Article

Molecular identification and antibiogram profiles of respiratory bacterial agents isolated from cattle reared in some selected areas of Mymensingh division, Bangladesh

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Abstract: Respiratory bacterial infections in cattle are very common all over Bangladesh causing high economic loss. This research was performed with a view to proper control of respiratory bacterial infections of cattle in Bangladesh. A total of 100 nasal samples were collected on the basis of clinical signs. From the collected samples isolation, identification and characterization of the bacterial agents was done using cultural, biochemical and molecular techniques. Antibiogram profiles of the isolated agents were studied by disc diffusion method. *Pasteurella multocida*, *Staphylococcus aureus and Escherichia coli* were successfully isolated and identified from the collected samples. The isolated *Pasteurella multocida* produced small, round, opaque colonies on blood agar; *Staphylococcus aureus* produced golden yellow colony in mannitol salt agar; *E. coli* produced black color colonies with metallic sheen on EMB agar. *Pasteurella multocida* showed Gram negative, bipolar rods. *Staphylococcus aureus* showed Gram positive, cocci shaped and *E. coli* showed Gram negative, small rod shaped. Among 100 nasal samples 16 were found to be positive for *Pasteurella multocida,* 21 for *Staphylococcus aureus* and 13 for *E. coli* on the basis of cultural and biochemical characteristics. The antibiogram study reflected that ciprofloxacin, tetracycline and chloramphenicol should be first choice of treatment of respiratory bacterial infections caused by the isolated 3 bacteria. *Pasteurella multocida* was further characterized by PCR where 16 isolates showed positive band at 460 bp and *Pasteurella multocida* type A at 1044 bp. The present research work covering antibiogram study is a preliminary report in the context of Bangladesh.

Keywords: respiratory diseases; cattle; *P. multocida*; *E. coli*; *S. aureus*; PCR; antibiogram

1. Introduction

Respiratory disease is among the most economically important diseases of cattle occurring in Bangladesh and all parts of the world. Annual losses to the US cattle industry are estimated to approach US$1 billion, whereas preventative and treatment costs are over US$3 billion annually (Griffin, 2006; Snowder et al., 2007). Respiratory diseases of cattle is typically not due to a single cause but is usually caused by a combination of several aspects, such as infectious viral and bacterial agents, as well as other factors that cause stress on the animal.

Pneumonia is the most frequently occurring respiratory infection in domestic animals, since the etiologic agents are bacteria, viruses or viruses complicated with bacteria (Allan *et al.,* 1991). *Pasteurella multocida* is the cause of various diseases in mammalian and avian species (Carter , 1967). *Pasteurella multocida* type A strains cause pneumonia in cattle, sheep and pigs, fowl cholera in birds, and "snuffles" in rabbits (Carter , 1967), strains

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of types B and E cause haemorrhagic septicaemia in cattle and buffaloes (Bain *et al.,* 1982) and type-D strains cause pneumonia in cattle and atrophic rhinitis in pigs (Rutter, 1985). *Staphylococus aureus*, *Streptococcus Pneumoniae* (Beiter *et al.,* 2006) and *E. coli* (Wessely *et al.,* 2005) are the other pneumonic pathogens-but less frequently–could be recovered from pneumonic lungs.

Bovine respiratory disease (BRD) causes increased death losses as well as medication costs, labor, and lost production. BRD accounts for approximately 75% of feedlot morbidity and 50 -70% of all feedlot mortality (Edwards, 2010). The percent of morbidity and mortality depends on the management system in place, prevention program and the kind of pathogens involved.

Different antibiotics are used in the treatment of respiratory diseases in cattle but antibiotic resistance among bacteria is creating a serious threat throughout the world. In the past two decades the rise in antibiotic resistance has been reported in many countries including Bangladesh (Kapil, 2004). It might be due to indiscriminate use of antimicrobial agents (Nazir *et al.,* 2005). This problem increases the importance of antibiotic sensitivity testing to identify accurate antibiotic for certain bacteria affecting respiratory system. To the best of our knowledge, not much work has been carried out in Bangladesh on molecular detection of the bacteria associated with respiratory diseases of cattle covering antibiogram study. This study was therefore designed to detect bacteria from cattle suffering with respiratory diseases using polymerase chain reaction (PCR) based approach including their antibiogram profiles.

2. Materials and Methods
2.1. Sample collection, primary isolation and identification of the bacteria by conventional methods
A total of 100 field samples (nasal secretion & swab) were collected from suspected cattle with respiratory problems of the study area including the dairy farm of BAU, Pirbari-Mymensingh Sadar, Poarkandi-Muktagacha, Dewangonj-Jamalpur, Mohangonj-Netrokona and Nakhla-Sherpur. The samples were collected aseptically by using sterilized cotton buds from the nostril, immediately after collection transported to the laboratory by inoculating into nutrient broth and incubated at 37^0C overnight for enrichment. The broth culture was then streaked onto Nutrient agar, Blood agar, MacConkey agar, Eosin methylene blue (EMB) agar and Mannitol salt agar (MSA) where all of the media were brought from the Indian company, HiMedia. Suspected colonies were further analyzed by Gram's staining technique and biochemical tests for preliminary isolation and identification of bacteria from respiratory diseases of cattle (Cheesbrough, 2006).

2.2. Molecular identification of the species and type A specific *P. multocida* by PCR
DNA was extracted from the isolated bacteria using conventional boiling method. PCR was performed to detect *P. multocida* using the species specific primers KTT72 and KTSP61 (sequences mentioned in Table 2) having amplicon size 620 bp and to detect *P. multocida* type A using type specific primers CAPA-FWD and CAPA-REV (sequences mentioned in Table 2) having amplicon size 1044bp. PCR reaction mixture (25 µl) was prepared by mixing 12.5 µL master mixtures (Maxima Hot start Green, USA), 1µL of each primer, 8.5 µL deionized water and 2 µL DNA template. Amplification was performed in a thermal cycler for *P. multocida* species specific primers as follows: initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for1 minute, annealing at 49°C for 1 minute, elongation at 72°C for 1 minute, and a final extension at 72°C for 7 min. Amplification for *P. multocida* type A specific primers was as follows: initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 0.3 minute, annealing at 55°C for 0.3 minute, elongation at 72°C for 1.3 minute, and a final extension at 72°C for 5 min. Electrophoresis was run at 100 Volt for 30 minutes on 1.5% agarose (Sigma-Aldrich, USA) gel after mixing PCR product with loading buffer along with 1-kbsize DNA marker (Promega, USA). Then agarose gel was stained with ethidium bromide (0.5µg/ml) and de-stained in distilled water and placed on the floor of UV transilluminator for visualization and image documentation.

2.3. Antibiotic sensitivity assay
The isolated bacterial species were subjected to antibiotic sensitivity test against 10 commonly used antibiotics of different groups by Kirby-Bauer disc diffusion method, as described by Clinical and Laboratory Standard Institute (CLSI, 2013). The antibiotics used were – Chloramphenicol (30µg/disc), Ciprofloxacin (5µg/disc), Erythromycin (15µg/disc), Tetracycline (30µg/disc), Streptomycin (10µg/disc), Sulphamethoxazole (25µg/disc), Amoxicillin (30µg/disc), Azithromycin (15µg/disc), Kanamycin (30µg/disc), Nalidixic acid (30µg/disc). Comparing with 0.5 McFarland standards, inocula were prepared and the test was done in freshly prepared Mueller Hinton agar (HiMedia, India). The results of the sensitivity test were interpreted as per the guidelines of Clinical and Laboratory Standard Institute (CLSI, 2013).

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3. Results and Discussion

Based on cultural (Table 1), staining and biochemical characteristics, among 100 nasal samples 16 were found to be positive for *P. multocida*, 21 for *S. aureus* and 13 for *E. coli*. The positive *P. multocida* isolates were further confirmed by polymerase chain reaction (PCR).

Table 1. Results of cultural, staining and morphological characteristics of the *P. multocida, S. aureus* and *E. coli* isolated from cattle.

Characteristics Cultural	P. multocida	S. aureus	E. coli
NA	Whitish, opaque, circular, translucent appearance	Gray, white or yellowish colony	Smooth, circular, white to grayish white colony
EMB	No growth	-	Smooth, circular, black color colonies with metallic sheen were produced (Figure 2)
MAC	No colony appears	-	Rose pink lactose fermented colony (Figure 3)
BA	Small, round, whitish, opaque colonies were produced with musty odor and there was no hemolysis (Figure 1)	White colony with hemolysis were produced	-
MSA	-	Yellow color colony (Figure 4)	-
Staining			
Stain	Gram negative	Gram positive	Gram negative
Shape	Bipolar rods	Cocci	Small rods
Arrangement	Single or pairs	Grape-like clusters	Single, pairs or short chain

Legends: NA = Nutrient agar, BA= Blood agar, EMB =Eosin Methylene Blue agar, MAC = MacConkey agar, MSA = Mannitol Salt agar

The results of sugar fermentation test showed that *P. multocida* fermented sucrose, mannitol and dextrose producing only acid, but did not ferment lactose and maltose whereas all five basic sugars were fermented by *E. coli* with the production of acid and gas, and *S. aureus* producing only acid. The results of other biochemical tests showed that *P. multocida* was positive to indole, catalase and oxidase test but negative to MR-VP test whereas both *E. coli* and *S. aureus* were positive to MR, indole and catalase test but negative to VP test.

DNA extracted from *P. multocida* isolates used in PCR assay for molecular identification. PCR with KMT1T7 and KMT1SP6 primers (species specific) identified 16 isolates as positive for *P. multocida* showing amplification of 460bp (Figure 5). PCR with CAPA-FWD and CAPA-REV primers identified 16 isolates as positive for *P. multocida* type A showing amplification of 1044bp (Figure 6).

From the antibiogram study, it was revealed that *P.multocida* were highly sensitive to ciprofloxacin, chloramphenicol, tetracycline and streptomycin, intermediate to erythromycin, azithromycin but resistant to amoxicillin. For *E.coli*, chloramphenicol, kanamycin, nalidixic acid and tetracycline were sensitive, intermediate to ciprofloxacin but resistant to amoxicillin and erythromycin. For *S. aureus*, ampicillin, amoxicillin, ciprofloxacin, tetracycline, erythromycin and gentamycin were sensitive but resistant to amoxicillin. The results of antibiogram profiles are presented in (Table 3; Figures 7, 8 and 9).

**Table 2. List of primers used for the detection of *Pasteurella* spp.**

Specificity	Primers	Sequence (5'-3')	Amplicon size (bp)
P. multocida	KMT1T7	ATC-CGC-TAT-TTA-CCC-AGT-GG	460 bp
	KMTISP6	GCT-GTA-AAC-GAA-CTC-GCC-AC	
P. multocida type A	CAPA-FWD	TGC-CCA-AAT-CGC-AGT-CAG	1044 bp
	CAPA-REV	TTG-CCA-TCA-TTG-TCA-GTG	

Legends: FWD=Forward; REV=Reverse; bp=Base Pair; Reference :(OIE Manual, 2012)

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Table 3. Results of antibiogram profile of *P. multocida, E. coli* and *S. aureus* isolated from cattle.

| Name of the antibiotic discs	*P. multocida*		*E. coli*		*S. aureus*	
	Zone of inhibition diameter (mm)	Interpretation	Zone of inhibition diameter (mm)	Interpretation	Zone of inhibition diameter (mm)	Interpretation
Amoxicillin (AML)	10	R	00	R	00	R
Ciprofloxacin (CIP)	30	S	21.5	I	22	S
Chloramphenicol (C)	35	S	22	S	-	-
Erythromycin (E)	15	I	16	R	-	-
Azithromycin(AZM)	14	I	-	-	-	-
Kanamycin (K)	-	-	17	S	-	-
Nalidixic acid (NA)	-	-	16.5	S	-	-
Tetracycline (TE)	30	S	10.5	S	25	S
Streptomycin(S)	20	S	-	-	-	-

Legends: S = Sensitive, I = Intermediate, R = Resistant
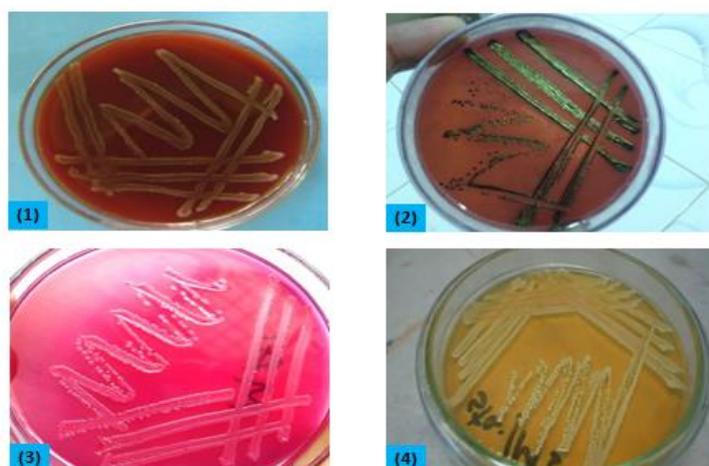
Figure (1) *P. multocida* colonies on Blood agar; (2) *E. coli* colonies on EMB agar; (3) *E. coli* colonies on MacConkey agar; (4) *S. aureus* colonies on MS agar.
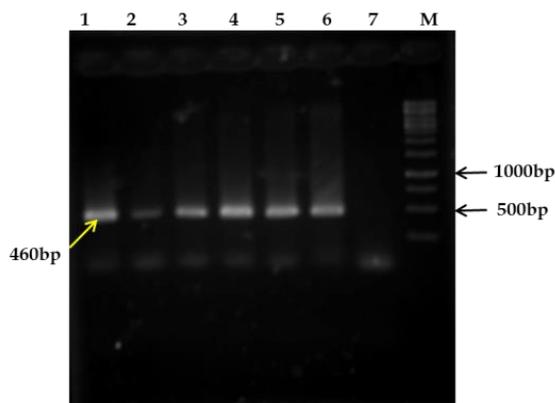
Figure 5. PCR image of *P. multocida* Lane M- 1k bp DNA ladder, Lane 7-Negative control, Lane 6: Positive control *species* and Lane 1-5: Isolated sample of *P. multocida*.
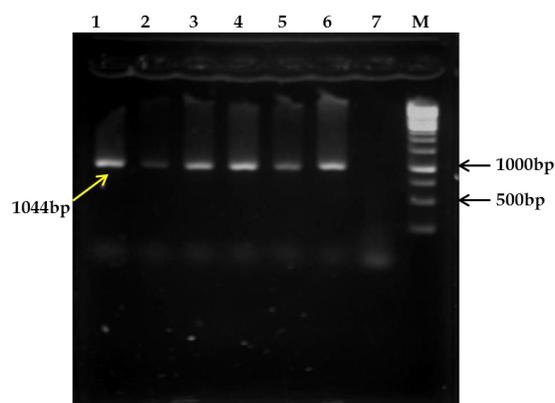
Figure 6. PCR image of *P. multocida* type A Lane M- 1k bp DNA ladder, Lane 7-Negative control, Lane 6: Positive control species and Lane 1-5: Isolated sample of *P. multocida* type A.

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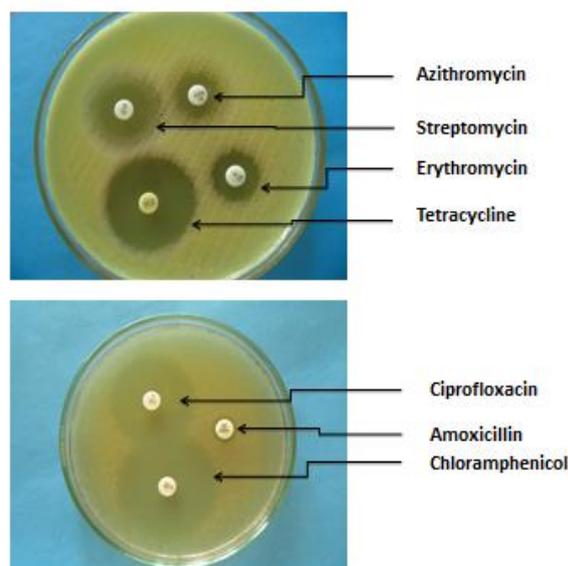
Figure 7. Antibiotic sensitivity pattern of *P. multocida*.

The objectives of the present research work were to isolate and identify the bacterial etiological agents using conventional as well as molecular techniques along with the antibiogram profile study of the isolated bacterial species from respiratory diseases of cattle in Bangladesh.

Colony characters of *P. multocida* from cattle on blood agar, nutrient agar were similar to the findings of Naz *et al.*, 2012; Ashraf *et al*., 2011; De Alwis, 1996. The morphology of the isolated *P. multocida* found Gram negative, bipolar rods, Single or paired in arrangement which was supported by Cowan, 1985 and Ashraf, 2011. The sugar fermentation test revealed all the *P. multocida* isolates as fermenter of dextrose, sucrose and mannitol and produced acid and non-fermenter of maltose and lactose, supported by Buxton and Fraser, 1977. The isolates were also found as negative to MR test, VP test and positive to indole, catalase and oxidase test, as reported by Buxton and Fraser, 1977. Also in this study, colony of the isolated *E. coli* observed on NA, EMB and MacConkey agar showed similarities to the findings of Kalin *et al.*, 2012 Nazir *et al.*, 2005. The *E. coli* isolates revealed a complete fermentation of 5 basic sugars by producing both acid and gas which was supported by Thomas *et al.*, 1998. The isolates also revealed positive reaction in MR test and indole test but negative reaction in VP test were similar to the statement of Buxton and Fraser, 1977. The morphology of the isolated *S. aureus* found in Gram's staining was supported by Kitai *et al.*, 2005. Isolates of *S. aureus* revealed a complete fermentation of 5 basic sugars and production of acid which was supported by Mckec *et al.,* 1995 and OIE Manual, 2012. All of the isolated *S. aureus* revealed positive reaction in catalase, Indole and MR test but negative reaction in VP test as reported by Cheesbrough, 2006.

In this study molecular detection, a 1044-bp band was seen in each lane with the product of the PCR for *P. multocida* type A. The isolates of *P. multocida* type A in this study were similar to the findings of the researcher Khalid *et al.,* 2017.

From the antibiogram study, it was revealed that *P.multocida* were highly sensitive to ciprofloxacin, chloramphenicol, tetracycline and streptomycin, intermediate to erythromycin, azithromycin but resistant to amoxicillin. For *E.coli*, chloramphenicol, kanamycin, nalidixic acid and tetracycline were sensitive, intermediate to ciprofloxacin but resistant to amoxicillin and erythromycin. Those findings were almost similar with the findings of Akond et al., 2009 and Jeyasanta et al., 2012. For *S. aureus*, ampicillin, amoxicillin, ciprofloxacin, tetracycline, erythromycin and gentamycin were sensitive but resistant to amoxicillin. Almost similar sensitivity to these antibiotics was found by Farzana *et al.,* 2004.

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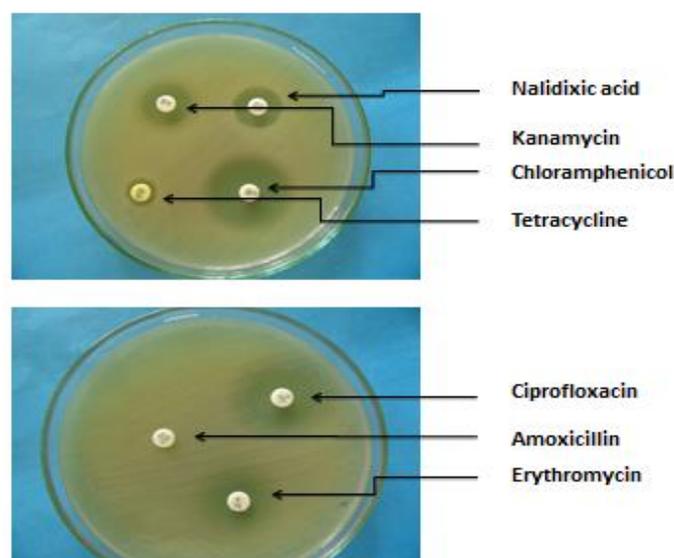
Figure 8. Antibiotic sensitivity pattern of *E. coli*.
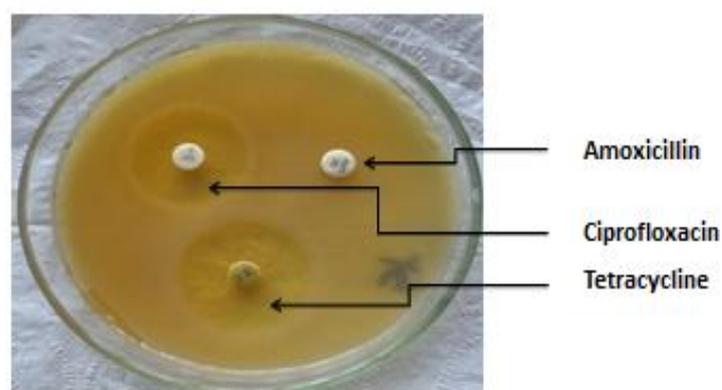
Figure 9. Antibiotic sensitivity pattern of *S. aureus*.

4. Conclusions

The findings of the present research work will certainly facilitate the field veterinarians to select the appropriate antibiotics against cattle respiratory diseases throughout the country and to overcome the bacterial antibiotic resistance issue. Considering the importance and impact of the respiratory disease in cattle and their drug resistance, steps should be taken by government to maintain strict hygienic measurement and proper use of antibiotics.

Conflict of interest

None to declare.

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