Frontiers in Environmental Microbiology
2022; 8(2): 19-24
http://www.sciencepublishinggroup.com/j/fem
doi: 10.11648/j.fem.20220802.11
ISSN: 2469-7869 (Print); ISSN: 2469-8067 (Online)

Antimicrobial Susceptibility Patterns of *Pasteurella multocida*, Mannhemia Haemolytica, and Bibersteina Trehalosi Bacterial Isolates

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To cite this article:
Dessie Abera. Antimicrobial Susceptibility Patterns of *Pasteurella multocida*, Mannhemia Haemolytica, and Bibersteina Trehalosi Bacterial Isolates. *Frontiers in Environmental Microbiology*. Vol. 8, No. 2, 2022, pp. 19-24. doi: 10.11648/j.fem.20220802.11

Received: April 15, 2022; **Accepted**: May 12, 2022; **Published**: May 24, 2022

Abstract: Pneumonic pasteurellosis causes a significant economic loss in small ruminant production but its control is complicated because of drug resistance development. It is multifactorial but the most frequently isolated bacterial species are *Pasteurella multocida* (*P. multocida*), *Mannheimia haemolytica* (*M. haemolytica*), and *Bibersteinia trehalosi* (*B. trehalosi)*. The antimicrobial susceptibility patterns of those commonly isolated respiratory pathogens of sheep and goats were determined to get insight for better antimicrobial therapy. The isolates were identified from pneumonic sheep and goats by Assosa Agricultural Research Center from November 2019 to April 2020 in Assosa and Bambasi woredas of Benishangul-Gumuz Regional State. 25 isolates from pneumonic sheep and 11 isolates from pneumonic goats preserved at Assosa Regional Veterinary Laboratory were tested against commonly used antimicrobial agents. Antimicrobial susceptibility profiles of the isolates were carried out using the disc diffusion method. The identified isolates were susceptible to Sulfonamide (94.4%) and Norfloxacin (91.7%). However, they were resistant to Streptomycin (88.9%) and Kanamycin (80.6%), and intermediate to Trimethoprim (55.6%). From all of the isolates, 42.4% of sheep and 40% of goat origin were resistant to all antimicrobial discs tested. About 31 out of 36 (86.6%) isolates were resistant to two or more discs and 69.4% of them were resistant to Streptomycin and Kanamycin. 81.8% (9/11) from goats and 64% (16/25) isolates from sheep were resistant to Streptomycin and Kanamycin. Eight out of thirty-six (22.2%) were resistant to three discs and none of them were resistant to four or more discs tested. Therefore, antimicrobial susceptibility monitoring programs for *P. multocida*, *M. haemolytica,* and *B. trehalosi* are crucial for proper antimicrobial therapy. Sulfonamide and Norfloxacin were preferred from the antimicrobial discs tested and are suggested to treat pneumonic sheep and goats. Further characterization of the isolates through a more specific diagnostic marker is recommended.

Keywords: Antimicrobial Susceptibility, Bibersteinia Trehalosi, Mannheimia Haemolytica, *Pasteurella multocida*, Small Ruminants

1. Introduction

Small ruminants constitute the second most important farm animals in Ethiopia [1, 2]. In Benishangul-Gumuz Regional State, sheep and goats population are 160, 029, and 602, 840 respectively [3]. Sheep and goat production is preferred since they require short generation intervals, high market demand, smaller space, and capital investment [4]. Efficient utilization and their contribution to the country's economy are restricted because of a combination of health problems, poor management systems, and malnutrition. Among different constraints, disease stands on the front line which causes mortality and poor reproductive performance of sheep and goats [1, 5-7].

From different types of diseases, pneumonia is the major disease restricting sheep and goat production in the tropics [8]. It is found as a major problem usually encountered in flocks, affecting groups or individuals of all ages and types of sheep and goats [9]. Respiratory infections of small ruminants contribute to 5.6 percent of the entire illness of sheep and goats globally [10, 11]. They occur due to the interaction of the host with different infectious agents and are

influenced by environmental factors [12, 13]. Bacterial pneumonia is the most common respiratory problem in small ruminants. It is regularly diagnosed and reported in veterinary clinics in Ethiopia and it is the leading cause of mortality [2, 8]. Respiratory illnesses are common as the aerosol spread is the primary way of transmission [8].

Pneumonia is the main cause of economic loss in producers of ruminants. Its economic loss is about 8% of production cost, including medical expenses, poor food conversion efficiency, increment in production costs, and decreased availability of human foods [14]. From all of the respiratory diseases of small ruminants, pasteurellosis is therefore a high-priority issue at the country level due to the significant economic losses it causes through mortality, morbidity, and the excessive price of treatment preparations [15-17]. The mortality rate of sheep and goats by ovine and caprine pasteurellosis is aggravated in lowland areas where infrastructures are limited and when the immune status of an animal is compromised by stressors [5, 18].

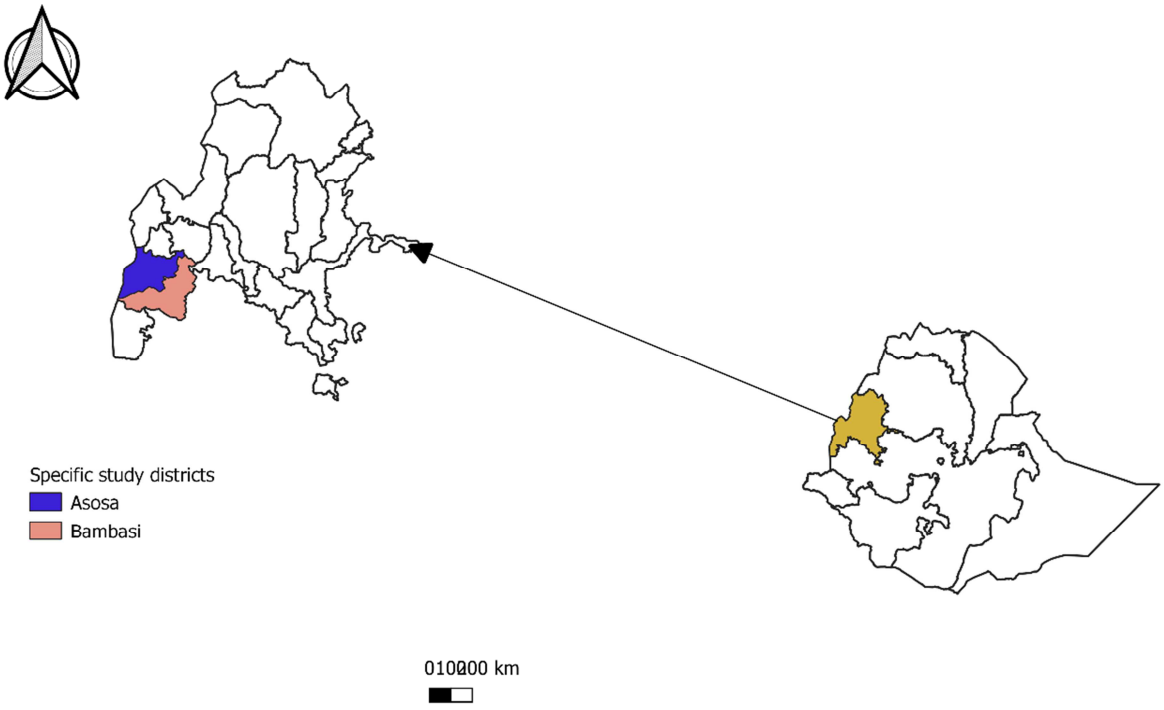
Pasteurellosis causes outbreaks of acute pneumonia in all age groups of sheep and goats [1]. And pneumonic pasteurellosis is the most important infectious disease which greatly affects the productivity of small ruminants [19-21]. Commonly isolated causative agents of pneumonic pasteurellosis are *M. haemolytica*, *P. multocida,* and *B. trehalosi*. These are involved in different types of infections of farm animals. They are frequently encountered in small ruminants as major pathogens in respiratory illnesses and result in heavy losses. They are regularly isolated from infected than non-pneumonic animals [9, 22, 23].

Control and prevention of pneumonic pasteurellosis is mandatory to improve sheep and goats' contribution to the development of the country [21, 22, 24]. However, control of pneumonic pasteurellosis is a difficult task because antimicrobial agents which were the most powerful tool to control this disease become unsuccessful because of the development of drug-resistant strains [21, 22]. Therefore, the study was conducted to determine the antimicrobial susceptibility pattern of *P. multocida, M. haemolytica*, and *B. trehalosi* bacterial isolates.

2. Materials and Methods

2.1. Study Area

The isolates were identified from the samples collected from Assosa and Bambasi woreda of Benishangul-Gumuz Regional State. Benishangul-Gumuz is located in the Western part of Ethiopia between latitude 10° and 04° N and longitude 34° and 31° E. The region has three administrative zones namely Assosa, Kamashi and Metekel divided into 18 woredas and two special woredas (Mao-komo and Pawe). About 86% of the total population are rural dwellers which depend on agriculture and related activities for livelihood. Assosa woreda has a livestock population of 36,916 cattle, 23,500 goats, 14,325 sheep, 5,890 equine and 35,125 poultry and Bambasi woreda has 38,964 cattle, 11,990 goats, 3,739 sheep, 4,467 equine, and 41,438 poultry (BGRBOA, 2018). The major management system practiced in the area is an extensive production system. They practice a mixed farming system and small ruminants are the predominant animal species kept in the area. Traditional housing and grazing of natural pasture are the predominant husbandry practices. The map showing the study woredas is indicated in Figure 1 below.



Source: Developed through QGIS version 2.4.0

Figure 1. *Map showing the study area.*

2.2. Study Design

A cross-sectional study design was employed. The bacterial isolates determined by Assosa Agricultural Research Center and preserved at Assosa Regional Veterinary Laboratory were used for the antimicrobial susceptibility test. The isolates were *P. multocida*, *M. haemolytica*, and *B. trehalosi* identified from nasal swab specimens of clinically suspected cases of sheep and goats. The nasal swab subculture may be predictive of the bacterial pathogen inside the lung. It is a reliable guide for practitioners in the treatment of pneumonic pasteurellosis and it may be used to decide on antibiotic susceptibility [25, 26]. The bacterial isolates tested were presented in Table 1.

Table 1. *Bacterial isolates of sheep and goats were tested for antimicrobial susceptibility.*

Animal species	Bacterial species isolated			Total isolates
	P. multocida	*M. haemolytica*	*B. trehalosi*	
Ovine	2	6	17	25
Caprine	2	1	8	11
Total	4	7	25	36

2.3. In Vitro Antimicrobial Susceptibility Test

All identified isolates of *P. multocida*, *M. haemolytica,* and *B. trehalosi* preserved were reconfirmed through morphological and biochemical characterization. And they had been subjected to antimicrobial susceptibility testing using commonly used antimicrobials and based on the availability of the discs. The test was performed by the disc diffusion technique as defined by Kirby Bauer [27]. Disc diffusion assay can yield clinically applicable and reliable information for figuring out remedy strategies [28]. The identified isolates were examined for sensitivity against five different antimicrobial discs. The antimicrobial discs used were in antimicrobial class: Aminoglycosides (Streptomycin (Himedia, India), and Kanamycin (Oxoid, England)); Quinolones (Norfloxacin); and Folate pathway inhibitors (Trimethoprim and Sulfonamides (Oxoid, England)) according to the procedures recommended by Quinn and his colleagues [29].

In the pure culture of the bacteria at Nutrient agar, at least 4-5 well-isolated colonies were selected and transferred to a tube containing 5 ml nutrient broth. It was incubated at 37°C until slightly visible turbidity appears. The turbidity was adjusted by comparison with a 0.5 McFarland turbidity standard. They were in similar 5 ml test tubes.

The bacterial suspension was spread to Muller Hinton agar using a sterile cotton swab and allowed to stand for 3-5 minutes before the antimicrobial disc was placed on the inoculated surface using sterile forceps. Complete contact is required between the discs and the agar surface by gently pressing with the point of the forceps. The plates were inverted upside down and incubated at 37 °C for 18 to 24 hours. The results were evaluated for clear zones produced by antimicrobial inhibition of bacterial growth surrounding the antimicrobial discs. The diameter of the inhibition zone was measured for each in mm using a measuring ruler by holding on to the back of the inverted Petri dish. The result was compared with the standard inhibition zone of each antimicrobial agent and interpreted as susceptible, intermediate, and resistant according to internationally approved breakpoints of the British Society for Antimicrobial Chemotherapy [30], and Clinical and Laboratory Standards Institute [31].

2.4. Data Management and Analysis

The data was entered into the Microsoft Excel spreadsheet and then it was analyzed using IBM SPSS Statistics version 20 software. Descriptive statistics employed for analysis.

3. Results

The antimicrobial susceptibility test result showed good susceptibility of identified isolates to various antimicrobials including fluoroquinolones and sulfonamides. However, the antimicrobial susceptibility test result of most isolates for the aminoglycoside family were resistant and they were intermediate for Trimethoprim. *In vitro* antimicrobial susceptibility test results indicated that three out of four (75%) of *P. multocida* isolates tested were resistant to Streptomycin and Kanamycin, whereas 75% of them were susceptible to Sulfonamide and Norfloxacin. All isolates of *M. haemolytica* and *B. trehalosi* were susceptible to Sulfonamide and Norfloxacin respectively. From the identified isolates, 88.9% and 80.6% of them were resistant to Streptomycin and Kanamycin respectively as shown in Table 2. The susceptible breakpoints are ≥ 17 mm for Norfloxacin (NOR_{10}), Kanamycin (K_{30}), and Sulfonamide ($S3_{300);}$ ≥ 16 mm for Trimethoprim (W_5), and ≥ 15 mm for Streptomycin (S_{10}) (Table 2).

Table 2. *Antimicrobial susceptibility pattern of isolated bacteria.*

Antimicrobial discs	Disc Concentration (µg / disc)	Performance	Diameter in mm	Species of bacteria			Total (36)
				P. multocida (4)	*M. haemolytica (7)*	*B. trehalosi (25)*	
Streptomycin (S_{10})	10	Resistant	≤11	3 (75%)	6 (85.7%)	23 (92%)	32 (88.9%)
		Intermediate	12-14	0 (0%)	1 (14.3%)	2 (8%)	3 (8.3%)
		Susceptible	≥15	1 (25%)	0 (0%)	0 (0%)	1(2.8%)
Norfloxacin (NOR_{10})	10	Resistant	≤12	0 (0%)	0 (0%)	0 (0%)	0 (0%)
		Intermediate	13-16	1 (25%)	2 (28.6%)	0 (0%)	3 (8.3%)
		Susceptible	≥17	3 (75%)	5 (71.4%)	25 (100%)	33 (91.7%)
Trimethoprim (W_5)	5	Resistant	≤10	2 (50%)	4 (57.1%)	8 (32%)	14 (38.9%)
		Intermediate	11-15	0 (0%)	3 (42.9%)	17 (68%)	20 (55.6%)
		Susceptible	≥16	2 (50%)	0 (0%)	0 (0%)	2 (5.6%)

Antimicrobial discs	Disc Concentration (µg / disc)	Performance	Diameter in mm	Species of bacteria			Total (36)
				P. multocida (4)	*M. haemolytica (7)*	*B. trehalosi (25)*	
Kanamycin (K_{30})	30	Resistant	≤14	3 (75%)	5 (71.4%)	21 (84%)	29 (80.6%)
		Intermediate	15-16	1 (25%)	1 (14.3%)	3 (12%)	5 (13.9%)
		Susceptible	≥17	0 (0%)	1 (14.3%)	1 (4%)	2 (5.6%)
Sulfonamide ($S3_{300}$)	300	Resistant	≤12	0 (0%)	0 (0%)	0 (0%)	0 (0%)
		Intermediate	13-16	1(25%)	0 (0%)	1 (4%)	2 (5.6%)
		Susceptible	≥17	3 (75%)	7 (100%)	24 (96%)	34 (94.4%)

From all the isolates tested, 42.4% and 40% of the isolates from sheep and goats were resistant to antimicrobial discs tested respectively. About 90.9% of goat isolates were resistant to Streptomycin and Kanamycin. Whereas sheep isolates were resistant to Streptomycin (88%) and Kanamycin (76%). These were described in Table 3.

Table 3. *Antimicrobial susceptibility of sheep and goat bacterial isolates.*

Species	Performance	Antimicrobial discs					Overall susceptibility
		Streptomycin (S_{10})	Norfloxacin (NOR_{10})	Trimethoprim (W_5)	Kanamycin (K_{30})	Sulfonamide ($S3_{300}$)	
Caprine	Susceptible	0 (0%)	10 (90.9%)	2 (18.2%)	0 (0%)	10 (90.9%)	22 (40%)
	Intermediate	1 (9.09%)	1 (9.09%)	7 (63.6%)	1 (9.09%)	1 (9.09%)	11 (20%)
	Resistant	10 (90.9%)	0 (0%)	2 (18.2%)	10(90.9%)	0 (0%)	22 (40%)
	Total	11 (100%)	11 (100%)	11 (100%)	11 (100%)	11 (100%)	55 (100%)
Ovine	Susceptible	1 (4%)	23 (92%)	0 (0%)	2 (8%)	24 (96%)	50 (40%)
	Intermediate	2 (8%)	2 (8%)	13 (52%)	4 (16%)	1 (4%)	22 (17.6%)
	Resistant	22 (88%)	0 (0%)	12 (48%)	19 (76%)	0 (0%)	53 (42.4%)
	Total	25 (100%)	25 (100%)	25 (100%)	25 (100%)	25 (100%)	125 (100%)

Most identified isolates were resistant to antimicrobial discs used in this antimicrobial susceptibility testing. 31 out of 36 (86.6%) identified isolates were resistant to two or more drugs tested and 69.4% of them were resistant to Streptomycin and Kanamycin. But isolates from goats (81.8%) and sheep (64%) were resistant to Streptomycin and Kanamycin. Eight out of thirty-six (22.2%) were resistant to three discs and none of them were resistant to four or more discs tested. It was indicated in Table 4.

Table 4. *Antimicrobial resistance profile for two or more discs tested.*

Resistance to two or more antimicrobial discs		Species of animal		Number of isolates (%) N=36
		Caprine (n=11)	Ovine (n= 25)	
For two discs	Streptomycin and Kanamycin	9 (81.8%)	16 (64%)	25 (69.4%)
	Streptomycin and Trimethoprim	2 (18.2%)	10 (40%)	12 (33.3%)
	Trimethoprim and Kanamycin	2 (18.2%)	8 (32%)	10 (27.8%)
For three discs	Streptomycin, Kanamycin, and Trimethoprim	2 (18.2%)	6 (24%)	8 (22.2%)

4. Discussion

In this antimicrobial sensitivity testing study, Sulfonamide and Norfloxacin were found to be the most effective antimicrobial agents since 94.4% and 91.7% of the isolates were susceptible respectively; while the rest of the antimicrobials were less effective. In agreement with this investigation, higher efficacy of fluoroquinolone antimicrobial agents against *P. multocida* has been reported by Kumar and his colleagues in an *in vitro* study conducted from *P. multocida* isolates of ruminant origin [32]. Other reports also indicated that fluoroquinolones and sulfonamides have shown good antimicrobial susceptibility patterns for ovine respiratory pathogens [33]. *In vitro* antimicrobial susceptibility test results can be used as preliminary information for further studies [34]. And, the antimicrobial susceptibility test result of identified isolates to Trimethoprim (55.6%) in this study was intermediate and they should be used in the treatment of pneumonic animals when they show susceptibility. Kehrenberg and his colleagues reported that Trimethoprim resistance of *Pasteurella* and *Mannheimia* isolates is commonly due to dihydrofolate reductases [22].

Drugs under class aminoglycosides: Streptomycin (88.9%) and Kanamycin (80.6%) were inefficient drugs in this study and this was in line with the finding of Marru *et al*. [1] who reported that Streptomycin and Gentamycin were inactive against 90.6% of the isolates. And also Streptomycin was ineffective for 72.3% of isolates in Haramaya and Harar districts, Eastern Ethiopia [35]. Moreover, other scholars reported that the disc diffusion test of kanamycin was not effective for 61.5% of the isolates in the Assosa and Bambasi districts, and 57% of *P. multocida* isolates of ruminant origin respectively [32, 36].

Resistance to the aminoglycoside drugs like Streptomycin could be related to its frequent and long-term use in Penstrep as a prophylactic and therapeutic measure for sheep and goats. Administration of drugs to animals is deviated from the standard recommendations of manufacturers because of problems in prescribing and diagnosis of diseases [37]. These will favor the emergence of resistant strains through selective

pressure [22]. Although Kanamycin is not normally utilized in animals, the level of resistance of isolates to this antimicrobial was relatively high. The resistance asset of *Pasteurella* related to a large conjugative R plasmid of about 113 kb which mediated resistance to streptomycin, kanamycin, and tetracycline has been recognized [22, 38]. The gene mediating streptomycin resistance, *strA*, codes for an aminoglycoside-3'-phosphotransferase which enzymatically inactivates streptomycin. This gene has been found to be a part of transposons, plasmids, or in the chromosome of a wide range of bacteria including *P. multocida* and *Mannheimia* species [22].

In this study, 22.2% of identified isolates were resistant to three discs tested. In the same study area, Bote and his colleagues from clinically sick cattle reported that 53.8% of *P. multocida* isolates were resistant to three drugs and 15.38% were resistant to four drugs, with overall multidrug resistance of 69.2% [36]. Multidrug resistance occurs by the accumulation of genes on plasmids or transposons with every coding for resistance to a specific agent, and/or by the action of multidrug efflux pumps, each can pump out a wide range of drug types [39].

The limitations of this study were that the isolates were not confirmed by genetic tools and subtyping of each species of bacteria till the serotype level was not made.

5. Conclusion and Recommendations

The isolates of *Mannhemia haemoltyica, Bibersteina trehalosi,* and *Pasteurella multocida* were sensitive to fluoroquinolones and sulfonamides but resistant to antimicrobial agents under the class aminoglycoside. Therefore, antimicrobial susceptibility monitoring programs for *P. multocida*, *M. haemolytica,* and *B. trehalosi* are essential for evidence-based antimicrobial therapy. Moreover, Characterization of the isolates through molecular markers for species and serotypes is required.

Conflict of Interest

The authors declare that there is no conflict of interest.

Acknowledgements

The authors thank Assosa Regional Veterinary Laboratory for preserving the bacterial isolates and staff members for their support during the laboratory work.

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