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Article

Isolation, identification and characterization of bacterial flora from the respiratory tract of apparently healthy sheep

Chandan Kumar Sarker[1], Anowar Hossen[2], Md. Abu Yousuf[3*], Md. Ala Uddin[4], Most. Sumona Akter[1], Mostafizur Rahman[5] and Md. Bahanur Rahman[5]

[1]Veterinary Surgeon, Department of Livestock Services, Farmgate, Dhaka, Bangladesh
[2]Poultry Disease Diagnostic Laboratory, Poultry Production Research Division, Bangladesh Livestock Research Institute, Savar, Dhaka, Bangladesh
[3] SAARC Regional Leading Diagnostic Laboratory for PPR, Animal Health Research Division, Bangladesh Livestock Research Institute, Savar, Dhaka, Bangladesh
[3]Poultry Production Research Division, Bangladesh Livestock Research Institute, Savar, Dhaka, Bangladesh
[4]Department of Animal Husbandry and Veterinary science, University of Rajshahi, Rajshahi, Bangladesh
[5]Department of Microbiology and Hygiene, Bangladesh Agricultural University, Mymensingh, Bangladesh

[*]Corresponding author: Dr. Md. Abu Yousuf, Scientific Officer, SAARC Regional Leading Diagnostic Laboratory for PPR, Animal Health Research Division, Bangladesh Livestock Research Institute, Savar, Dhaka-1341, Bangladesh. Mobile: +8801717449845; E-mail: mayousuf_82yahoo.com

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Abstract: Sheep is the common name for a group of grazing mammals that may be either wild or domesticated; the domesticated varieties are amongst the most widely distributed types of domestic animal, found in nearly all countries. Bangladesh is a densely populated developing country and its economy is primarily based on agriculture. The current study was designed for isolation, identification and characterization of bacterial flora from the upper respiratory tract of sheep. Thirty (30) apparently healthy sheep were selected at the adjacent areas of Bangladesh Agricultural University (BAU) for this experiment. Swab samples were collected from nasal swabs (10), lung swabs (10) and tracheal swabs (10). All samples were subjected into inoculated on to bacteriological media (nutrient broth, nutrient agar, Salmonella-Shigella agar, MacConkey agar, blood agar, brilliant green agar). Furthermore, all of the bacterial isolates were characterized by Gram's staining, biochemical tests (sugar fermentation tests, catalase test, coagulase test, indole test, MR-VP test), antibiotics sensitivity tests and pathogenicity tests. None of the isolated *Bacillus*, *E. coli* and *Staphylococcus* spp. was found to be pathogenic. Isolated *Pasteurella* spp. were found to be pathogenic as observed in different experimental models and showed a degree of variation in antibiotic drug sensitivity test. Ciprofloxacillin was sensitive to all of the isolated bacteria. Through the bacteria that were isolated from various organs of apparently healthy sheep is normal micro flora, however these may act as primary pathogen and may produce diseases when the sheep are immunologically suppressed due to severe stress conditions.

Keywords: sheep; respiratory tract; bacteria; antibiotics

1. Introduction

Bangladesh is a densely populated developing country and its economy is primarily based on agriculture. Agriculture is composed of crop, livestock, fisheries and forestry. The livestock population in Bangladesh is composed of 22.90 million cattle, 1.26 million buffaloes, 21.56 million goats and 2.78 million sheep (BBS, 2008). Respiratory disease is commonly encountered in sheep herd, affecting groups or individuals (Sultan, 1995). The sheep has remarkable ability to adapt to diverse environmental conditions; it plays an important role in traditional smallholder farming systems of Bangladesh but this progress has been hampered by the several

types of bacterial and viral infection which causes a great loss in our national economy. The most affecting site of sheep caused by bacteria is respiratory system (Ajuwape *et. al.,* 2002). The most common diseases of respiratory system are pneumonia is caused by several organisms. Sheep respiratory infections appear as differing clinical syndromes. Mild, acute infections are usually due to parainfluenza 3 (PI3) viruses (Richard, 1986). The prevalence of six different bacterial species was greater in the lungs of unhealthy animals, namely *Actinomyces pyogenes*, *Erysipelothrix* spp., *P. haemolytica*, *Pasteurella ureae*, *Staphylococcus aureus*, and *Staphylococcus epidermidis*, which could be risk factors in the complexity of the prevalent respiratory diseases of the animals surveyed. *Pasteurella* spp. (particularly *P. haemolytica*) are also responsible for the most severe and acute forms of ovine pneumonia. The disease is an acute exudative pneumonia with septicemia and very high mortality rates. Difficulties in reproducing the disease experimentally with pure cultures of *Pasteurella* spp. suggest that a previous infection with a virus or mycoplasma is usual in field cases (Barbour 1997 and Davies *et al.,* 1981). Information generated from this research may help to reduce the mortality rate of sheep from bacterial diseases, minimize cost of healthy sheep production, reduce economic loss which is spent for the treatment of sheep diseases resulting more establishments of sheep farms that creates employment opportunity and will increase animal protein production.

2. Materials and Methods

2.1. Experimental animals and samples

Thirty (30) apparently healthy sheep were selected at the adjacent areas of Bangladesh Agricultural University (BAU) for this experiment and under the Department of Microbiology and Hygiene. Swab samples were collected from Nasal swabs (10), Lung swabs (10) and Tracheal swabs (10). Nasal swabs, tracheal swabs and lungs swabs were collected using sterilized cotton with aseptically. Then the swabs were inoculated into NB and selenite broth and incubated at 37^0C for 24 hours. After incubation it was streaked on to NA and MC agar medium.

2.2. Selection of day-old suckling mice

Bacterial pathogen free healthy day-old Swiss Albino suckling mice were selected for this experiment. All the mice were supplied by the laboratory experimental animal house of the Department of Microbiology and Hygiene, Bangladesh Agricultural University, Mymensingh. After inoculation of the inoculum (bacteria, crude toxin and both bacteria and crude toxin) the laboratory animals were kept in an isolation room of the animal shed of the Department of Microbiology and Hygiene providing appropriate temperature.

2.3. The experimental design

The entire study was divided into three major steps: The first step included selection of sources, collection of samples, isolation, identification and characterization of isolates organisms on the basis of their colony morphology, staining property, motility and biochemical characteristics. In the second step, pathogenicity study of the isolates organisms was performed in different experimental model such as in day-old chicks and in day-old suckling mice to observe their lethal effect. In the third step, the current status of drug sensitivity and resistance pattern of the bacterial isolates from nasal, tracheal and lung swabs was determined.

2.4. Bacteriological media

a. Agar media

Agar media used for bacteriological analysis were Nutrient agar (NA), Salmonella Shigella agar (SSA), MacConkey (MC) agar, Eosin methylene blue (EMB) agar, and Brilliant green (BG) and agar Blood agar (BA) that was made according to the manufacturing instruction.

b. Liquid media (broth)

The liquid media used for this study were Nutrient broth, Peptone broth, Methyl-Red and Voges-Proskauer broth (MR-VP broth) and Sugar media (dextrose, maltose, lactose, sucrose and mannitol) that was made according to the manufacturing instruction.

c. Chemicals and reagents

The chemicals and reagents were used for this study , e.g. 0.1% Peptone water, Phosphate buffered saline (PBS), reagents for Gram's staining (Crystal Violate, Gram's iodine, Safranin, Acetone alcohol), 3% Hydrogen

peroxide, Phenol red, Methyl red, 10% Potassium hydroxide, Kovac's indole reagent (4-dimethylamino-benzaldehyde, concentrated HCL), Mineral oil, Normal saline and other common laboratory chemicals and reagents.

2.5. Antimicrobial discs

To determine the drug sensitivity and resistance pattern and to interpret their disease potential commercially available antimicrobial discs (Becton, Dickinson and Company, USA) were used. This method allowed for the rapid detection of the efficacy of drugs against the test organisms by measuring the diameter of the zone of inhibition that resulted from diffusion of the agent into the medium surrounding the discs inhibiting the growth of the organisms. The following antimicrobial agents with their disc concentration (Gentamycin 10 µg /disc, Erythromycin 15 µg /disc, Tetracyclillin 30 µg /disc, Azithromycin 15 µg /disc, Ciprofloxacin 5 µg /disc, Amoxicillin 10 µg /disc, Ampicillin 10 µg /disc, and Metronidazole 80 µg /disc) were used to test the sensitivity and resistance pattern of the selected bacterial isolates from nasal, tracheal and lung swab of sheep.

2.6. Isolation and identification of *Pasteurella* spp.

Pasteurella spp. was isolated on the basis of the morphology, cultural characteristics and biochemical characteristics. The colonies of *Pasteurella* spp. were round, moderate size and grayish on BA. A characteristic musty odor was present in the fresh cultural. The organisms were Gram-nagative, non-motile, bipolar rod occurring in pairs, singly or in chains and were negative to indole, MR and VP (Merchant and Packer, 1967).

2.7. Isolation and identification of *Bacillus* spp.

The samples were first inoculated in to NB and then it was inoculated on to BA and NA and then incubated at 37^0C for 24-48 hrs for the isolation and identification of *Bacillus* spp. On Gram's staining the Gram-positive large, sporulated, rod-shaped bacteria in chain from, indicated Bacillus spp. It was confirmed by biochemical tests. Many rod-shaped bacilli produced β-hemolysis on BA (Marchant and Packer, 1967).

2.8. Gram's staining method

Gram's staining was performed as per recommendation of Merchant and Packer (1976) to determine the size, shape and arrangement of bacteria. The procedure was as follows-

A small colony was picked up with a bacteriological loop, smeared on a glass slide and fixed by gentle heating. Crystal violet solution was then applied on the smear to stain for two minutes and then washed through running tap water. Gram's iodine was then added to act as a mordent for one minute and then again washed with running water. Acetone alcohol was then added, which act as a decolorizer. After washing with water, safranine was added as counter stain and allowed to stain for 2 minutes. The slide was then washed with water, blotted and air dried and then examined under microscope with high power objective (100X) using immersion oil.

2.9. Motility test for bacterial isolates

The motility test was performed according to the method described by Cowan, 1985 to differentiate the motile bacteria from the non-motile one. Before performing the test, a pure culture of the test organism was allowed to grow in nutrient broth. One drop of cultured broth was placed on the cover slip and was placed inverted condition over the concave depression of the hanging drop slide to make hanging drop preparation. Vaseline was used around the concave depression of the hanging drop slide for better attachment of the cover slip to prevent air current and evaporation of the fluid. The hanging drop slide was then examined carefully fewer than 100X power objective of a compound microscope using immersion oil. The motile and non motile organisms were identified by observing motility in contrasting with to and fro movement of bacteria.

3. Results and Discussion

The results presented below demonstrated the isolation and identification of bacterial flora from respiratory tract of apparently healthy sheep. Bacterial floras which were isolated from nasal, tracheal and lung swab of apparently healthy sheep were recorded as *Pasteurella* spp., and *Bacillus* spp.

3.1. Bacteria isolated from nasal swabs

Pasteurella spp. was isolated from nasal swab of apparently healthy sheep. The result of bacteria isolated from nasal swab was 9 (90%) Whereas, Barbour *et al. (*1997) isolated *Pasteurella* spp. from respiratory tract of healthy and unhealthy sheep.

3.2. Bacteria isolated from tracheal swabs

Pasteurella spp. *and Bacillus* spp. were isolated from tracheal swab of apparently healthy sheep. The result of bacteria isolated from tracheal swab was *Pasteurella* spp. 8 (80%) and *Bacillus* spp. 9 (90%). Whereas, Tatum *et al. (*2005) observed that *Pasteurella* spp. can occur as a commensal in the nasopharyngeal reason of apparently healthy animal.

3.3. Bacteria isolated from lung swabs

Pasteurella spp. and *Bacillus* spp. were isolated from lung swab of apparently healthy sheep. The result of bacteria isolated from lung swab was *Pasteurella* spp. 7 (70%) and *Bacillus* spp. 7 (70%). Whereas, Mohammed (1999) isolated *Bacillus* spp. from trachea and lung of sheep and goats although Shemsedin (2002) isolated only from camel's lung.

3.4. Isolation and identification of *Pasteurella* spp. by different bacteriological methods

Nutrient broth was inoculated separately with the nasal, tracheal and lungs swab and incubated at 37°C for 24 hrs. The growth *of P. multocida* in NB was characterized by diffused turbidity and no pellicle was found to be formed. The culture of organism on Nutrient broth (NA) yielded small colonies. The other characteristics of these colonies included whitish, opaque circular and translucent appearance (Table 1). The culture of organism on (Blood agar) BA yielded small colonies. The other characteristics of these colonies included whitish, opaque circular and translucent appearance. No hemolysis was noticed on blood agar (Table 1 and Figure 1). Culture *of P. multocida* on EMB agar yielded small, circular, smooth, convex, translucent, glistening colonies which had tendency to coalesce. On EMB agar metallic sheen was absent (Table 1). No colonies produced by the organisms on MacConkey agar after overnight incubation was tentatively confirmed as *P. multocida* (Table 1). Culture of *P. multocida* on SS agar yielded small, circular, smooth, convex, translucent, glistening colonies which had tendency to coalesce (Table 1). Culture of *P. multocida* on Brilliant Green agar (BGA) yielded small, circular, smooth, convex, translucent, glistening colonies which had tendency to coalesce. In case of Gram's staining method, Gram's staining was performed on smears of samples showed the presence of gram negative, coccobacillary organism and arranged singly or in paired (Table 1). In Leishman's staining, presence of bipolar characters of *P. multocida* organisms was manifested on Leishman's method of staining. In case of organisms grown on artifical media, the presence of metachromatic granules providing bipolar characters were not a regular phenomenon. The isolates were found to be non-motile when examined under the hanging drop preparation (Table 1).The morphology of the isolated *Pasteurella* spp. in Gram's staining exhibited gram negative, coccobacillary organism and arranged singly or in paired which was supported by several authors (Buxton and Fraser 1977; Freeman, 1985 and Jones *et al.*, 1987).

Table 1. Results of cultural, morphological and motility characteristics of the isolated *P. multocida* from apparently healthy sheep.

Colony characteristics					Staining characters	Motility
Nutrient Agar	**Blood agar**	**EMB agar**	**MC agar**	**SS agar**		
Whitish, opaque, circular, translucent appearance	Whitish, opaque, circular, translucent appearances, and no haemolysis	Small circular, convex, glistening colonies, no metallic sheen.	No colony appears	Same as EMBagar.	Gram negative. Bipolar, coccobacillary	Non motile

Legends

EMB = Eosin Methylene Blue; MC= MacConkey; SS = Salmonella Shigella; BG= Brilliant Green

In case biochemical tests, the biochemical tests conducted with all the isolates inoculated into various sugar media such as dextrose, maltose, lactose, sucrose and mannitol. All the isolates fermented dextrose, sucrose and mannitol with production of acid but did not ferment lactose and maltose (Table 2). In MR test, persistence of red

colour indicated positive test while appearance of yellow colour indicated negative test. In VP test, the appearance of pink colour indicated the positive test. Both MR and VP tests were found to be negative for the isolates of *P. multocida* (Table 2). In Indole test, the appearance of red colour indicated the positive test. Indole test was found to be negative for the isolates of *P. multocida* (Table 2). Results of *Pasteurella* spp. isolates were positive as reported by Buxton and Fraser 1977; Honda *et al.,* 1982. The isolates also revealed negative reaction in MR, VP and Indole test which was supported by several authors (Buxton and Fraser 1977; Honda *et al.,* 1982).

Table 2. Results of biochemical test of the *P. multocida* isolates.

Fermentation reaction with five basic sugars	Results
Dextrose	A
Sucrose	A
Lactose	-
Maltose	-
Mannitol	A
MR	-
VP	-
Indole	-
Catalase test	+

3.6. Isolation and identification of *Bacillus* spp. by different bacteriological methods

Nutrient broth was inoculated separately with the nasal, tracheal, lungs swab and incubated at 37°C for 24 hrs. The presence of turbidity indicates growth of bacteria (Table 3). In nutrient agar, Culture on nutrient agar plates with the organism revealed the growth of bacteria after 24 hrs of incubation at 37°C aerobically and was indicated by the growth of thick, grayish-white or cream colored colonies. (Table 3), In case of Blood agar (BA), plates were streaked separately with the organism and incubated at 37°C aerobically for 24 hrs. Large, creamy colonies with ß hemolysis were produced (Table 3 and Figure 2). Gram's stained smear from NA and BA were examined microscopically which revealed Grain-positive, large rod shaped organisms arranged in chain (Table 3). In motile test, all the isolates were found to be motile with hanging drop slide preparation (Table 3)**.** In case of biochemical tests, the isolates fermented the five basic sugars (dextrose, maltose, lactose, sucrose and mannitol) with the production of acid. Acid production was indicated by the change of color from reddish to yellow (Table 3). In Methyl red (MR) test, persistence of red colour indicated positive test while appearance of yellow colour indicated negative test. In Voges- Proskauer (VP) test, the appearance of pink colour indicated the positive test. MR test negative and VP tests were found to be positive for the isolates of *Bacillus* spp. (Table 3). In Indole test, the appearance of red colour indicated the negative test. Indole test was found to be positive for the isolates of *Bacillus* spp. (Table 3). In Gram's staining, the morphology of the *Bacillus* spp. exhibited Gram-positive, large rod shaped organisms arranged in chain which was supported by several authors (Buxton and Fraser 1977; Freeman, 1985 and Jones *et al.,* 1987). All the isolates fermented dextrose, sucrose, lactose, maltose and mannitol with the production of acid within 24h-48h of incubation. Results of *Bacillus* spp. were positive as reported by Buxton and Fraser 1977. The isolates also revealed positive reaction in VP test, negative reaction in MR and Indole test which was supported by several authors (Honda *et al.,* 1982).

Table 3. Cultural, morphological and biochemical characteristics of isolated *Bacillus* spp.

Cultural characteristics		Biochemical characteristics		Staining and morphological characteristics
BA	**NA**	**Tests**	**Results**	**Staining properties**
Large, creamy colonies with P hemolysis were produced.	Thick, grayish-white or cream colored colonies were produced.	Dextrose	A	Gram-positive, large rod shaped organisms arranged in chain.
		Maltose	A	
		Lactose	A	
		Sucrose	A	
		Mannitol	A	
		MR	-	
		VP	+	
		Indole	-	

3.7. Pathogenicity test of *Pasteurella* spp. *and Bacillus* spp. in mice

Pathogenicity test was conducted with the *Pasteurella* spp. isolated from sheep. The organism was inoculated intramuscularly (IM) in three adult mice and another three mice were kept as control. The isolates of *Pasteurella* spp. caused death of the mice within 48 hours in the test group and were categorized as pathogenic. Characteristic lesions in different organs were produced following the experimental inoculation of *Pasteurella* spp. in adult mice. White necrotic foci were present in the liver. There were hemorrhages in the lungs, trachea, spleen and liver. The organisms were re-isolated from the heart blood sample of experimentally inoculated mice. Also pathogenicity test was done with the selected strains of *Bacillus* spp. isolated from sheep. Three 1-4 days old mice were inoculated orally with the *Bacillus* spp. isolates. The experimental mice were alive after 48 hours of inoculation of isolates of *Bacillus* spp. in the test group and were categorized as non pathogenic as they did not produce any clinical disease.

3.8. Results of antibiogram study of the isolated bacteria

Five *Pasteurella* spp. and five *Bacillus* spp. were tested for the antibiotic sensitivity and resistance against commonly used antibiotics. The results of sensitivity against antibiotic discs (zone of inhibition) were categorized as resistant (-), less sensitive (+), moderately sensitive (++) and highly sensitive (+++). The results of sensitivity tests are given in Table 4.

Table 4. Results of antibiotics sensitivity tests.

Name of bacteria	Total isolates	CIP	AZM	E	TE	GM	A	LVX	MET
Pasteurella spp.	05	++	+++	++	++	++	+	-	-
		++	++	++	++	+++	++	+	+
		+++	++	+++	+	+	++	++	+
		+++	+++	++	++	++	+	-	-
		++	+++	++	+++	++	+	+	+
Bacillus spp.	05	+++	+	++	+++	++	-	+++	-
		++	-	+	+++	++	+	++	+
		+++	++	++	++	+	+	++	+
		+++	+	+	++	++	-	+++	-
		++	+	+	+++	+	-	++	-

Legends: CIP = Ciprofloxacin; AZM = Azithromycin; E = Erythromycin; TE =Tetracycline; GN = Gentamycin; LVX = Levofloxacin; A = Amoxicillin; MET = Metronidazole; - = Resistant; + = Less sensitive; ++ = Moderately sensitive; and +++ = Highly sensitive

3.9. Antibiotic sensitivity pattern of *Pasteurella* spp. *and Bacillus* spp.

Among the 5 isolates of *Pasteurella* spp**.,** 40% were resistant to Levofloxacin and Metronidazole. Sixty percent were less sensitive to Amoxicillin and Metronidazole, 40% to Levofloxacin and 20% to Tetracycline and Gentamycin. Among the isolates 80% were moderately sensitive to Erythromycin, 60% to Ciprofloxacin, Tetracycline and Gentamycin, 40% to Amoxycillin and 20% to Levofloxacin. The organism showed 60% high sensitive to Azithromycin and 40% to Ciprofloxacin (Table 5 and Figure 3). Among the 5 isolates of *Bacillus* spp**.,** 60% were resistant to Amoxycillin and Metronidazole and 20% to Azithromycin. Sixty percent were less sensitive to Azithromycin, Erythromycin and Gentamycin, 40% to Amoxycillin and Metronidazole. Among the isolated *Bacillus* spp., 40% were moderately sensitive to Ciprofloxacin, Erythromycin, Tetracycline and Levofloxacin and 20% to Azithromycin and 60% were highly sensitive to Ciprofloxacin, Tetracycline and Levofloxacin (Table 5 and Figure 4).

Table 5. Results of antibiotic sensitivity tests in percent.

Name of bacteria	Total isolates	Sensitivity/ resistance	% of isolated strains sensitive/ resistance to various antibiotics							
			CIP	AZM	E	TE	GM	A	LVX	MET
Pasteurella spp.	05	Resistance	00	00	00	00	00	00	40	40
		Less sensitive	00	00	00	20	20	60	40	60
		Moderately sensitive	60	40	80	60	60	40	20	0
		Highly sensitive	40	60	20	20	20	00	00	00
Bacillus spp.	05	Resistance	00	20	00	00	0	60	00	60
		Less sensitive	00	60	60	00	60	40	00	40
		Moderately sensitive	40	20	40	40	40	00	40	00
		Highly sensitive	60	00	00	60	00	00	60	00

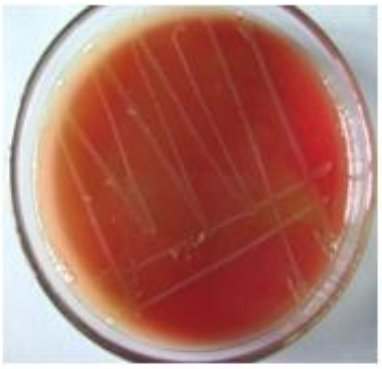


Figure 1. Whitish opaque circular colony of *Pasturella* spp. on blood agar.

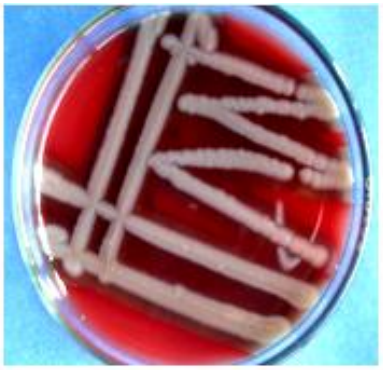
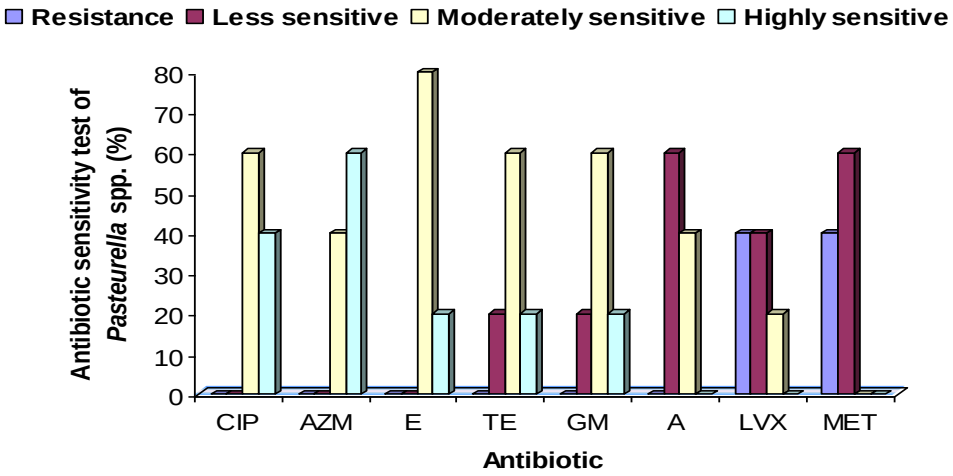
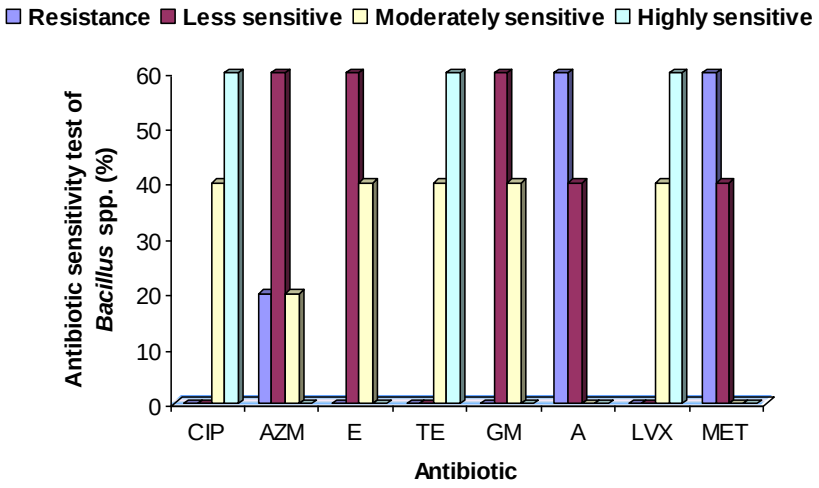


Figure 2. Creamy-yellow coloured colony of *Bacillus* spp. on blood agar media and hemolysis of the media.



CIP = Ciprofloxacin; AZM = Azithromycin; E = Erythromycin; TE=Tetracycline
A = Amoxicillin; LVX = Levofloxacin; MET = Metronidazole; GM = Gentamycin

Figure 3. Diagrammatic presentation of antibodies sensitivity pattern of isolated *Pasteurella* spp.



CIP = Ciprofloxacillin; AZM = Azithromycin; E = Erythromycin; TE=Tetracycline
A = Amoxicillin; LVX = Levofloxacin; MET = Metronidazole; GM = Gentamycin

Figure 4. Diagrammatic presentation of antibodies sensitivity pattern of isolated *Bacillus* spp.

4. Conclusions

The present study was undertaken for the isolation, identification, and determination of biochemical properties of the bacteria, isolated from nasal, tracheal and lung swabs of apparently healthy sheep. A comparative study to determine the sensitivity and resistance pattern of the isolated bacteria to different antimicrobial agents was also performed. From the present study it may be concluded that among all the isolates the percentage of the *Pasteurella* is higher than *Bacillus*. None of the isolated *Bacillus* was found to be pathogenic. Isolated *Pasteurella* spp. were found to be pathogenic as observed in different experimental models and showed a degree of variation in antibiotic drug sensitivity test. Ciprofloxacillin were sensitive to all of the isolated bacteria .Through the bacteria that were isolated from various organ of apparently healthy sheep are normal micro flora, however these may act as primary pathogen and may produce diseases when the sheep are immunologically suppressed due to severe stress conditions.

Conflict of interest

None to declare

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