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Prevalence and antibiotic sensitivity of bacteria isolated from respiratory system of cattle

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ABSTRACT

The present study was conducted for isolation, identification, determination of biochemical properties, frequency distribution, pathogenicity and antibiotic sensitivity of the bacteria, isolated from nasal and lung swab from healthy and sick cattle around the Bangladesh Agricultural University (BAU) campus and brought for the treatment in the Veterinary Clinic, BAU, Mymensingh with a history of suffering from acute respiratory problem. A total of 40 nasal and lung swabs were collected. A series of tests were performed for the isolation and identification of different types of bacteria and to determine the frequency distribution, pathogenicity and antibiotic sensitivity of those isolates to different antibiotics. Different types of ordinary, enriched and selective media such as Nutrient agar, Blood agar, MacConkey agar, Eosin Methylene Blue agar, Salmonella-Shigella agar, Triple Sugar Iron agar, Crystal Violet Blood agar, Nutrient agar slant and Triple Sugar Iron agar slant etc .were used for the determination of the cultural characteristics of the different types of isolated bacteria. Biochemical properties of the isolated bacteria were studied by sugar fermentation, catalase, coagulase and IMViC utilization tests. As the isolates of the bacteria were different so the patterns of reactivity of the isolates with various biochemical tests were also different. On the basis of morphology, staining, cultural and biochemical characteristics, the isolated organisms were classified as *E. coli*, *Staphylococcus*, *Bacillus.* Out of 40 samples only 20 samples were found positive for *Staphylococcus*, 10 samples were positive for *Bacillus* species. The organisms *E. coli* was isolated from 6 samples and 4 samples were found negative for bacteria. The pathogenicity of the 3 different types of bacteria isolated from cattle was studied by inoculation individually in day old mice (*Staphylococcus*, *Bacillus*, and *E. coli*) after 24 hrs all the mice were died. *Staphylococcus* spp. was found highly sensitive to erythromycin, moderately sensitive to ampicillin, amoxycillin, mild sensitive to penicillin, nalidixic acid, azithromycin and resistant to trimethoprim and metronidazole respectively. *Bacillus* spp. was found highly sensitive to nalidixic acid, moderately sensitive to erythromycin and azithromycin and resistant to amoxicillin metronidazole, penicillin, trimethroprim and ampicillin respectively. Whereas *E coli* was highly sensitive to ciprofloxacin, norfloxacillin, enrofloxacin moderately sensitive to amoxycillin, pefloxacin, furazolidone and mild sensitive ampicillin and gentamycin respectively. The pneumonia was severe in case of mixed infection where two or more types of organisms involved. The study suggests that there might be some mutual exchanges of virulent factors between the bacteria which make the neonatal calf more susceptible to pneumonia. From this study it is observed that pneumonia is more prominent in young, female those are poorly nourished and in unhygienic condition with high humidity in rainy season and faulty managemental practices. The result of this study speculates that ciprofloxacin and amoxycillin may have the preference in clinical control of cattle pneumonia.

INTRODUCTION

In Bangladesh there are many barriers exist in cattle production. Among all problems pneumonia and other respiratory problems are predominant in rainy and winter season. In every year a large number of cattle populations die due to pneumonia at the early stage of their lives. This poses a great

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economic threat to the cattle rearer each year. Pneumonia sometimes called shipping fever is one of the most common problems encountered in cattle today. In cattle herd pneumonia increases production costs associated with expensive treatments. Early diagnosis and proper treatment are essential for effective treatment and prevention and control of the diseases. A thorough study in relation to its causal agents, host factors, environmental factors that favour for the occurrence and dissemination of the infection should be carried out to provide appropriate prevention and control of the diseases. Though viral, fungal and other causal agents responsible for the cattle pneumonia, the present study is aimed at to focus only bacterial causes. Medium and large farmers interested in commercial cattle farming are managed either in intensive or semi-intensive conditions. This system of cattle rearing inherently incurs different diseases which intern reduces profitability of farming by treatment costs, reducing productivity and by mortality. Till now no systematic observation has been made on incidence of different diseases, mortality or different factors related with calf mortality in intensively or semi-intensively reared cattle Respiratory Bacteria are ubiquitous in nature and are normal inhabitants of the nasopharynx of cattle. This often creates difficulty in interpreting microbiological findings during an outbreak of respiratory diseases. Cattle respiratory infections exhibit various clinical syndromes. Respiratory problem in calf under 1 year old is thought to be association with *Pasteurella* and PI3 (Radostits et al., 1994). Acute bacterial pneumonia usually results from infection with *Pasteurella* of biotype A. Infection with PI3 can initiate invasion by *Pasteurella*. . Serotypes of biotype T *P. haemolytica* cause an acute septicaemia (Martin, 1996).

Stressful management practices may be a predisposing factor. The present study would give the appropriate identification of bacterial causes of cattle pneumonia, agents, host and environmental factors that may play the role for the occurrence and dissemination of the infection. In addition the present study would give a suggestion for the use of appropriate antibiotic therapy, hygiene measures, etc. for the prevention and control of cattle pneumonia in Bangladesh. For the prevention, control and treatment of bacterial pneumonia, epidemiological pattern, isolation and characterization of bacteria are essential. In view of the above discussion, the research work was undertaken to determine the epidemiological pattern of cattle pneumonia based on age, sex, environment, season, housing and hygiene following isolation and identification of bacteria from respiratory system of healthy and sick cattle, and to study the antibiotic sensitivity of the isolated bacteria in order to formulate prevention and control measures of cattle pneumonia in Bangladesh.

MATERIALS AND METHODS

Experimental animals and Samples

Fourty (healthy and sick) cattle were selected at the adjacent areas of Bangladesh Agricultural University (BAU) for this experiment. Nasal and lung swab samples were collected.

Bacteriological 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). 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).

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.

Sample collection

Nasal swabs were collected by inserting the sterilized cotton swabs directly into the nostrils. Lungs swabs were collected with sterilized loop by incising the lungs with a sterile scalpel. Then the swabs were inoculated separately into NB and selenite/ salinity broth and incubated at 37^0C for 24 hours. After incubation it was streaked on to NA and MC agar medium.

Isolation and identification of organism

E. coli

The samples were first inoculated in to NB and then it was inoculated on to NA, EMB agar, MC agar and then incubated at 37^0C for 24-48 hrs for the isolation and identification of E. coli. On Gram's staining the Gram-negative, pink colored, rod shaped organisms arranged as single or in pair, indicated E. coli. It was confirmed by biochemical tests (Merchant and Packer, 1967).

***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 (Merchant and Packer, 1967).

***Staphylococcus* spp.**

Staphylococcus spp. was isolated on the basis of the morphology, cultural characteristics and biochemical characteristics. The colonies of staphylococci were round, glistening convex, smooth and opaque on NA and BA. They were Gram-positive cocci arranged in clusters and catalyses test was performed for the identification of the pathogenic *Staphylococcus* spp. from the nonpathogenic one (Merchant and Packer, 1967).

Hemolytic activity of bacterial isolates

Isolated organisms from different sources were inoculated on blood agar media and incubated at 37ºC for 24 hours to determine their hemolytic property. The colony developed on the blood agar was examined for various types of hemolysis. The hemolytic pattern of the bacterial isolates was categorized according to the types of hemolysis produced on the blood agar and this was made as per recommendation of Carter (1986) who described Alpha (α) hemolysis as a zone of greenish discoloration around the colony manifested by partial hemolysis, Beta (β) hemolysis as a clear zone of complete hemolysis around the bacterial colony and Gamma (γ) hemolysis as no detectable hemolysis.

Gram's staining

For identification of bacterial isolates from the suspected colonies by Gram's staining method was performed as per recommendation of Merchant and Packer (1976).

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.

Biochemical tests

Several biochemical tests were performed for confirmation of bacterial isolates. Carbohydrate fermentation test, Catalase test, Methyl Red test, Voges-Proskauer (V-P) test, Indole test were performed according to the methods described by Cheesbrough, 1985. Coagulase test (Carter, 1986) according to the standard method described by Cowan (1985).

Animal experiment

A total of 10, day-old mice of both sexes were used for experimental production of pneumonia. They were divided into six groups, consisting of ten mice in each of five experimental groups. Remaining one group contained fifteen mice which served as control. Four experimental groups of mice were inoculated with individual species of bacteria (*Staphylococcus, Bacillus, E .coli*) and the remaining experimental group was infected with mixed organisms (*Staphylococcus, Bacillus, E. coli*). Inoculum of each isolate was prepared by culturing the organisms in Nutrient broth and

harvesting after 24 hours of culture. The viable counts of the bacterium in these preparations was 5 x 10^6 CFU per ml. Inoculation was made by orally one drop of bacterial suspension singly and combinedly (*Staphylococcus, Bacillus, E. coli*) in each mice of different experimental groups. The mice were observed for every 6 hours interval for 7 days for the manifestation of clinical symptoms of pneumonia Isolation and identification of organisms from the experimentally infected mice was made on several days after inoculation. Mice which developed pneumonia were sacrificed and pathological changes were recorded.

Antimicrobial sensitivity test

A total of 36 isolates collected from nasal, and lung swab were used for disk sensitivity testing. The antimicrobial sensitivity testing of each isolate was carried out by the Bauser Kirby Disc Diffusion Method according to National Committee for Clinical Laboratory Standards (NCCLS) procedures. Gentamycin (10 µg), Erythromycin (15 µg), Enrofloxacin (5 µg), Azithromycin (15 µg), Ciprofloxacin (5 µg), Amoxicillin (25 µg), Ampicillin (10 µg), Metronidazole (80 µg), Penicillin (10 µg), Trimethoprim (5 µg), Nalidixic acid (30 µg), Furazolidone (50 µg), Norfloxacin (10 µg), Pefloxacin (10 µg) disc ((Becton, Dickinson and Company, USA) were used to test the sensitivity and resistance pattern of the selected bacterial isolates from nasal, and lung swab of cattle.

RESULTS AND DISCUSSION

Isolation and identification of *E .coli*

Nutrient broth was inoculated separately with the nasal, tracheal and lungs swab and incubated at 37°C for 24 hrs. The presence of turbidity indicated the growth of bacteria. NA plates streaked separately with the organism revealed the growth of bacteria after 24 hrs of incubation at 37°C aerobically and was indicated by the growth of circular, smooth, white to grayish or white colony with feted odor (Table 2).

Cultural characteristics

Greenish colonies with metallic sheen produced by the organisms on EMB agar after overnight incubation were tentatively confirmed as *E. coli* (Table 2). Bright pink colored colonies on MacConkey agar produced by the organisms after overnight incubation were presumptively selected as *E. coli* (Table 2).

Gram's staining

Light microscopic examination after Gram's staining revealed Gram-negative, pink colored, rod shaped organisms arranged as single or in pair (Table 2).

Biochemical tests

All the *E. coli* isolates fermented five basic sugars with the production of acid and gas. Acid production was indicated by the color change of the sugar media from reddish to yellow and the gas production was noted by the accumulation of gas bubbles in the inverted Durham's tube (Table 3). The organism *E coli* was found positive for MR and Indole but negative for VP test (Table 3). The catalase test was performed to differentiate catalase enzyme producing *E. coli* from those of non-catalase producing one. *E. coli* revealed positive reaction (Table 3).

Isolation and identification of *Staphylococcus* spp and *Bacillus* spp

Cultural characteristics

The *Staphylococcus spp.* isolated from the samples showed grey-white to yellowish colony on Nutrient agar and white to golden yellow on Blood agar and small, pale pink coloured colony on MacConkey agar. The *Bacillus spp.* produced thick, greyish white or cream coloured colony with uneven surface on Nutrient agar and abundant growth with creamy yellow coloured colony and produced haemolysis on Blood agar media (Table 2, Figure 1).

Staining characteristics

Stained smear of the slide revealed gram positive cocci arranged in cluster indicating *Staphylococcus.* The organisms found as gram positive rods with spores arranged in pair and also in long chain indicating *Bacillus* (Figure 2).

Biochemical tests

The results of biochemical tests are presented in Table 2. The isolates of *Staphylococci* were allowed to react with five basic sugars i.e. dextrose, maltose, lactose, sucrose and mannitol (Table 3). It was observed that the organisms were able to ferment all the sugars completely with the production of acid to their corresponding sugars without gas. The *Bacilli* organisms when allowed to ferment the basic five sugars, they produced only acid from dextrose, maltose and sucrose but partial or incomplete fermentation with small amount of gas production was observed in case of mannitol and lactose.

In Catalase test *Staphylococcus,* and *Bacillus* revealed positive reaction, in coagulase test *Staphylococcus* showed positive reaction and *Staphyloccus spp* and *Bacillus* spp was found negative for VP and indole test but positive for Methyl red test (Table 3).

Frequency distribution of isolated bacteria

The results of frequency distribution of different bacterial isolates are presented in Table 4. A total of 40 nasal and lung samples were examined for the isolation of bacteria of which 10 samples were found positive for *Bacillus spp*. (25%), 6 for *E coli* (15%), 20 for *Staphylococcus spp*. (50%).

Pathogenicity test in mice

To determine the pathogenicity of the isolated bacteria 0.5ml nutrient broth containing bacteria were administered orally into day old mice (four) and observation after 24 hrs. The observations were revealed that all (four) day old mice died.

Antibiotic sensitivity

The antibiotic sensitivity of bacteria isolated from pneumonic cattle is presented in Table 5. The results of antibiotic sensitivity of *Staphylococcus spp*. revealed that highly sensitive to erythromycin, moderately sensitive to ampicillin, amoxicillin, mild sensitive to penicillin, nalidixic acid, azithromycin and resistant to trimethoprim and metronidazole respectively and *Bacillus spp*. revealed that highly sensitive to nalidixic acid, moderately sensitive to erythromycin and azithromycin and resistant to amoxicillin metronidazole, penicillin, trimethroprim and ampicillin respectively and *E coli* revealed that highly sensitive to ciprofloxacin, norfloxacillin, enrofloxacin moderately sensitive to amoxycillin, peafloxacin, furazolidone and mild sensitive ampicillinand and gentamycin respectively.

Table 1
Prevalence of cattle pneumonia in the study areas.

Epidemiological pattern	Level of pattern	No. of animal examination	No. of affected Animal	Prevalence
Age	1-6 month	200	4	2%
	6-12 months	300	4	1.33%
	12-18 month	250	2	0.8%
Sex	Male	300	2	0.66%
	Female	300	3	1%
Season	Rainy	450	10	2.2%
Hygiene status	Poor	150	3	2%
	Moderate	150	1	0.67%
	Good	50	0	0
Health status	Poor	400	8	2%
	Moderate	250	2	0.8%
	Good	200	1	0.5%
Humidity	High	200	3	1.5%

Table 2
Result of cultural and staining characteristics of bacteria isolated from respiratory system of cattle.

Cultural characteristics					Staining characteristic			Identified organism
Nutrient agar	Blood agar	MC agar	EMB agar	SS agar	Shape	Arrangement	Gram's staining (+,-)	
Gray white or yellowish colony	Whitish to golden colony	No growth	No growth	No growth	Cocci	Cluster	Gram positive	*Staphylococcus spp*
Thick grayish white or cream coloured colony	Abundant growth, Cream yellow colored colony and heamolysis of the media	No growth	No growth	No growth	Rod with square end	Single ,paired and in long chain	Gram positive	*Bacillus spp*
Smooth, circular, whitish to grayish white colony , peculiar fitted odor	Produce heamolysis	Rose pink lactose fermented	Moist circular colonies with dark centers yellow green metallic sheen	Pink color colony	Short plump rods	Single ,paired or in short chain	Gram negative	*Escherichia coli*

Table3
Result of biochemical characteristic of isolated bacteria from respiratory system of cattle.

Isolated bacteria	Fermentation properties with Carbohydrate					Catalase test	Cogulase test	Indole test	Methyle-Red test	Voges-prokauer test	Citrate utilization test
	D	ML	L	S	MN						
Staphylococcus spp	+ A	+ A	+ A	+ A	+ A	+	+	-	-	-	-
Bacillus spp	+ A	+ A	+ A	+ A	+ A	+	-	-	-	-	-
Escherichia coli	+ AG	+ AG	+ AG	+ AG	+ AG	+	-	+	-	-	-

Legends: D=Dextrose, ML=Maltose, L= Lactose, S=Sucrose, MN= Manitol, A= Production of acid only
AG= Production of acid and gas, + =Positive reaction, - = Negative reaction

Table 4
Frequency distribution of the isolated bacteria from the respiratory system of cattle.

Isolated bacteria	No of samples examined	No. of positive samples	Frequency distribution (%)
Staphylococcus spp	40	20	50
Bacillus spp	40	10	25
Escherichia coli	40	6	15

Table 5
Antibiotic sensitivity pattern of isolated bacteria from respiratory system of cattle.

Bacteria isolates	No. of isolated tested	Antibiotic sensitivity							
		AP	P	E	W	NA	A	AZM	MET
Staphylococcus spp	20	++	+	+++	-	+	++	+	-
Bacillus spp	10	-	-	++	-	+++	-	++	-
Escherichia coli	4	CIP	AML	PEF	AMP	NOR	ENR	GM	FR
		+++	++	++	+	+++	+++	+	++

Legends: AP/AMP = Ampicillin, GM = Gentamycin, E= Erythromycin, P = Penicillin, NA = Nalidixic acid, W = Trimethoprim, AZM = Azithromycin, A/AML = Amoxycillin, CIP = Ciprofloxacin, MET = Metronidazole, NOR = Norfloxacin, PEF = Pefloxacin, ENR = Enrofloxacin, FR = Furazolidone;
+++ = Highly sensitive, ++ = Moderately sensitive, + = Less sensitive, - = Resistant

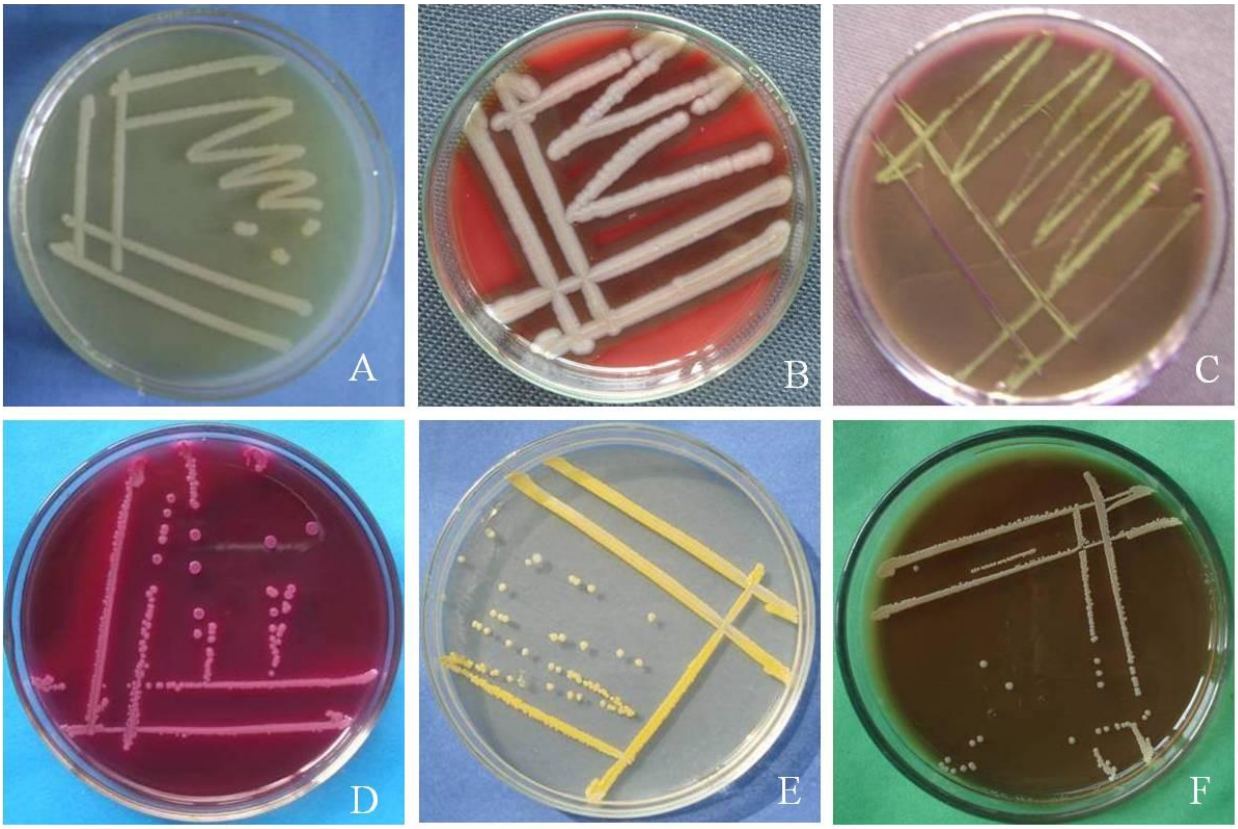


Figure 1
A. Thick grayish white colony *Bacillus spp* on Nutrient agar media. B. Creamy-yellow coloured colony of *Bacillus spp on* Blood agar media and hemolysis of the media. C. Metallic sheen colony of E. coli on EMB agar media. D. Rose pink lactose fermented colony of *E. coli* on MacConkey agar media. E- Yellowish colony of *Staphylococcus* spp on Nutrient agar media. F. Golden yellow colony of Staphylococcus spp on Blood agar media.

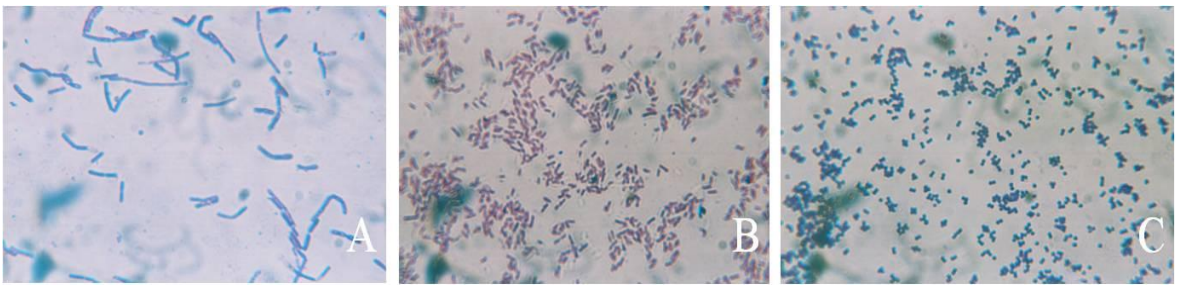
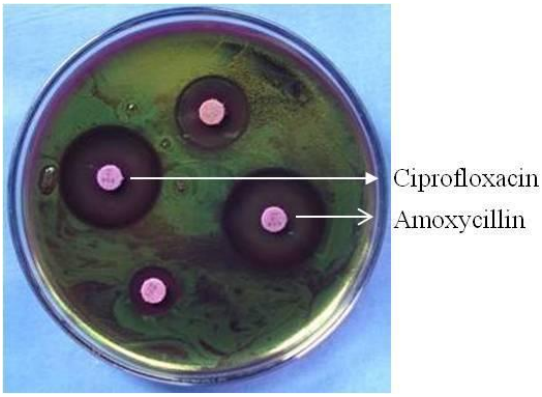
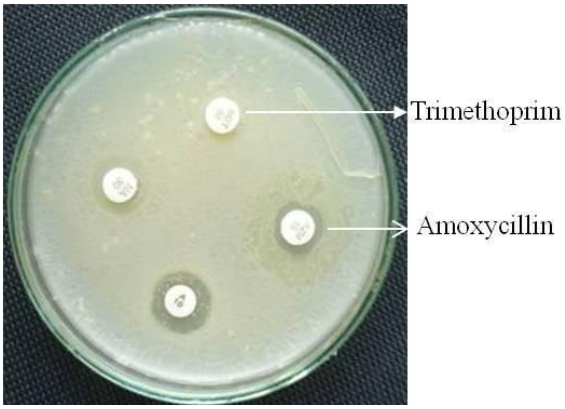


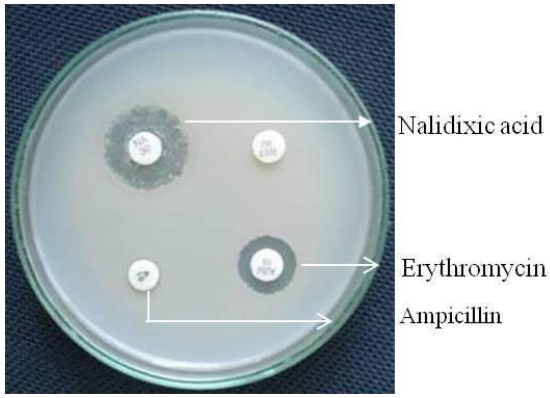
Figure 2
A. Gram-positive single, paired, and also long chain of *Bacillus spp* (Gram's stain). B. Gram-negative single, pair or in short chain of *E. coli* (Gram's stain). C Gram-positive cluster form of *Staphylococcus spp* (Gram's stain).



A. Antibiotic sensitivity test for *E. coli*



B. Antibiotic sensitivity test for *Staphylococcus spp*



C. Antibiotic sensitivity test for *Bacillus spp*

Figure 3
Antibiotic sensitivity test for different isolates.

In this study, 3 different types of bacteria were isolated from a total 40 respiratory sample collected from healthy and sick cattle. The isolated bacteria were *Staphylococcus*, *Bacillus*, and *E. coli*. Out of 40 respiratory samples only 6 samples positive for *E. coli*, 20 samples positive for *Staphylococcus* and 10 samples positive for *Bacillus*. None of the bacteria were isolated from remaining 4 samples. The frequency distribution of different bacteria isolates in different respiratory samples were variable. Result of the present study indicate that all the 3 different types bacteria were not present in the same samples collected from healthy and sick cattle . The incidence of different bacteria isolated from cattle correlated with the findings of Ajuwape et al., (2004), with slight variation.

The different isolates of *Staphylococcus*, *Bacillus*, and *E. coli* showed identical results in different biochemical tests including sugar fermentation, catalase, coagulase and IMVIC utilization tests. The actual cause for which the manifestation of an identical result in biochemical tests by the three groups of known identified isolates were not clear. It is not unlikely that almost all isolates in the present study possess some common genetic materials which are responsible for the manifestation of similar type of biochemical reaction as reported by Amaechi *et al.*, (2006).

The pathogenicity of the 3 different types of bacteria isolated from cattle was studied by inoculation individually in day old mice (*Staphylococcus, Bacillus*, and *E. coli*). After 24 hrs all the mice were died.

The results of antibiotic sensitivity of *Staphylococcus spp*. revealed that highly sensitive to erythromycin, moderately sensitive to ampicillin, amoxicillin, mild sensitive to penicillin, nalidixic acid, azithromycin and resistant to trimethoprim and metronidazole respectively and *Bacillus spp*. revealed that highly sensitive to nalidixic acid, moderately sensitive to erythromycin and azithromycin and resistant to amoxicillin metronidazole, penicillin, trimethroprim and ampicillin respectively and *E coli* revealed that highly sensitive to ciprofloxacin, norfloxacillin, enrofloxacin moderately sensitive to amoxycillin, pefloxacin, furazolidone and mild sensitive ampicillin and gentamycin respectively. The findings are in agreement with the result of Catry et al., (2002) and Amaechi et al., (2006).

The variation in the sensitivity of antibiotics of the respiratory isolates may be due to the out come of choice and also the indiscriminate use of antibiotic in different disease stage to various species of animals. The results of isolation, identification, biochemical test, frequency distribution, pathogenicity and antibiotic sensitivity of the bacteria isolated from respiratory swab of cattle in the present study indicated that the microbial factors might play an important role for the development of cattle pneumonia.

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