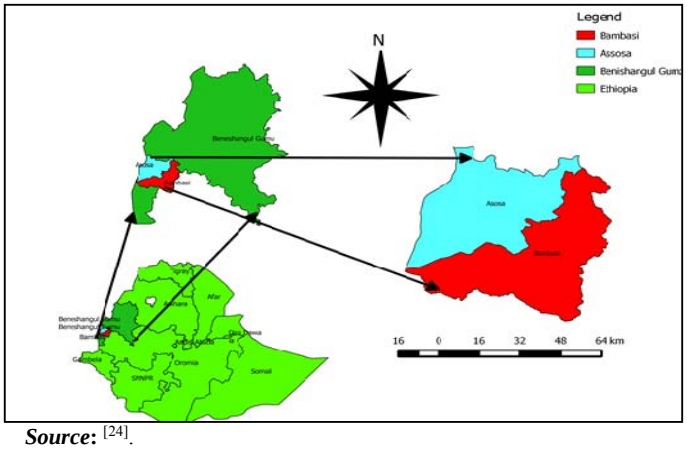
2. Materials and Methods

2.1. 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 4 assosa town "kebeles" [2] which is located at 9.60^0 and 10.45^0 N and 34.20^0 and 34.58^0E longitude. The altitude of Assosa ranges from 580 to over 1544 meter above sea level [25]. The total area of the district is 2317 Km^2 of which area is characterized by low land plane agro-ecology according to National Meterology Service Agency [25]. 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 [2] and personal communication).

Bambasi district is located in Benshnigul Regional State Southernwest of the Assosa zone and 616 km West of Addis Ababa at 9.45- 9.75^0N and 34.35-34.88^0E 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 23^0C and maximum temperature is 32^0C. 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 [3].

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 (Personal communication).



***Source*:** [24].

Fig 1: Map of the study area showing relative location of Assosa and Bambasi districts in Benshangul Gumuz Regional State

2.2 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.

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

2.4 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.

2.5 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.

2.6 Study methods
2.6.1 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 coducted. In the laboratory nasal swabs were incubated immediately at 37^0C 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 [1].

2.6.2 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 37^0C 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 37^0C 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 [15].
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 pasteurelloceae 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 MacConkey agar were observed.

2.6.3 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 H_2O_2 were added onto the smear. It was mixed with a tooth pick. Since the metal loop gives false positive result when we

use with H_2O_2, it was avoided metal loop or needle use with H_2O_2 in this test. A positive result was shown the rapid evolution of O_2 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 ringat the top of broth.384 So among 384 sample tested only 13were indole positive. *P. multocida* was Indole positive and *Actinobacillus* spp and *Manhhemia haemolytica* were Indole negative [19].

2.6.4 *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*. 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 37^0C 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 [21].

2.6.5 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 or B2 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 $+4^0$C 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 100^0C. 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 interfer 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 3µlx8=24µl for total reaction, Primer-KTT 72-Fow-5pm/µl 5'AGGCTCGTTT-GGATTATGA- AG-3'2µlx8=16µl, Primer KTSP 61-REV-5pm/µl 5'-ATCCGCTAACAC ACTCTC-3' 2µlx8=16µl, IQ Super mix 10µl x8= 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 95^0C for 5 minutes), annealing (at 55^0C for 1 minute and extension/elongation (at 72^0C for 1 minute) which requires 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.

2.7 Data management and Analysis

For interpretation of the results, the whole data was entered into the Microsoft Excel shee 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 (x^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.

3. 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 on blood agar and didn't grow on MacConkey agar were grouped as *P. multocida*.

3.1 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. 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.

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

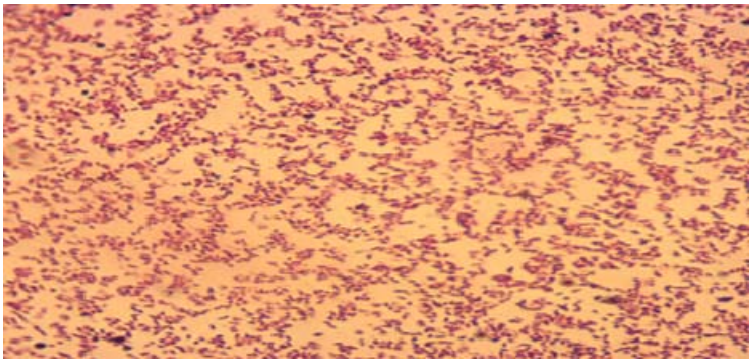


Fig 3: Gram stained isolate of *P. multocida*

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

3.2. Biochemical activities of the isolates

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

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

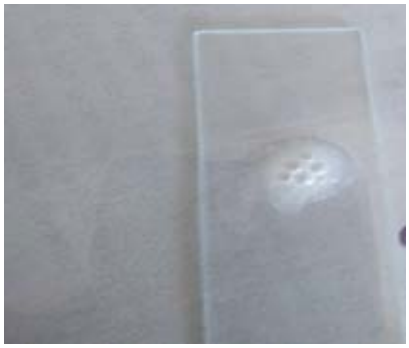
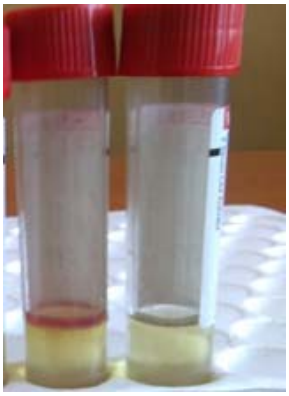


Fig 6: positive catalase test of *P. multocida*



A B

Fig 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 eexamined	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	Nonvaccinted	126	8 (6.35%)	160.2	0.000
	Vaccinated	258	5(1.94%)		

3.3 Antimicrobial susceptibility testing

Table 3: Antimicrobial 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%)
Chloramphincol	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 NCCLSs' Standard on zone size interpretation chart. 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 interpreted accordingly.

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 chloramphincol (61.5%) intermediately susceptible.

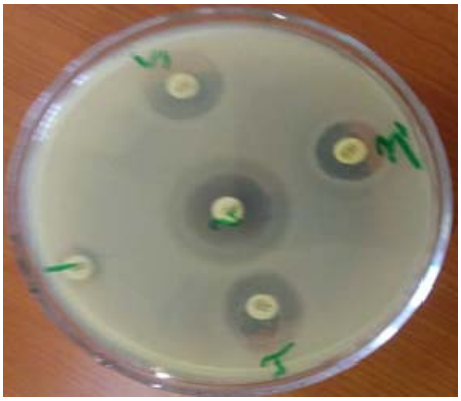


Fig 8: Antimicrobial drug sensitivity test on sample from Assosa and Bambasi districts. 1) TTC, 2) Sterptomycin, 3) Clindamycin, 4) Gentamycin, 5) Kanamycin

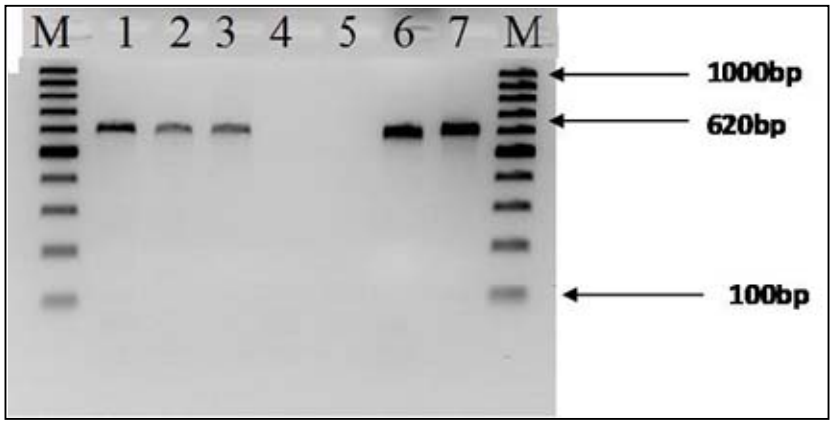
Table 4: Multi drug resistance of isolates (MDR) of *P. multocida*

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%) 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.

3.4 Molecular detection of P. multocida isolates



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.

Fig 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

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.

4. 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 [7] 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 [8] and Rahman [27]. 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 [31] also reported similar biochemical characteristics for *P. multocida* type B. characteristics colonies of *P. multocida*, as described by Choudhury [8].

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%).

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 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=0.07) But the status of the isolation rate 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 to 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 [12].

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 [13] 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 [14]. 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 [30]. 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 [11].

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 [35] in Iraq. Whereas susceptible to Sterptomycin (61.5%) and clindamycin (53.8%). This study also agreed with De Alwis [10]; Shivachandra [32] in Sri-lanka, Refsdal [28] in Siwa Oasis, Egypt. General Tetracycine, 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* 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 [5] gradual development of multi-drug resistance in Asia. Generally, Out of 13 isolates, 7 (53.8%) isolates showed resistance for drugs but only 2 (15.38%) developed resistance for 4 drugs. This study has shown that multi drug resistance which was similar with kumer [22] 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 [34] PCR was carried out to confirm the isolate as *P. multocida* type B using the specific primers pairs KTT72 ′ and KTSP61.

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 [33] 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.

5. 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 have shown resistance to some of the antibiotic dicks. 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.

6. References

1. Ashraf A, Tariq H, Shah MS, Nadeem S, Manzoor I, Ali S, *et al*. Characterization of Pasteurella multocida strains isolated from cattle and buffaloes in Karachi, Pakistan. African Journals Microbiology Research. 2011; 5(26):4673-4677.
2. Assosa district office of livestock and fishery development, 2013.
3. Bamibasi district office of livestock and fishery development, 2015.
4. Blackall P. Bojesen A. Christensen H, Bisgaard M. Reclassification of [*Pasteurella*] *trehalosi* as Bibersteinia trehalosi. Systematic and Evolutionary Micr- obiology. 2007; 57:666-674.
5. Biswas, Shivachndra SB, Saxena MK, Kumar AA, Singh VP, Srivastava SK. Molecular variability among strains of *P. multocida* isolated from an outbreak of haemorrhagic septicaemia in India. Veterinary Research and Commu-nication. 2004; 28:287-298.
6. Catry B. Pasteurella and Mannheimia species from calves:

differentiation and antimicrobial resistance, PhD thesis, Ghent University, Merelbeke, Belgium, 2005.
7. Cheesbrough M. District Laboratory Practice in Tropical Countries.Part 2.2nd edition, Cambridge University Press, New York. USA. 2006, 45-62.
8. Choudhury KA, Amin MM, Rahman A, Ali MR. Investigation of natural outbreak of fowl cholera. Bangladesh Vetterinary Journal. 1985; 19:49-56.
9. Clinical and laboratory standard institute (CLSI), Performance standared for antimicrobial susceptibility testing; Tw. Sec. info. Sup. 2012; 32:100-522.
10. De Alwis MCL. Hemorrhagic septicemia. Canberra: Austeralian Center for International Agricaltural Research. 1999, 1-10.
11. De Alwis MCL. Sumanadasa MA. Naturally acquired immunity to haemorrhagic septicaemia among cattle and buffaloes in Sri Lanka. Tropical Animal Health and Production. 1982; 14:27-28.
12. De Alwis MCL, Kodituwakku AO, Kodituwakku S. Hemorrhagic septicemia an analysis of two outbreaks of disease among buffaloes Ceylon veterinary Journals. 1976; 24:18-21.
13. Dereje A, Tesfaye S, Tadesse B. Isolation and Identification of Mannhemia and Pasteurela species from pneumonic and apparently health cattle and their antibiogram susceptibility pattern in Bedelle district, Western Ethiopia. Journal of Bacteriology Res- earch. 2013; 6(5):32-41.
14. Dowling A, Hodgson JC, Dagleish MP, Eckersall PD. Pathology, Veterinary Immunology, Sale Journal. 2004; 100:197-207.
15. Environmental Agency. The Microbiology of Drinking Water, (2002). Methods for Examination Water and Associated Materials. 2002, 10-14.
16. Ethiopia animal health yearbook. Summary of disease outbreak reports during 2009-2010 Fiscal Year. 2011, 41.
17. Garner G, Saville P, Fediaevsky A. Manual for the recognition of exotic diseasesof livestock: A reference guide for animal health staff [online] Food and Agriculture Organization of the United Nations [FAO]; Hemorrhagic septicemia, 2003.
18. Hawari AD, Hassawi DS, Sweiss M. Isolation and Identification of Mannheimia haemolytica and pasteurella multocida in Sheep and Goats using Biochemical Tests and Random Amplified Polymorphic DNA (RAPD) Analysis Journals Biological Science. 2008; 8(7):1251-1254.
19. Heath protection agency. Indole test. National Standard Method BSOP. 2010; 19(2).
20. Katherine O. Pasteurella multocida. Pasteurellosis. 2008; 7:26-45.
21. Kehrenberg C, Schulze-Tanzil G, Martel JL, Dancla EC, Schwarz S. Antimi- crobial resistance in *Pasteurella* and *Mannheimia*: epidemiology and genetic basis. Veterinay Research. 2001; 32:323-339.
22. Kumar AA, Shivachandra SB, Biswas AV, Singh P, Singh Vijendra P, Srivastava SK. Prevalent serotypes of Pasteurella multocida isolated from different animal and avian species in India. Canadian Journal Veterinary Research Community. 2004; 28:657-667.
23. Michael W. Pasteurellosis Transmission Risks between Domestic and Wild Sheep. CAST Commentary. 2008, 325.
24. Mulaw S, Addis M, Fromsa A. Study on the Prevalence of Major Trypanosomes Affecting Bovine in Tsetse Infested Asosa District of Benishangul Gumuz Regional State, Western Ethiopia. Global Veterinaria. 2011; 4:330-336.
25. National Meterology Service Agency NMSA, 2007.
26. Ragy S. Major Pathogenic Components of Pasteurella multocida and Mannheimia (Pasteurella) haemolytica Isolated From Animal Origin. Veterinary Microbiology. 2005; 17:312-356.
27. Rahman MH, Shahiduzzaman ANM, Haque ME, Nazir KHMNH, Rahman Development of Experimental Oil based Inactivated HS Vaccine from field Isolates of *Pasteurella multocida* from Cattle in Bangladesh. Inerination Journal Vaccines and Vaccination. 2016; 2(2):15406.
28. Refsdal AO. To treat or not to treat:a proper use of hormones and antibiotics. Animals Reproduction Science. 2000; 60-61:109-119.
29. Richard W. Outer Membrane Proteomics of *Pasteurella multocida* Isolates to Identify Putative Host-Specificity Determinants. Bioscience Horizons. 2009; 2(1):1-12.
30. Sheikh MA, Anzam M, Shakoori AR. Observations on haemorrhagic septicaemia in Pakistan livestock. Journal of Veterinary Medicine Series. 1996; 43:293-304.
31. Shivachandra SB, Viswas KN, Kumar AA. A review of hemorrhagic septicemia in cattle and buffalo. Animal Health Research Review. 2011; 12:67-82.
32. Shivachandra SB, Kumar AA, Gautam R, Singh VP, Chaudhuri P, Srivastava SK. PCR assay for rapid detection of Pasteurella multocida serogroup-A in morbid tissue materials from experimentally induced Fowl cholera in chicks. Veterinary Journal. 2004; 168:349-352.
33. Townsen KJ, Boyc J, Chung A, Frost Adler B. Genetic organization of Pasturella multocida cap loci and development of multiplex capsular PCR typing system. Jouinal Clinical and Microbiology. 2001; 39:924-929. Pasteurella multocida. Journal of Clinical Microbiology. 36:1096-1100.
34. Townsend KM, Frost AJ, Lee CW, Papadimitriou JM, Dawkins HJS. Development of PCR assays for species and type specific identification, 1998.
35. Zuber I. Epidemiological Study of Pasteurellosis in Lambs in Erbil Governorate, Northern Iraq. UDC. 2009; 619:579.84.