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Biochemical and Antigenic Characterization of *Mannheimia*, *pasteurella* and *Mycoplasma* Species from Naturally Infected Pneumonic Sheep and Goats, Bishoftu, Ethiopia

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Abstract: The total isolation rate of *Mannheimia haemolytica*, *Pasteurella trehalose* and *Pasteurella multocida* was 28, 3.9 and 2.2%, respectively. The isolation rate of *Mannheimia haemolytica* from sheep was higher than goats with statistically significant difference (X^2=7.87 and P< 0.05). *Pasteurella trehalose* was isolated from pneumonic sheep but not from pneumonic goats and *Pasteurella multocida* was isolated only from pneumonic goats. *Mannheimia haemolytica* serotype A2 (21.5%), A8 (20.5%) and A1 (17.7%) were the three dominant serotypes from total isolates of sheep and goats. Serotype A2 (26.3%), A1 (17.5%), A 8 (15.8%) were dominant serotypes from sheep and A8 (33.3%), A1 (19%) and A11 (14%) were dominate isolates from goat. A5, A7 and A12 were not isolated from goats. Thirty-five mycoplasmas were isolated and characterized from pneumonic lungs of sheep and goats. Four *Mycoplasma* species namely, *Mycoplasma ovipneumoniae* (nine isolates); *Mycoplasma mycoides* subspecies *mycoides* Large colony (*Mmm*LC), two isolates; *Mycoplasma mycoides capri* (Mmc), one isolate and *Mycoplasma arginini,* two isolates were identified from sheep where as five Mycoplasma species namely, MmmLC (nine isolates); *Mycoplasma Capricolum* subspecies *capricolum* (Mcc), three isolates; *Mycoplasma Capricolum subspecies Capripneumonae* (Mccp),three isoaltes; *Mmc* (four isolates) and *M. arginini* (two isolates) were isolated and identified from goats. The concurrent isolation rate of *Mannheimia haemolytica* and *Mycoplasma* species was 24.6% in sheep and 9.5% in goats. *Mycoplasma ovipneumoniae* was isolated at 15.8% rate from *Mannheimia haemolytica* infected sheep. *Mmm*LC, *Mmc* and *Mycoplasma arginini* were also isolated from *Mannheimia haemolytica* positive sheep. Generally, the isolation rate of *Mycoplasma* and *Mannheimia haemolytica* together was significantly lower in goats than in sheep.

Key words: Ethiopia · Mannheimia · Mycoplasma · Pasteurella · Serotype

INTRODUCTION

Ethiopia owns huge number of small ruminants, estimated to be 42 million heads of sheep and goats [1, 2]. Sheep and goats play a significant role in the nation's economy. Meat and milk are major sources of protein while hides, live animals and carcasses account for a significant proportion of exports [3]. This rich potential, however, is not properly exploited due to constraints of which diseases come in the forefront. Among these, respiratory diseases represent a serious threat to small ruminant production [2]. It has been suggested that *Mycoplasma* interaction with the host´s cilia prevents normal ciliary activity, facilitating the invasion of the lower respiratory tract by other organisms, including *Mannheimia haemolytica* and *Pasteurella multocida* [4, 5].

In Ethiopia, pneumonic pastuerellosis and mannheimiosis have been investigated in sheep particularly in highland areas but little is known about the role of other Mycoplasmas as an etiology of pneumonia in small ruminants in Ethiopia. There are also no well written documents about the concurrent occurrence of *Mycoplasma*, *Pasteurella* and *Mannheimia* in Ethiopia. Therefore, the objectives of this work are: To study the concurrent occurrence of *Mannheimia, Pasteurella* and *Mycoplasma* involved in pneumonia of sheep and goats with comparing the isolation rate between sheep and

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goats. Serotyping of *Mannheimia haemolytica* isolated from pneumonic lungs and comparing them with *Mannheimia haemolytica* serotypes distributed in sera of the same animals

MATERIALS AND METHODS

Sample Collection: Blood samples were collected from the jugular vein and kept for some times to allow clotting and finally the sera were separated and kept at -20°C until tested. The whole lung affected by pneumonia was collected separately in sterile zip lock bags (sheep, n=167 and goats n=112), labeled and transported to the laboratory in icebox [6]. For histopathology, the tissue samples (about 5cm) were collected in 10% buffered formalin [7].

Sample Processing: The bacteriological standard procedure of Sisay and Zerihun [8] and OIE [9] were followed for isolation and identification of *Mannheimia* and *Pasteurella* species. Isolates serotyping and serotypes distribution of *Mannheimia haemolytica* was performed by the IHA technique described by Baberistein [10] and modified by Kawamoto *et al.* [11]. A positive and a negative control were included in each test. *Mycoplasma* species isolation was done according to OIE [9] and biochemical characterization was done according to Sabry *et al.* [12]; Poveda [13]; and Odugbo *et al.* [12-14]. *Mycoplasma* species growth inhibition test was done according to Clyde [15]. The monoclonal antibodies for mycoplasma species identification were RAS No 43 for MmmLc (F30), NCTC10137 RAS for Mmc, CCTC10151 RAS for M. ovipneumonia, Mcc NCTC 10154 RAS and Mccp NCTC 10152 RAS (All were from Veterinary Central Laboratories of UK, Courtesy of Robin Nicholas).

RESULTS

The total isolation rate of *Mannheimia haemolytica* from sheep and goats was 28%. The details were depicted on Table 1. The isolation rate of *Mannheimia haemolytica* from sheep was higher than goats with statistically significant difference (X^2=7.87 and P< 0.05). *Pasteurella trehalose* was isolated from pneumonic sheep but not from pneumonic goats and *Pasteurella multocida* was isolated only from pneumonic goats (Table 1).

***Mannheimia Haemolytica* Serotyping (Strain Characterization):** Because of the absence of monoclonal antibodies for *Pasteurella trehalose* and *Pasteurella multocida* they were not serotyped. *Mannheimia haemolytica* serotypes A14, A16 and A17 were also not available and strain characterization was done only for 10 serotypes (Table 2).Serotypes A2, A1 and A 8 were repeatedly isolated from many pneumonic sheep and were the dominant serotype while serotypes A5 and A7 were isolated only from few pneumonic sheep. The details were shown by Table 2. Monoclonal antibody for serotypes A9 and A13 were not related with any *Mannheimia haemolytica* isolates. Serotypes A8 and A1 were repeatedly isolated from many pneumonic goats and serotypes A5, A7 and A13 were not isolated from goats in this study. Serotype A13 was isolated neither from sheep nor from goats in the present study.

Comparison of *Mannheimia Haemolytica* Lung Isolate Serotypes with Serotypes Detected in Sheep Sera: Total of 167 sheep sera for which all pneumonic lungs were cultured for bacterial isolation were tested for serotype specific antibodies by IHA test against 10 known *Mannheimia haemolytica* antigens at the dilutions of 1/20, 1/40, 1/80 and 1/160. Antigens of serotypes A14, A16 were not available and then not tested. All the 10 known *Mannheimia haemolytica* antigens reacts to the number of sera with variable titer results and no serum was found negative to at least one of these antigens (Table 3). Those sheep sera showed haemagglutination reaction at 1/40 dilution and above were reported as positive [11].

Of the 167 sera tested, 96 showed multiple reactions to more than one *Mannheimia haemolytica* antigens particularly to serotypes A1 (30%) and A8 (29.3%). Serotype A2 had shown the highest mean antibody titer (1/7437) and A13 (1/40) the least mean positive antibody titers (Table 3). When comparing the dominance of *Mannheimia haemolytica* serotypes in sheep sera with that of lung isolates of the same sheep great variation was observed. For instance, serotypes A9 and A13 were not isolated from pneumonic lung but from the same sheep sera serotype A9 was positive at 4.2% and serotype A13 at 9%. The details of comparison are indicated in tables 2and3. The dominance of serotypes A1, A2 and A8 were almost similar both from pneumonic lung and in the sera of the same sheep.

Isolation and Identification of Mycoplasma

Colony Characteristics and Biochemical Reactions: For assessing concurrent occurrence of *Mannheimia haemolytica* and mycoplasma species 126 lungs samples (57 from sheep previously positive for *Mannheimia*

Table 1: Isolation rates of *Mannheimia haemolytica*, *Pasteurella trehalose* and *Pasteurella multocida* from pneumonic lungs of sheep and goats.

Animal Species	Pneumonic lungs	*Mannheimia haemolytica* No. Positive, (%)	*Pasteurella trehalose* No. Positive,(%)	*Pasteurella multocida* No. Positive, (%)	Total, (%)
Sheep	167	57, (34.1%)	11, (6.6%)	NI	68(40.7%)
Goat	112	21, (18.8%)	NI	6, (5.4%)	27, (24.1%)
Total	279	78, (28%)	11, (3.9%)	6(2.2%)	95(34.1%)

NI= Not Isolated in this study

Table 2: *Mannheimia haemolytica* serotypes isolated from pneumonic lung of sheep and goats.

Spp.	Total	A1 No, (%)	A2 No, (%)	A5 No,(%)	A6 No(%)	A7 No(%)	A8 No,(%)	A9 No,(%)	A11 No,(%)	A12 No,(%)	A13 No(%)
Sheep	57	10 (18)	15(26.3)	2(3.5)	8(14)	2(3.5)	9(15.8)	NI	5(8.8)	6(10.6)	NI
Goat	21	4(19)	2 (9.5)	NI	3(14)	NI	7(33.3)	2(9.5)	3(14)	NI	NI
Total	78	14(17.9)	17(21.8)	2(2.6)	11(14)	2(2.6)	16(21)	2(2.6)	8(10.2)	6(7.7)	NI

NI= Not Isolated in this study; Spp. = animal species

Table 3: The distribution of *Mannheimia haemolytica* serotypes in sampled sheep sera

Dilutions	*Frequency of Mannheimia haemolytica* serotypes antibody in sheep sera A1	A2	A5	A6	A7	A8	A9	A11	A12	A13
1/20	46	14	10	33	31	33	9	16	26	24
1/40	26	12	6	18	22	29	4	6	16	11
1/80	12	5	4	3	14	10	3	NA	12	2
1/160	12	5	NA	3	6	10	NA	NA	4	2
No.Pos=1/40	50	22	10	24	32	49	7	6	32	15
% Pos=1/40	30**	13.2	6	14.4*	19.2*	29.3**	4.2	3.6	19.2*	9

NA = Antibody is weak at this specific dilution and no agglutination; ** most frequent serotypes;* moderately repeated serotypes

Table 4: Biochemical characteristic of *Mycoplasma* species isolated from sheep and goats.

List of biochemical tests utilized with results obtained							
			Tetrazolium reduction				
Species Isolated	Glucose	Arginine hydrolysis	Aerobic	Anaerobic	Phosphate	Serum digestion	Digitonin sensitivity
MmmLC	+	-	-	v	-	+	+
Mcc	+	+/v	+	+	+	+	+
Mccp	+	-	+	+	-	+	+
Mmc	+	-	+	+	-	+	+
M.ovp	+	-	+	+	-	-	+
M.a	-	+	-	-	-	-	+

+ = positive, - = negative and v = variable

haemolytica) and 69 goats (21 previously positive for *Mannheimia haemolytica*) were cultured. Colonies with characteristic "fried egg" appearance (Fig. 1) except *M. ovipneumoniae* were sub-cultured for further biochemical tests. The details of biochemical reactions are illustrated in Table 4. Four *Mycoplasma* species namely, *Mmm*LC, *Mmc*, *Mcc* and *Mccp* digested insipissated serum with *Mmm*Lc the most digester and *Mccp* the least (Fig. 2). *M. ovipneumoniae* and *M. arginini* did not digest insipissated serum at all. *M.arginini* and *Mcc* were positive for arginine hydrolysis but while *Mcc* is positive for phosphatase production, *M. arginini* was negative

Growth Inhibition Test: Using mycoplasma species specific monoclonal antibody growth inhibition test (Fig. 3), nine *Mycoplasma ovipneumoniae,* two *Mmm*LC, one *Mmc* and two *Mycoplasma arginini* were identified from sheep (Table 5). A total of 21 *Mycoplasma* (summarized in Table 6) isolates were identified and characterized from goats using biochemical and monoclonal antibody growth inhibition tests.

Concurrent Occurrence of *Mannheimia Haemolytica* and *Mycoplasma* Species: Out of the 57 sheep pneumonic lungs which were positive for *Mannheimia*

Table 5: Ovine *Mycoplasma* species identification by growth inhibition against specific hyper immune serum

	Hyperimmune sera used for growth inhibition test					
Species isolated	*MmLc*	*Mmc*	*Mcc*	*Mccp*	*M.ovp*	*M.a*
MmmLc	2/14	0/14	0/14	0/14	0/14	0/14
Mmc	0/14	1/14	0/14	0/14	0/14	0/14
Mcc	0/14	0/14	0/14	0/14	0/14	0/14
Mccp	0/14	0/14	0/14	0/14	0/14	0/14
M.ovp	0/14	0/14	0/14	0/14	9/14	0/14
M.a	0/14	0/14	0/14	0/14	0/14	2/14*

*=biochemically isolated species

Table 6: Caprine *Mycoplasma* species identification by growth inhibition against specific hyper immune sera

	Hyperimmune sera used for growth inhibition test					
Species isolated	*MmmLc*	*Mmc*	*Mcc*	*Mccp*	*M.ovp*	M.a
MmmLc	9/21	0/21	0/21	0/21	0/21	0/21
Mmc	0/21	4/21	0/21	0/21	0/21	0/21
Mcc	0/21	0/21	3/21	0/21	0/21	0/21
Mccp	0/21	0/21	0/21	3/21	0/21	0/21
M.ovp	0/21	0/21	0/21	0/21	0/21	0/21
M.a	0/21	0/21	0/21	0/21	0/21	2/21*

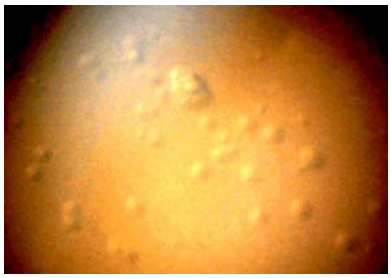

Fig. 1: *Mycoplasma* colonies with the typical characteristic "fried egg" appearance (dense center or nipple shaped morphology).

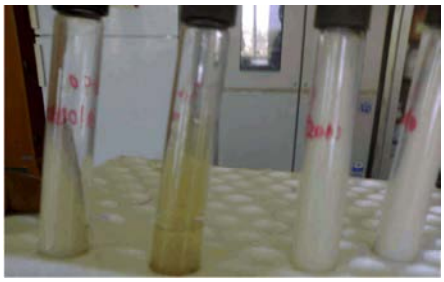

Fig. 2: Serum digestion test. Left to right, *Mmc*, *Mmm*LC, *Mcc* and *Mccp* inoculated serum. Note that *Mmm*LC almost liquefied the serum. Mccp digested the least (only on long incubation).

haemolytica 24.6% were also positive for mycoplasma species and of 21 goats' pneumonic lungs, which were positive for *Mannheimia haemolytica,* 9.5% were also positive *Mycoplasma* species. *M. ovipneumoniae* was

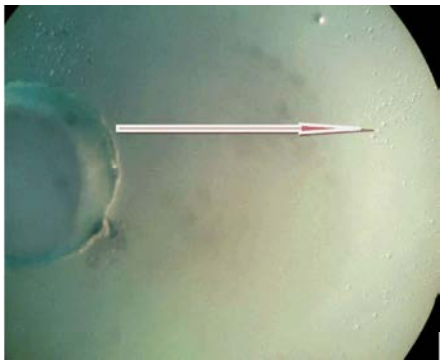

Fig. 3: Growth inhibition test. *Mccp* specific monoclonal antibody (NCTC 10152 RAS, CVL, UK) inhibited the growth of the isolated *Mccp* colonies over a large distance (arrow).

isolated at 15.8% rate with *Mannheimia haemolytica* in sheep. *Mmm*LC *and Mycoplasma arginini* were both isolated at 3.5% from *Mannheimia haemolytica* positive sheep and the least *Mycoplasma* associated with *Mannheimia haemolytica* in sheep was *Mmc*, which was isolated at 1.8% from *Mannheimia haemolytica* positive lungs. Generally, the isolation rate of *Mycoplasma* and *Mannheimia haemolytica* together was significantly lower in goats than in sheep. The only *Mycoplasma* species isolated together with *Mannheimia haemolytica* from goats was *Mmc*, which was isolated at 9.5% from *Mannheimia haemolytica* positive goats' pneumonic lung.

Histopathological Lesion Characterization: Histologically all the lesions detected were of inflammatory nature and the detected lesions were categorized as exudative, proliferative and exudative-proliferative (broncho-interstitial bronchopneumonia) with, exudative bronchopneumonia constituted the majority of these lesions. The histological lesions were not consistent for any of the pathogens isolated. However the majority of animals which were positive for *Mannheimia and Pasteurella* showed suppurative broncho-pneumonia that was characterized by presence of inflammatory cells mainly of neutrophils within the lumen of bronchiol and in the bronchiolar wall. Fibrino-suppurative bronchopneumonia that was characterized by fibrin mass admixed with number of neutrophils and macrophages and interstitial pneumonia that was characterized by focal epithelial hyperplasia of secondary and tertiary bronchi, smooth muscle hypertrophy in the wall of bronchi and connective tissue proliferation in the interstitial spaces were the subsequent lesion types

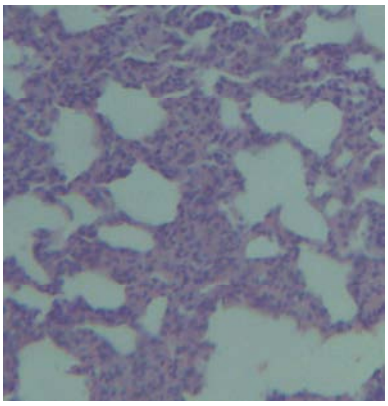

Fig. 4: Interstitial pneumonia with epithelial hyperplasia, smooth muscle hypertrophy and connective tissue proliferation in the interstitial spaces.

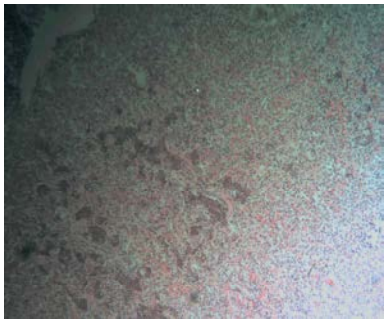

Fig. 5: Caseonecrotic bronchopneumonia with lysed cells at center of necrotic area (right bottom corner) and inflammatory cells especially of macrophages at the periphery of the necrotic center.

(Fig 4). Most of the lesions from animals positive for mycoplasmal isolation were characterized by subacute or chronic bronchopneumonia with foci of caseous necrosis and these lesions had more advanced necrosis and fibroplasias in chronic cases. Milder and probably earliest histologic lesions of caseonecrotic bronchopneumonia affected primarily the smaller bronchioles, with accumulation of necrotic leukocytes in the bronchiolar lumen (Fig 5).Necrotized cells had altered cellular outlines with homogeneously eosinophilic cytoplasm) and. Neutrophils and macrophages were recognizable at the periphery of the necrotic material, adjacent to intact bronchiolar epithelium (Fig 5).

DISCUSSIONS

In present investigation *Mannheimia haemolytica, Pasteurella and Mycoplasma* species were isolated from pneumonic lungs of sheep and goats. This indicates that these are among the important causes of clinical pulmonary diseases in sheep and goats in the study area. To the knowledge of the authors this is the first to isolated large number of *Mycoplasma* species from sheep and goats in Ethiopia so they should be considered in the diagnosis of ovine and caprine respiratory diseases in Ethiopia.

Mannheimia haemolytica serotypes from isolates and as well as *Mannheimia haemolytica* serotypes distribution in serum were not constant and showed great variation. The variation in serotype distribution also holds true anywhere else in the world [16, 17]. Gilmour [18] stated that members of the genus *Mannheimia* are phenotypically and genotypically very heterogeneous. The variation in serotype distribution in this study may be of significant in the formulation of vaccines against *Mannheimia haemolytica* serotypes in Ethiopia. Antigenic materials derived from all the dominant serotypes like serotypes A1, A2, A6, A7 and A8 (multivalent vaccine) should be comprised in sheep vaccine in Ethiopia. In line with this, OIE [9] inferred serotype tropism to lung and ability of causing lung injury varies greatly.

In addition to *Mycoplasma ovipneumoniae*, in present study *Mmm*LC, *Mmc* and *Mycoplasma arginini* were isolated from *Mannheimia haemolytica* positive pneumonic sheep. *Mycoplasma* mycoides clusters, which are the most pathogenic mycoplasma of ruminants' particularly of goats, were also isolated from sheep and this is in agreement with previous scholars studies [20- 22]. *Mmm*LC was isolated from sheep and goats. It can be concluded that *Mmm*LC is pathogenic to sheep and goats nevertheless it is not host specific [14]. *Mmm*LC is one of the most important causes of disease in herds of goats [20, 23, 24]. *Mmc* together with *Mmm*LC produced a pleuropneumonia in small ruminant that resembled CCPP [25, 26]. *Mcc* was isolated from pneumonic goats but not from sheep in this work. Thiaucourt and Roger [20] isolated *Mcc* from pneumonic goats and not from sheep while Ikheloa *et al*. [23] isolated *Mcc* from both pneumonic goats and sheep in Nigeria. *Mccp was* not isolated both from sheep as well as from *Mannheimia haemolytica* positive goats in this study but goats (32.6%) and sheep (6.5%) were found positive to *Mccp* antibodies by CFT. Shiferaw *et al* [27] isolated *Mccp* from *Mannheimia haemolytica* positive goats and Anonymous [1] isolated *Mccp* both from sheep and from goats. This study indicates that *Mannheimia*, *Pasteurella* and *Mycoplasma* species should be considered in the epidemiology of sheep respiratory diseases particularly in the study area and sheep vaccines should include stains

that commonly prevalent. Study that covers large area and large population should be conducted and trial polyvalent vaccines should be produced and evaluated.

Even if some histologic lesion of *Mycoplama, Mannheimia* and *Pasteurella* positive animals followed similar trends, considerable overlap of the different lesion types were observed and generally, it was difficult to conclude the relationship between the causative agent isolated and the lesion type detected.

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