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Isolation and Identification of Aerobic Bacteria and Influenza Virus 3 from Nasal Passageway of Camels and Outbreak Investigation in Borana, Southern Ethiopia

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Abstract

A cross-sectional study was conducted from December 2013 to March 2014 A total of 175 camels (140 apparently health and 35 clinically sick) were sampled. The samples from sick camels were used both for bacterial and viral isolation. The risk factors such as geographical location (districts), sex and age were assessed for their association with the respiratory diseases in the study area. Univariate logistic regression was used to test the association of the risk factors with the respiratory problems. Accordingly, 80% of the samples yielded at least one type of bacterial colony. A total of 274 isolates from 140 swab samples were obtained. The most frequently isolated pathogens significantly associated with respiratory signs ($p \leq 0.05$) were *Pasteurella maltocida* (22.3%), *Staphylococcus aureus* (21.7%), *E. coli* (20.6%), *Streptococcus pyogenes* (14.3%) and *Mannhemia hemolytica* (12.6%). Out of 35 nasal swabs from clinically sick camels, 13 (42.9%) samples exhibited morphologic alterations or cytopathic effect (CPE) on VERO cell monolayer. Five of these CPE positive samples were checked for the presence of Parainfluenza virus 3 (PIV 3) and were all positive using RT-PCR. The isolation of the bacteria from both health and clinically sick camels could be the indicative for the residence of the microflora in upper respiratory tract and involve in causing diseases under some difficult conditions such as viral infection. The isolation of PIV3 from camels with respiratory signs is suggestive for the distribution of the virus in the study area and the involvement of it in causing respiratory disease outbreak in camels of Borana Zone.

Keywords: Bacteria; Virus, Borana Zone; Camels; Respiratory diseases; Nasal swabs

Introduction

Camel (*Camelus dromedaries*) is reared in South, Southeast, East and Northeast of Ethiopia as primary livelihood and means of subsistence for pastoralists. It provides food (milk and meat), transportation services, long-term saving and are means of investment and source of prestige for their owners [1]. However, its production in the country has been constrained by various obstacles among which poor management systems and diseases are the most important.

Recently new cases marked by severe symptoms, high mortality and impossibility to form a precise diagnosis have been emerging in several countries [2]. Most of the diseases threatening the population manifest respiratory symptoms and have been becoming an important and serious problem of camel production in most African countries causing economic losses due to death, decreased performance and increase treatment costs [3].

Diseases outbreaks involving respiratory system and characterized by high rate of morbidity, a variable rate of mortality with sometimes sudden death have occurred in different parts of the country over times. Nevertheless, investigations of the causes of these outbreaks, made by a number of veterinary institutions and laboratories have failed to isolate the exact etiological agents of the disease [4].

As varieties of viruses, fungi, bacteria and parasites are the possible causes of respiratory outbreaks [5]. The previous works in Ethiopia have identified the etiological agents of camel respiratory diseases from field sero-epidemiological and abattoir surveys. However, complimentary information and data might be needed from isolation of causative agents from samples collected at level of residential areas.

In addition, in spite of the continuous occurrence of camel sudden death outbreaks and existence of respiratory related clinical signs, so far, in depth microbiological examination based on isolation of bacteria and virus from clinically sick and apparently health animals has not been carried out particularly in Borona pastoral area.

Therefore the objectives of the current study are isolation and identification of aerobic bacteria and PIV 3 and assessment of potential risk factors for viruses and opportunistic bacterial

infections in camels of Borana Zone, Oromia regional state, Ethioia.

Materials and Methods

Study area

The study was conducted in Borana Zone of Oromia Regional State from November 2013 to June 2014. Borana Zone is found about 600 km South of Addis Ababa, the Capital city of the country. It is located at a latitude and longitude of 03°37' 23.8" to 05° 02' 52.4" North and 37° 56' 49.4" to 39° 01' 101"East respectively. It represents a vast lowland area of southern Ethiopia covering an area of about 95,000 km2 bordering with Kenya to the South, with Somali region of Ethiopia to the East, with Guji zone to the North and with Southern Nations and Nationalities and People region (SNNPR) of Ethiopia to the West. Its altitude ranges from 970 meter above sea level (masl) in the South bordering Kenya to 1693 masl in the Northeast.

The climate is generally semi-arid with annual rainfall ranging from 300 mm in the South to over 700 mm in the North. The rainfall pattern is bimodal type with the main rainy season locally known as "ganna" extending from March to May and small rainy season called "haggaya" extending from mid-September to mid-November. The other two seasons are the cool dry season "adoleessa" extending from June to August and the warmer dry season "bonna" from December to February. The annual mean daily temperature of the area varies from 19ºC to 24ºC with moderate seasonal variation.

Surface water is a serious problem in the area. There are only two known Permanente rivers namely Dawa and Genale in the area. Traditional deep wells "ellas", large ponds (machine excavated) are used for both human and livestock during dry seasons.

Study animal

According to data of 2013 obtained from Borana zone plan and development Office, there were total of 199,993 camels in Borana zone and hence were considered as study population for the current study. Of these, 23,326 camels were found in Yabello, 35,165 camels in Arero, 19,286 camels in Dire and 13,305 camels in Moyale districts and these were considered as study frame for the research.

Study design

A cross-sectional study was conducted from December 2013 to March 2014. Four districts were selected purposively due to ease of accessibility, camel population, and security. Similarly, peasant associations (PA's) were also purposively selected for similar reasons. Households and camels were randomly selected.

Sample collections and laboratory analysis

Nasal swab collection: Camels from randomly selected herds were examined for presence of respiratory signs and their histories were obtained from the owners. A camel was considered as diseased if one or more respiratory signs including coughing, dyspenia, nasal discharge, and increased respiratory rate (18-20 per minutes) were observed. All clinically sick camels with no history of antimicrobial treatment were sampled and their sex and age were recorded.

Apparently health camels were sampled randomly for bacterial isolation. However, from camels with respiratory signs, pair of nasal swab samples, one for bacterial isolation and the other for viral isolation was collected. The sample collection was conducted in such a way that the external part of the nose of the animal to be sampled was cleaned using cotton wool moistened with 70% alcohol. A 15-inch long sterile swab was gently inserted into the nasal cavity. It was then rubbed and gently rotated against the wall of the nostril.

The sample for bacterial isolation was put into tube containing 3 ml Stourts transport medium and sample for viral isolation was put into tube containig similar amount of viral transporting medium. The tube was labeled with the date and sample ID and other information were recorded on the format prepared for the purpose. The samples were kept in icebox containing ice pack, and transported to Yabelo Regional Veterinary Laboratory within four to six hours. The samples were stored according to the protocol stated in OIE until processed [6].

The samples for bacteriological examinations were transported to the Microbiology Laboratory of College of Veterinary Medicine and Agriculture (CVMA) of Addis Ababa University (AAU); whereas samples for viral isolation were taken to the Virology Laboratory of National Veterinary Institute (NVI) at Bishoftu.

Bacteriological isolation and identification: Isolation and identification of the bacteria were conducted following standard procedures described by Quinn et al. The swab samples were inoculated into Brain Heart Infusion (BHI) broth for the primary enrichment and then incubated aerobically at 37°C for 24 hours. The growth in BHI was streaked onto sheep blood agar enriched with 5%-7% (V/V) sheep blood in such a way that cultured broth samples were thoroughly agitated and mixed. A loop full of the cultured broth was streaked onto agar plate.

The inoculated plates were checked for bacterial growth within 18-24 hours interval and for a maximum of two days. Mixed colonies were sub-cultured on blood agar to obtain pure culture. Primary bacterial identification of the isolates was conducted based on colony characteristics such as colony morphology, color, size, elevation, consistency and types of haemolysis on blood agar. Gram stain characteristics of the pure colony were examined in order to determine the Gram reaction, cellular morphology and arrangement of the isolate.

The Gram negative rod bacteria were subcultured on MacConkey agar for further identification. Primary biochemical tests such as Catalase test, Oxidase test, Oxidation-Fermentation tests, and Coagulase test were also used to identify the isolates to genera level. Some isolates were further identified based on their characteristics in secondary biochemical tests. Accordingly, different tests on agar media, and/or broth sugar and salts have

been used for biochemical tests to reach on definitive species of certain genera.

Viral isolation: Virus isolation was established under laminar air flow class II cabinet. The VERO cell cultures were propagated as monolayer cultures using minimal essential medium (MEM) with phosphate buffer saline (PBS) and supplemented with 10% fetal bovine serum (FBS). A suspension was prepared by adding 1 ml of the sample to 9 ml of antibiotics (penicillin, streptomycin and Amphotericin B solution) medium.

The media from monolayer formed cells was discarded and 0.5 ml from sample suspension was inoculated, into 25 cm2 VERO cell culture flasks respectively. The inoculated flasks were incubated at 37°C for one hour for adsorption of the viruses on to the cell then infected cell was added with 8 ml of maintenance media (2% MEM) and incubated at 37°C.

Inoculated samples were daily observed by using inverted microscope for any contamination or growth. After 2 days, the media was changed and repeated every two days for growth negative samples. Viral growth on the cell was checked every day with the microscope and was waited until 10 days. The cultures were read and the interpreted.

Molecular identification: *Parainfluenza* virus was identified by molecular techniques to determine the presence and types of the virus involved in the respiratory diseases of camels in the study area. Accordingly, five viral cultures with CPE, which were indicative for the presence of *parainfluenza* virus, were tested using polymerase chain reaction (PCR). The viral nucleic acid was extracted mainly based on viral capsid purification techniques described previously [7,8].

Briefly, the growth medium was removed from the cell culture and 0.6 ml lysis buffer with 2-mercaptoethanol was added. The cell pellet was vortexed until the pellets were dispersed and the cells appear lysed. The lysates were homogenized at room temperature using rotor-stator homogenizer for at maximum speed of 45 minutes.

The homogenates were centrifuged at 26000 x g for 5 minutes and then the supernatant was transferred to RNase-free tube. The RNA was purified by adding equal amount of 70% ethanol to the volume of cell homogenate. The alcohol-homogenate suspension was vortexed thoroughly to mix and disperse any visible precipitate that may form after ethanol addition. Seven-hundred µl of the sample including any remaining precipitate was transferred to the spin cartridge.

Then it was centrifuged at 12000 × g for 15 seconds at room temperature and the flow-through was discarded and the pin cartridge was reinserted into the same collection tube. This step was repeated until the entire sample was processed. The spin cartridges were washed by wash buffer II three times.

The RNA was eluted by adding 100 µl RNase-free water to the center of the spin cartridge, and incubated at room temperature for 1 minute. The spin cartridge was centrifuged for 2 minutes at 12000 × g at room temperature to elute the RNA from the membrane into the recovery tube. The purified RNA was stored at -80°C until PCR amplification process was carried out.

Complementary DNA synthesis

The following procedure was used to convert viral RNA into first strand cDNA. The components (sample, primer, dNTP mix and Diethyl pyrocarbonate (DEPC) treated water) were mixed and briefly centrifuged before use. The mixture was incubated at 65°C for 5 minutes and then placed on ice for about 1 minute.

The cDNA synthesis mix was prepared by adding in the order of 10XRT buffer, 25 mM MgCl2, 0.1 MDTT, RNase OUTTM (40 U/µg), and SuperScriptTM IIIRT. cDNA synthesis mix was added to RNA/primer mixture, mixed gently and collected by brief centrifugation. It was then incubated at 50°C for 50 minutes.

The reactions were terminated by placing at 85°C for 5 minutes and chilled on ice. The reactions were collected by brief centrifugation. RNase H was added and to each tube and incubated for 20 minutes at 37°C. cDNA synthesis reaction was stored at -20°C until used for PCR.

cDNA amplification

Parainfluenza virus 3 (PIV3) was investigated using PIV3 specific forward (PIV3PR5) and reverse (PIV3PR3) primers sequences: 5‟-GATCCACTGTGTCACCGCTCAATACC -3‟ and 3‟-ACCAGGAAACTATGCTGCAGAACGGC-5‟), respectively [9]. PCR reaction was carried out in a total volume of 20µl in a 0.2 ml reaction tube containing 2 µl RNase free water, iQTM supermix (2x) which contained antibody mediated hot-start Taq DNA polymerase, dNTPs, MgCl2, enhancers and stabilizers, forward and reverse PIV3 primers 2 µl each, and Template (cDNA) 4 µl.

Briefly, the iQTM supermix (2x) and other frozen reaction components were thawed to room temperature and were thoroughly mixed and centrifuged briefly to collect solution at the bottom of the tubes and were stored on ice protecting from light. Assay master mix was prepared one for all samples by adding all required components together except the DNA template according to the aforementioned volume. The assay master mix was thoroughly mixed to ensure homogeneity and equal aliquots were dispensed into each quantitative PCR (qPCR) tube.

In all of the work good pipetting practice has been employed to ensure assay precision and accuracy. DNA templates were then added to the PCR tubes and the tubes were sealed with flat caps. The tubes were vortexed for at least 30 seconds to ensure thorough mixing of the reaction components. The tubes were spinned to remove any air bubbles and the reaction mixture was collected in the vessel bottom.

The thermal cycling protocol on the PCR instrument (machine) was programmed in such a way that first polymerase activation and DNA denaturation was carried out at 95°C for 5 minutes for 1st cycle. Denaturation at 95°C, annealing at 65°C and extension at 72°C for 30 seconds was carried out. These steps were repeated for 15 cycles. Lastly, denaturation at 95°C and annealing at 62°C were carried out for 30 seconds each and extension was done at 72°C for 5 minutes. These steps were in turn repeated for 20 cycles.

PCR product analysis by gel electrophoresis

The PCR products were analyzed with 1.5% agarose gel containing specified volume of gel red. Briefly, 10 µl of PCR products mixed with loading buffer and loaded to wells in pre-prepared gel and run at 130 volt for 1:10 hours in parallel with DNA 100 bp molecular weight marker in electrophoresis apparatus using 1 x TAE buffer. The DNA band was visualized by UV illumination and the size was determined by the DNA molecular weight marker standard. The expected size of the amplicon was 234 bp.

Data entry and analysis

Data were entered in to Microsoft Excel 2007 spreadsheets and coded. Data were analysed using SPSS (statistical package software ver. 20 for Windows, SPSS Inc, Chicago, IL). The data were first validated, and then descriptive analysis was conducted. To study association between dependent variables (isolation rate) and independent variables (potential risk factors) Chi-square test and logistic regression analysis were used. The confidence level was set at 95% (A=0.05).

Results

Case history and clinical observation

Total of of 175 nasal swabs for bacteriological study and 35 nasal swab samples for viral isolation were collected. Of 175 camels 50 camels were from Yabelo, 16 camels were from Arero, 71 camels were from Dirre and 38 camels were from Moyale. Some of the animal with the respiratory problem signs were in poor body condition, weak and remained lay down.

Of the examined camels, 37.7% of them were from the mid-high land (Yabelo and Arero) and the rest 62.3% were from the low-land areas (Dirre and Moyale). The case history and visual inspections indicated that 33.7% of the samples were from diseased camels and the rest 66.3% were from apparently health camels. **Table 1** summarizes the association of the respiratory signs with the risk factors.

Table 1: Results of logistic regression analysis of the association between observed respiratory signs and potential risk factors in the study areas.

				95% CI	
Variable	**Category**	**Number of camels examined (n=175)**	**Number of camels with respiratory signs (%)**	**Odds ratio (95% CI)**	**p value**
Districts	Dirre	71	16 (22.5)	Ref	
	Yabelo	50	20 (40.0)	2.29 (1.03-5.06)	0.041
	Arero	16	9 (56.2)	4.42 (1.42-13.73)	0.010
	Moyale	38	14 (36.8)	2.00(0.84-4.75)	0.114
Age (years)	<3	58	15 (25.9)	Ref	
	3-8	69	25 (36.2)	1.62(0.75-3.50)	0.212
	>8	48	19 (39.6)	1.87(0.82-4.28)	0.134
Sex	Male	40	14 (35.0)	Ref	
	Female	135	45 (33.3)	1.07(0.44-1.95)	0.845

Isolated and identified bacteria

Detailed investigation was carried out to determine bacterial composition inhabiting the upper respiratory tract of apparently healthy and sick camels. Out of 175 nasal swab samples, 140 (80%) yielded at least one type of bacteria. As the result, a total of 274 isolates (representative of 274 different colony morphologies) were obtained from 140 samples.

That is at least about two bacterial types have been isolated from a single sample. Out of 116 samples collected from apparently healthy camels, 82 (70.7%) of them yielded bacterial growth. However, of 59 samples from clinically sick camels, 58 (98.3%) of them were positive for bacterial growth.

Both Gram negative and Gram positive bacterial genera were isolated in this study. Accordingly, out of 157 bacterial isolates from camels with respiratory signs, 97 (61.8%) were Gram positive and 60 (38.2%) were Gram negative. However, of 117 bacteria isolated from apparently healthy camels, 66(56.4%) were Gram positive and 51(43.6%) were Gram negative.

The current study isolated Pasteurella maltocida, *Streptococci equi subspp equi* and *E.coli* from one active case of sudden death in Kawa pastoralist association of Arero district. *Staphylococcus spp., Streptococcus spp.* and *Pasteurella spp.* were identified to species level. The likelihood of isolation of S. *equi subsp equi* was five times higher from sick camels with

respiratory signs as compared to apparently healthy camels **(Table 2).**

Table 2: Bacterial isolates identified from camels with (n=59) and without (n=116) respiratory problems.

Bacteria	Respiratory symptoms	No. of isolates (%)	Odds ratio (95% CI)	p value
S. equi subsp.equi	Healthy	3 (2.6)	Ref	0.022
	Sick	7 (11.9)	5.07(1.26-20.39)	
S. equi subsp. zooepidemicus	Healthy	6 (5.2)	Ref	0.015
	Sick	10 (16.9)	3.74(1.29-10.87)	
S.pnuemoniae	Healthy	2 (1.7)	Ref.	0.025
	Sick	6 (10.2)	6.45(1.26-33.03)	
S. pyogenes	Healthy	10 (8.6)	Ref	0.004
	Sick	15(25.4)	3.61(1.51-8.66)	
Pasteurella spp.	Healthy	4 (3.4)	Ref	0.168
	Sick	5 (8.5)	2.59(0.67-10.04)	
Proteus spp.	Healthy	4 (3.4)	Ref	0.519
	Sick	1 (1.7)	0.48 (0.53-4.42)	
S. aureus	Healthy	10 (8.6)	Ref	0.000
	Sick	28 (47.5)	9.57(4.19-21.86)	
S. hiycus	Healthy	3 (2.6)	Ref	0.046
	Sick	6 (10.2)	4.26(1.03-17.71)	
S. epidermidis	Healthy	11 (9.5)	Ref	0.318
	Sick	3 (5.1)	0.53(0.14-1.91)	
Corynebacterium spp.	Healthy	6 (5.2)	Ref	0.007
	Sick	11 (18.6)	4.201(1.47-12.02)	
Bacillus spp.	Healthy	4 (3.4)	Ref	0.604
	Sick	3 (5.1)	1.500(0.33-6.93)	
Actinomyces spp.	Healthy	5 (4.3)	Ref	0.488
	Sick	4 (6.8)	1.615(0.42-6.25)	
Mannhemia hemolytica	Healthy	10 (8.6)	Ref	0.031
	Sick	12 (20.3)	2.706(1.09-6.70)	
Pasteurella multocida	Healthy	17 (14.7)	Ref	0.001
	Sick	22 (37.3)	3.463(1.46-7.24)	
E. coli	Healthy	16 (13.8)	Ref	0.002
	Sick	20 (33.9)	3.205(1.51-6.82)	
Micrococcus spp.	Healthy	6 (5.2)	Ref	0.666
	Sick	4 (6.8)	1.333(0.36-4.92)	

Viral isolation

Thirty five samples taken from camels with respiratory signs were cultured for viral isolation on confluent grown VERO cell monolayer. Thirteen of them (42.9%) exhibited morphologic alterations (CPE) on VERO cell monolayer **(Table 3).** The presence of virus in the nasal swab samples was evidenced by initial swelling and rounding of infected VERO cells. The most

predominant and frequently observed type of CPE was the aggregation of infected cells with syncytia formation **(Figure 1).**

Table 3: Results of viral isolation on VERO cell monolayer with CPE based on sex and age of camels.

Factors		Tested samples	CPE positive (%)	Person Chi-square	df	p value
Sex	Male	11	4 (26.7)	0.276	1	0.599
	Female	24	11 (73.3)			
Age	<3 years	10	4(26.7)	1.789	2	0.471
	3<x< 8 years	15	5 (33.3)			
	>8 years	10	6 (40)			

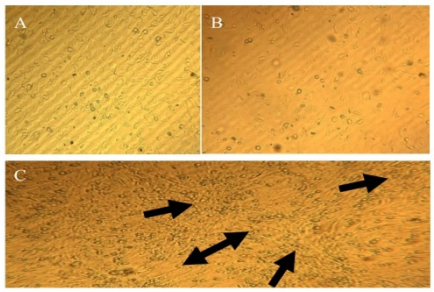

Figure 1: Digital photography of CPE formation of the viruses on VERO cell monolayer. (A) Not inoculated (B) No CPE formation (C) CPE positive: sample (→) syncytial formation, (↔) ploughing.

Molecular identification of isolated viruses: A total of five CPE positive samples were tested for the presence of *Parainfluenza* virus 3 genome. All samples tested were positive for *Parainfluenza* viruses 3 **(Figure 2).**

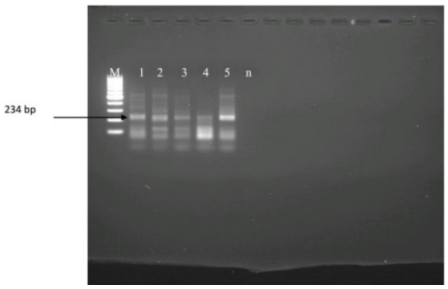

Figure 2: Digital photography of Gel red stained agarose gel electrophoreses PCR amplified PIV3 products samples analyzed by UV. M=Molecular Weight Ladder, 1-5=Samples, n=negative control.

Discussion

Case history and clinical observation

The symptoms were found to be associated with districts ($p<0.05$) but not with other risk factors as sex and age. However, despite it was insignificant, the disease rates seemed to increase with age (26% in<3years old, 36% in 3-8 years old and 40% in >8 years old) of camels. The reasons could be either due to the fact that the young camels might not develop an infectious component to the pathogen or as the result of prevalence of reactive airway obstruction associated with allergy to molds and spores, which increases with age [10].

Significant association was observed between the respiratory diseases and districts in which Dirre camels were less affected compared with the camels of other districts. Moyales', however, were roughly as two times affected as Dirres'. The crowding of the camels around limited watering points during dry season has been suspected to contribute to the spread of respiratory pathogens. Hence prevalence difference can occur among camels living in similar ecology as camels from some areas often use open water sources. Other risk factors including sudden climatic changes as well as the stress of migration during early rainfall were reported to be associated with respiratory problems of camel in Borana [11].

The frequently observed camel respiratory signs were coughing, sneezing, dyspenia, unilateral or bilateral nasal discharge, and increase in respiratory rate (18-20 per minute). From the case history, we determined that the disease appeared as acute and chronic forms. The acute form, locally called "Furi", could show mild or severe symptoms. The mild form is characterized by sneezing and serous nasal discharge whereas the sever form is characterized by coughing with profusion of purulent nasal discharge. The chronic form locally named as "Dhukuda" revealed as long time coughing, decreasing feed intake and emaciation. Similar clinical signs have been observed by previous researchers. For instance, Bekele reported that respiratory infections are common in Borana camels and the same words ("Furri" and "Dhukuda") were used to categories the clinical signs to indicate the severity of the disease [11].

The respiratory problems in camels of Ethiopia have repeatedly been reported by researchers in different times. As examples, respiratory symptoms have been observed by Bekele in Somali region of Ethiopia on an outbreak of camel disease [12]. Similarly Roger et al. reported the outbreak of respiratory disorders concurrent with high fiver with 90% morbidity and 5%-70% mortality in camels of Ethiopia [13].

Different agents have been proposed to cause respiratory diseases with various clinical signs in camels. The clinical signs are cough, bronchitis, mucopurulent discharge, inappetance, fever, pulmonary consolidation and rales on auscultation observed in camels of the Saudi Arabia was found to be caused by fungal infection [14]. However, an outbreak of camel respiratory diseases with signs as dullness and depression, nasal blockage with thick scar formation on nasal orifice, opaque in color nasal discharge, coughing, mouth breathing and gradual losing of body weight in Pakistan in 2011 was found to be caused by viral infection with bacterial complication of which definitive diagnoses could only be reached by laboratory techniques [15].

Bacterial isolates

The invariability of the isolation rate observed between apparently healthy and clinically sick camel reflects the possibility of the agents to involve in respiratory syndromes. Furthermore, majority of the isolates obtained in clinically sick camels were similar with those isolated from apparently health camels. This might, in turn, be indicative for the fact that the normal flora in upper respiratory tracts of healthy individuals can be altered to pathogenic by several factors such as the nutritional and immunological status of the animal or by the environment. That is, bacteria in apparently healthy animal can become opportunistic pathogens during stress to animals and thus resulting in endogenous infection. Azizollah et al., suggested that the suppression of the host immunity frequently allows the advancement of opportunistic bacteria to potential pathogens, leading to the presentation of a variety of pathologies [16].

The bacterial yield of the current finding is higher than the previous bacterial isolation rate from camel respiratory tracts by Moustafa who isolated bacteria from camel with respiratory problem and the normal ones at rates of 88% and 28.88% respectively. Even though the recovery rates of Moustafa was less than the current isolation rates, higher proportion of bacteria were recorded in clinically sick than the healthy ones [17]. The result was also in agreement with previous findings of Yimer et al. who recovered different bacteria from sheep nasal swab and got similar result with the current research [18].

In general, the isolation rate of Gram positive bacteria (60%) was significantly higher than Gram negative bacteria (48.6%) ($p<0.05$). The Predominance of Gram-positive bacteria from diseased camels might show their significant role in being opportunistic pathogens in camel respiratory diseases as similar to equines stated by Mir et al., [19]. The current finding is in agreement with previous reports done by Azizollah et al., from camel respiratory passage way, Mir et al., and Omer et al., from equines and sheep nasal swabs respectively. The results of this study showed the presence of P. multocida and/or M. haemolytica [16,19,20]

Staphylococcus spp., *Streptococcus spp*. and other bacteria in healthy and clinically sick camels. Awol et al., also reported the involvement of multiple agents in causing camel pneumonia in Ethiopia. It has also been repeatedly determined that bacterial infections are among the main causes of pneumonia in camels [21].

Moreover, in conjunction with viruses such as *parainfluenza* viruses, influenza viruses and morbili viruses, most of these bacteria have previously been incriminated to cause camel sudden death (CSD) [12,22]. El-Moez et al. also stated that respiratory disease causing bacteria that have been incriminated to cause sudden death in animals include Gram positive bacteria such as *Staphylococcus aureus, Streptococcus equi subsp. equi and acid fast bacilli,* and Gram negative bacteria such as *P. multocida, P. pneumonia* and *E. coli* [23].

The predominant species among the isolates from nasal swab samples were *Streptococcus pyogenes* (14.3%), *Staphylococcus aureus* (21.7%), *Mannhemia hemolytica* (12.6%), *Pasteurella maltocida* (22.3%) and *E. coli* (20.6%). In addition, *S. epidrmidis*, *S. intermedius*, *S. hyicus*, *Micrococcus spp*., *Streptococcus equi subsp equi*, *S. pneumonia*, *Corynebaxterium spp*., *Actynomyces spp*. *Proteus* and *Klebseilla spp.* were also isolated in less frequent. Previous studies have also isolated similar bacteria from nasal passage way of camels [16,24].

The odds ratio of the sick camels to have *S. aureus* is 4 times more than that of the healthy one. The predominance of *Staphylococcus aureus* in this study was in agreement with other findings that have isolated it from different respiratory organs of camels. In their research on pneumonic camel lung Abo-Elnaga et al., have isolated *Staphylococcus aureus* predominantly [25,26]. The high isolation rate of the bacteria summed up with the possibility to be recovered from the healthy one is indicative for the opportunistic involvement in camel respiratory diseases.

There were also the situations in which mixed infections have been observed in the findings. Accordingly, *S. aureus* was isolated concurrent with *S. equi subsp equi* (10.7%), *S. equi subsp zooepidemicus* (21.4%), *S. pneumoniae* (14.3), *S. pyogenes* (35.7%), *Corynebacterium spp.* (28.6%), *Actinomyces spp.* (7.1%), *Mannhemia hemolytica* (10.7%), *P. maltocida* (35.7%) and *E. coli* (32.1%). Significant correlation between the presences of bacterial species consistent with clinical observations was explained by Wood et al. that mixed infections in the presence of disease were common in equine respiratory disease symptoms [27].

Other *Staphylococcus spp*. viz. *Staphylococcus hyicus*, *S. intermedius* and *S. epidermidis* were also isolated in the present study. The coagulase positive *S. intermedius*, and the coagulase-variable *S. hyicus* are important pathogens of domestic animals [28]. However, except *S. aureus*, most of *Staphylococcus spp.* isolated from camel respiratory tracts in previous study has been reported as a general *Staphylococcus spp*. and this made the comparison of the significance of each species with previous studies difficult. *S. hyicus* was isolated at rate of 5.1% from nasal swab samples. Abdul-Gader et al., has isolated *S. hyicus* from clinical mastitic cases of Ethiopian camels. *S. hyicus* has also been isolated from camel urine [29,30].

Micrococcus spp. was isolated only from 5.1% of camels. The recovery rate of the bacterium from apparently healthy and clinically sick camels were 5.2% and 6.8% respectively. Al-Ani et al. also isolated the bacteria from nasal cavity of both clinically ill and apparently healthy camels [31]. Although Micrococcus species were isolated from nasal cavities of camels and normal

rabbits, nasal cavity and tonsils of goats and from nasal cavity of camels in the current study, they are assumed to be nonpathogenic in veterinary microbiology [28,32-34]. However, from the fact that this bacterium has been frequently isolated from lesioned lungs of camels in previous studies and in clinically sick camels in the current study, it could have a role in the development of respiratory infections [4,26].

As far as *Streptococcus spps.*, are concerned, they were isolated at rates of 25.7%. The recovery rate of the species from sick camels was about 3.5 times more than that of from healthy ones (17.2% in healthy camels and 42.4% in clinically sick camels); which is indicative of their association with respiratory problem. In contrast to the present finding Azizollah et al. isolated *Streptococcus spp*. from nasal cavity of apparently healthy camels, the finding was at higher rate than the current report (35%). *S. pyogenes* was found significantly associated with respiratory diseases symptoms ($p<0.05$) that it was isolated from 25.4% of sick camels but from only 8.6% of the apparently healthy ones [16]. These results were much higher than that of Abo-Elnaga and Osman, who recovered *Streptococcus pyogens* at rates of 2.9% from condemned lungs of camels. This might be due to the anatomical difference in sample collection [25].

Streptococcus equi subsp equi was other *Streptococcus spp.* isolated from nasal swab samples. It was recovered from 5.7% of the sample in which was isolated significantly at higher rate (11.9%) from clinically sick camels than from apparently healthy ones ($p<0.05$, OR=5.07). Previous studies reported that the bacterium had been isolated from camels with respiratory outbreak in Ethiopia. Moreover, *Streptococcus equi sub-species equi* have been strongly incriminated as cause of acute camel respiratory disease outbreaks occurred in Ethiopia in 1995 [12,22].

S. pneumonia was also isolated at rate of 4.6% of the nasal swab samples from camels and was found significantly associated with respiratory symptoms observed ($p<0.05$). Similarly, Abo-Elnaga and Osman recovered the bacterium from pneumonic lung in camel at rate of 8.6% [25]. Even though the difference is insignificant, the isolation rate of *S. pneumonia* was found to decrease with increment of the age of camels in that it was isolated from 6.9% of camels of ≤ 3 years as compared to 2.1% isolation from camels>8 years old.

Moreover, the result of the current study also revealed that significantly high rate of *Corynebacterium spp.* were isolated from the samples of camels with respiratory signs (18.2%) and compared to healthy camels (9.7%) ($p<0.05$). The isolation rate from sick camels is nearly 4 times higher than healthy camels. Similarly, Abubakar et al. (2010) isolated *Corynebacterium spp.* from normal and pneumonic lung in camel at rates of 2.2% and 10.9% respectively [26]. Abo-Elnaga and Osman also isolated *C. pyogenes* in 5.7% of pneumonic lesions [25]. The pathogen has been incriminated to involve in pneumonia of camels under stress condition, poor sanitation and immunosuppression.

In the study *M. hemolytica* (12.6%) and *Pasteurella maltocida* (22.3%) were isolated from camel nasal swab samples. *M. hemolytica* was recovery at rates of 8.6% in apparently healthy and 20.3% in clinically sick camels whereas *Pasteurella maltocida* was recovered at rates of 8.6% and 20.3% in apparently healthy and in clinically sick camels respectively. Both were found significantly associated with respiratory problem signs in the study area ($p<0.05$). *Mannhemia hemolytica* has been reported as a cause of primary and secondary pneumonia and a number of non-specific inflammatory lesions in various species of domestic animals [28,31]. The bacterium has also been isolated from normal and pneumonic lungs of camel by different researchers. Accordingly, Seddek, Abo-Elnaga and Osman and Al-Tarazi et al. reported isolation rates of 1.17%, 1.4% and 6.6% from camel lungs, respectively [21,25,32]. Shemsedin (2002) also reported that 8.7% of the total bacterial isolates from camel lung tissue were *M. haemolytica* [26]. Al-Rawashdeh et al., isolated *M. haemolytica* from 56% of pneumonic lungs of slaughtered camels [33-35].

Seroprevalence study on camels with respiratory signs conducted in North Gondar and North Wollo in Ethiopia reveled that, *M. haemolytica* was positive in 56.5% and 8.7% respectively [5]. *Camel pasteurellosis*, due to *M. haemolytica* has long been reported from lung and whole blood of febrile camels in Shinnelle zone of Somali National Regional State of Ethiopia [12,36]. In most active respiratory disease problem *M. haemolytica* can be the predominant isolates [37-39]. Even though both *P. multocida* and *M. haemolytica* are considered as important contributory pathogens in enzootic or primary pneumonia in different animals, *M. haemolytica* assumes greater prominence than *Pasteurella spp*., in the same environment both in terms of infection intensity and pathogenicity [20,40].

Escherichia coli were another isolate recovered from 20.6% of the samples. It was isolated from healthy camels at rate of 13.8 % and from camels with pulmonary disease symptoms at rate of 33.9%. The isolation rate from sick camels was about 3 times greater than that of healthy camels. The result is comparable with result of Zubair et al. who recovered it as 3% from camel lung lesion but lower than the report of Al-Tarazi et al., Mahmoud et al., Awol et al., and Abo-Elnaga and Osman, who recovered *E.coli* at a rates of 26.7%, 11.1%, 17.5% and 8.6% respectively [4,25,38,41,42] E. coli has also been reported as predominant commensal isolates residing in nasopharyngeal microflora and are capable of causing infection when the body defense mechanisms are impaired [18,43].

Viral isolation

The study showed that the overall proportion of camels infected with virsuwas 20% (35/175). Ayelet et al. (2013), in their investigation of camel respiratory problems, reported associated with viral agents in 8.9% of slaughtered camels [44]. With duration of cytopathic effect (CPE) development from 7 to 14 days ranges, the CPE were observed with characteristics such as many scattered, rounded, refractory cells and syncytia formation. Out of 35 samples inoculated on VERO cell culture, 15 (42.9%) showed more than 50% CPE. Similarly, Shaker (2003) reported isolation of *Parainfluenza* virus 3 (PIV3) from camel lungs in Egypt [45]. Intisar et al. isolated PIV3 from camel lung specimens inMadin Darby bovine kidney (MDBK) cell cultures

observing the typical CPE of PIV3 i.e., rounded retractile cells, cell elongation and sloughing with some syncytia formation, which was similar with early description given by [46,47].

In the current study, five of CPE positive samples tested for *Parainfluenza virus* gave visible positive PCR results as *Parainfluenza virus* 3 (PIV3) which showed that *Parainfluenza virus* 3 plays a role in camel respiratory disease syndrome. The contribution of the present study is that *Parainfluenza virus* 3 was isolated for the first time from Ethiopian camels. *Parainfluenza virus* 3 is one of the viruses known to cause respiratory infection. As opposed to the present findings, Ayelet et al. did not find PIV3 in CPE positive samples during PCR amplification using PIV3 specific primers [44]. At the time they suggested that the absence of a PCR product could be due to the use of VERO cell lines for virus culture in the study instead of the commonly used MDBK cell by other researchers which is more susceptible for *Parainfluenza viruses*.

Parainfluenza type 3 (PIV3) in combination with other viruses such as Bovine viral diarrhea (BVD) and *Bovine herpesvirus* type 1 (BHV-1) viruses has been incriminated to cause acute respiratory diseases [48]. Although, PIV-3 is the viruse known to cause respiratory infection in camel, geographical distribution of the virus in camels have not been well studied so far. However, there have been reports from many camel rearing countries. Serological findings reported from Nigeria and Ethiopia were the early indicative of the common occurrence of *Parainfluenza* 1 and 2 virus infection in camel rearing areas [49,50]. Olaleye et al., who investigated camel respiratory disease in Northeastern Nigeria, determined that out of 150 camel sera samples, tested by complement fixation test, 22.3% were positive to *Parainfluenza*-1, 18.5% to *Parainfulenza*-3 virus and 12.7% to influenza virus B. Schwartz reported that *Parainfluenza* 1, 2 and 3 were common and widely distributed in most camel rearing areas [49,51]. In addition, Dioli and Stimmelmayr reported that viruses associated with respiratory infections in camels are *Parainfluenza* virus 3 (PIV-3), influenza virus A and B, *adenovirus*, *respiratory syncytial virus* (RSV) and infectious bovine rhinotracheitis (IBR) [52].

In Ethiopia, the evidence of a study conducted by Kebede and Gelaye was suggestive for *Parainfluenza* virus-3 being the primary pathogenic agent in the camel respiratory disease outbreak in the country [5]. During the study they demonstrated that there was significant difference in positive proportion of sera from surveyed areas with outbreak (70.5%) and without outbreak (6.8%). There was also high antibody titer level in outbreak area than the surveyed area without outbreak. In the current study, some bacteria were isolated concurrently with virus. The isolation of *E. coli* and *Staphylococcus spp.* from viral positive camels was about 8 and 2 times higher respectively than those from which virus was not isolated. Other bacteria found associated with virus infection were *Manhemia hemolytica*, *Pasteurella maltocida*, *Actinomyces spp.* and *Corynebacterium spp*. This is supportive for the fact that bacteria residing in upper respiratory tracts of animals can cause diseases as secondary invaders in viral infection. Kebede and Gelaye stated that a number of microbial agents involved as a primary or secondary infection of camel respiratory disease. *Parainfluenza* virus concurrently occurs with *Pasteurella spp.* as a secondary invader in camel pulmonary diseases [5]. The association of *Parainfluenza* with pasteurellosis causes serious respiratory disease complication and even death in camels if they were not treated in early time of infection. This is because pasteurellosis usually related with various debilitating factors, it appeared as a secondary bacterial complication following *parainfluenza* exposure.

Parainfluenza viruses are important respiratory tract pathogens of human as well as mammals and birds [53]. Theyreplicate in the epithelial cells lining the respiratory tract, causing rhinitis, pharyngitis, laryngitis, tracheobronchitis, bronchiolitis, and pneumonia. Early during *parainfluenza* virus infection, the mucous membranes of the nose and throat are involved [54]. Transmission of *Parainfluenza* viruses is by direct animal-to-animal contact or by large-droplet spread. However, the viruses do not persist long in the environment. The high rate of infection early in life, coupled with the frequency of reinfection, suggests that these viruses spread readily from animal to animal [55-58].

Conclusion

In the present study, high percentage of various types of aerobic bacteria were isolated from nasal swabs of apparently healthy and sick camels in Borana Zone.. They include the bacteria which have been incriminated to cause camel sudden death (CSD). The isolation of pneumotropic virus, *Parainfluenza* virus 3 from clinical cases concurrent with opportunistic bacteria is indicative for the distribution of the virus in study area that favors the involvement of microflora in camel respiratory disease outbreaks.

Based on the above conclusion, the following recommendations are forwarded.

Detailed molecular based investigation of bacterial isolates from camel respiratory tracts should be conducted to characterize the types of bacteria involved in respiratory diseases

The epidemiology of *parainfluenza* virus and other camel respiratory disease causing viruses should be studied in order to design specific prevention and control methods

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