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*Article*

# Isolation and molecular detection of respiratory bacterial agents from buffalo reared in some selected areas of Bangladesh

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**Abstract:** In buffalo respiratory bacterial infection is very common which occurs sporadically or enzootically all over Bangladesh causing high economic loss. The study was performed with a view to proper control of respiratory bacterial infection in Buffalo. A total of 40 samples were collected on the basis of clinical signs. The samples were then subjected to isolation, identification and characterization of the bacterial agents using cultural, biochemical and molecular techniques. Antibiogram profiles of the isolated agents were studied by disc diffusion method. *Pasteurella multocida*, *Staphylococcus aureus* and *E. 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. On the basis of cultural and biochemical characteristics, among 40 nasal samples 5 were found to be positive for *Pasteurella multocida*, 4 for *Staphylococcus aureus* and 3 for *E. coli*. *Pasteurella multocida* was further confirmed by PCR where isolates showed positive band at 620 bp. The antibiogram study concluded that amoxicillin, gentamicin, & ciprofloxacin should be the first choice of treatment for respiratory bacterial infection caused by the isolated 3 bacteria.

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

## 1. Introduction

Respiratory diseases are generally the most important in all species of domestic animals (Thomas, 1988). Like other animals, the respiratory system of buffaloes is commonly exposed to various deleterious agents like bacteria, virus, fungi etc. due to its exposure to the external environment. Among various respiratory infections, pasteurellosis is one of the most common buffalo respiratory diseases caused by *Pasteurella (P) multocida* which is one of the nasopharyngeal commensal and commonly isolated from bovine respiratory diseases (Dabo *et al.*, 2007). Other pneumonic pathogens (but less frequently) could be recovered from pneumonic lungs are *Staphylococcus aureus*, *Streptococcus pneumoniae* (Beiter *et al.*, 2006) and *E. coli* (Wessely *et al.*, 2005).

Bovine respiratory disease (BRD) is the most costly disease of cattle and buffalo in Asia. In a study in Bangladesh, Ahmed (1996) made an attempt to compute the economic losses resulting from three important endemic diseases, anthrax, black quarter and haemorrhagic septicemia (HS). It was found that the direct losses which took into account the market value of the animals that died and the cost of treatment was 2.3 million US dollars.

Antibiotic resistance among bacteria is becoming more and more serious problem throughout the world. The rise in antibiotic resistance has been reported in the past two decade in many countries including Bangladesh

(Kapil, 2004). It might be due to indiscriminate use of antimicrobial agents (Nazir *et al*., 2005). 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 buffalo. This study was therefore designed to detect bacteria from buffalo suffering with respiratory diseases using polymerase chain reaction (PCR) based approach including their antibiogram.

## 2. Materials and methods

### 2.1. Sampling, preliminary isolation and identification of bacteria

A total of 40 field samples (nasal swab & secretion) were collected from suspected buffalo with respiratory problems of the dairy farm of BAU, Kanihari-Trishal, Bakshigonj-Jamalpur, Jaintapur and Bianibajar-Sylhet and Suhilpur–Brahmmanbaria during the period of October, 2014 to November, 2015. The samples were collected aseptically by using sterilized cotton buds from the nostril of the buffalo. The swab samples were then inoculated into nutrient broth immediately after collection transported to the laboratory and incubated at $37^0$C 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). Suspected colonies were further analyzed by Gram's staining technique and biochemical tests for preliminary isolation and identification of bacteria from respiratory diseases of buffalo (Cheesbrough, 2006).

### 2.2. Molecular detection of *P. multocida* by PCR

Conventional boiling method was used for DNA extraction from the isolated bacteria. PCR was performed to detect *P. multocida* using the species specific primers KTT72 and KTSP61 (sequences mentioned in Table 1) having amplicon size 620 bp. PCR reaction mixture (25 µl) was prepared by mixing 12.5 µL master mixtures (Promega, USA), 1µL of each primer, 8.5 µL Nuclease free water and 2 µL DNA template. Amplification was performed in a thermal cycler as follows: initial denaturation at 95°C for 5 min, followed by 29 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. 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 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 8 commonly used antibiotics of different groups by disc diffusion method, as described by Clinical and Laboratory Standard Institute (CLSI, 2012). The antibiotics used were - Sulphamethoxazole (25µg/disc), Ciprofloxacin (5µg/disc), Erythromycin (15µg/disc), Tetracycline (30µg/disc), Amoxicillin (30µg/disc), Ampicillin (10µg/disc), Kanamycin (30µg/disc), Gentamicin (10µg/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 expressed as either resistant or sensitive as per the guidelines of Clinical and Laboratory Standard Institute (CLSI, 2012).

## 3. Results and Discussion

On the basis of cultural and biochemical characteristics, among 40 nasal samples 5 were found to be positive for *Pasteurella multocida*, 4 for *Staphylococcus aureus and* 3 for *E. coli*. *Pasteurella multocida* was further confirmed by polymerase chain reaction (PCR). On nutrient agar *P. multocida* formed whitish, opaque, circular, translucent colonies; *E. coli* formed smooth, circular, whitish to grayish white colonies; *S. aureus* formed gray, white or yellowish colonies. On blood agar *P. multocida* formed small, round, whitish, opaque colonies with musty odor and there was no hemolysis (Figure 1) *S. aureus* formed white colony with hemolysis. On MSA agar *S. aureus* formed yellow colored colonies (Figure 2). On EMB agar *P. multocida* showed no growth; *E. coli* formed smooth, circular, black colored colonies with metallic sheen (Figure 3). On MacConkey agar *P. multocida* showed no growth, *E. coli* formed rose pink lactose fermented colonies (Figure 4). After Gram's staining *P. multocida* showed Gram negative, pink colored, bipolar rods arranged in single or pairs, *E. coli* showed Gram negative, pink colored, small rod shaped organisms arranged in single, pairs or short chain, *S. aureus* showed Gram positive, cocci arranged in grapes like clusters. Carbohydrate fermentation test showed that *P. multocida* fermented dextrose, sucrose and mannitol producing only acid, but did not ferment maltose and lactose. *E. coli* fermented all 5 basic sugars with the production of acid and gas. *S. aureus* fermented all 5 basic sugars producing only acid. *P. multocida* showed positive reaction to indole, catalase and oxidase test but

negative reaction to MR-VP test. Both of the *E. coli* and *S. aureus* showed positive reaction to MR, indole and catalase test but negative reaction to VP test.

DNA extracted from *P. multocida* isolates used in PCR assay. Polymerase chain reaction (PCR) with primers KMT1T7 and KMT1SP6 identified 5 isolates as positive for *P. multocida* type B showing amplification of 620-bp (Figure 5).

Antibiogram study of *P. multocida* isolates revealed highly sensitive to ampicillin, amoxicillin, gentamicin and ciprofloxacin, intermediate to kanamycin but resistant to tetracycline, sulphamethoxazole and erythromycin. For *E. coli*, ampicillin, ciprofloxacin, gentamicin, tetracycline and sulphamethoxazole were sensitive, intermediate to amoxicillin but resistant to kanamycin, erythromycin. For *Staph. aureus*, ampicillin, amoxicillin, ciprofloxacin, erythromycin & gentamicin were sensitive, intermediate to kanamycin but resistant to sulphamethoxazole, tetracycline. The results of antibiogram profiles are presented in (Table 2 and Figure 6, Figure 7 and Figure 8).

**Table 1. List of primers used for the detection of *P. multocida*.**

| Specificity | Primers | Sequence (5'-3') | Amplicon size (bp) |
|---|---|---|---|
| *P. multocida type B* | KTT72<br>KTSP61 | AGG-CTC-GTT-TGG-ATT-ATG-AAG<br>ATC-CGC-TAA-CAC-ACT-CTC | 620 bp |

**Legend:** bp=Base Pair

**Table 2. Results of antibiogram profile of *P. multocida, E. coli and S. aureus* isolated from buffalo.**

| 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 |
| Ampicillin (AMP) | 20 | S | 21 | S | 30 | S |
| Amoxicillin (AML) | 25 | S | 16 | I | 30 | S |
| Ciprofloxacin (CIP) | 23 | S | 24 | S | 25 | S |
| Erythromycin (E) | 8 | R | - | R | 29 | S |
| Gentamicin (GEN) | 20 | S | 20 | S | 30 | S |
| Kanamycin (K) | 17 | I | - | R | 16 | I |
| Sulphamethoxazole (SXT) | - | R | 23 | S | - | R |
| Tetracycline (TE) | 6 | R | 25 | S | - | R |

**Legends: S = Sensitive, I = Intermediate, R = Resistant**

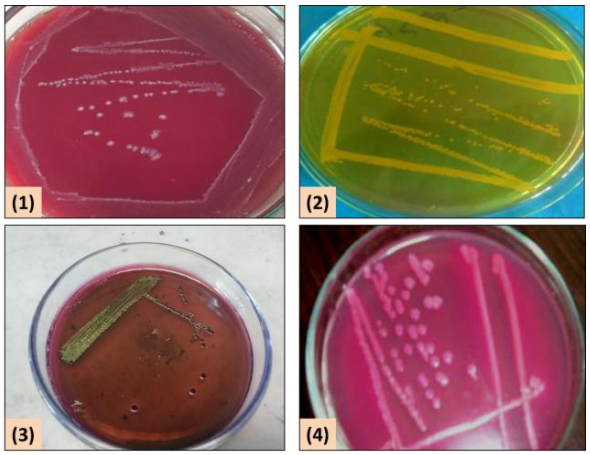


**Figure (1) *P. multocida* colonies on Blood agar; (2) *S. aureus* colonies on MSA agar; (3) *E. coli* colonies on EMB agar; (4) *E. coli* colonies on MacConkey agar.**

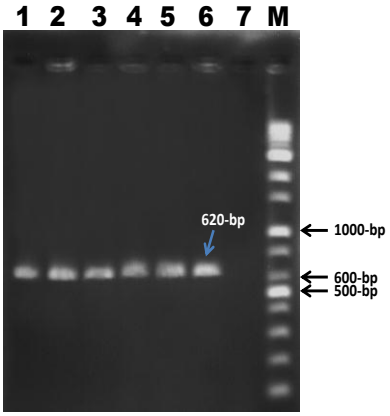


**Figure 5. PCR image of *P. multocida* Lane M- 1k bp DNA ladder, Lane 7-Negative control, Lane 6: Positive control and Lane 1-5: Isolated sample of *P. multocida* type B.**

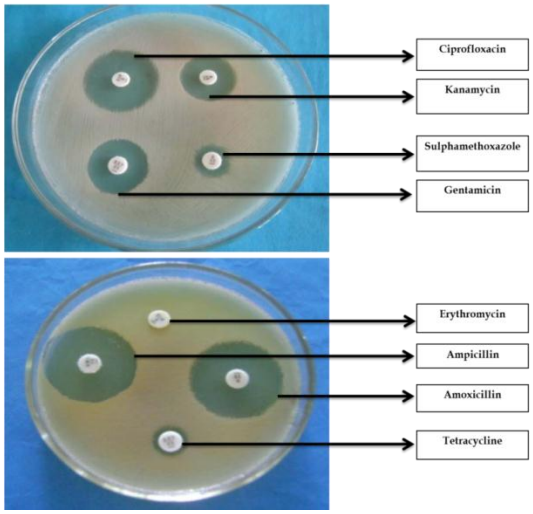


**Figure 6. Antibiotic sensitivity pattern of *P. multocida.***

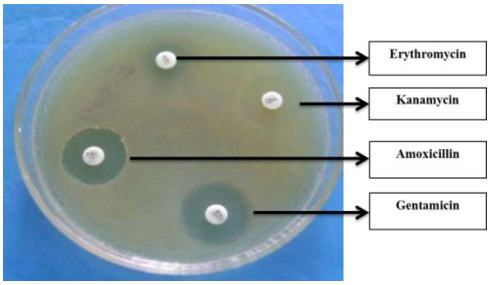


**Figure 7. Antibiotic sensitivity pattern of *E. coli.***

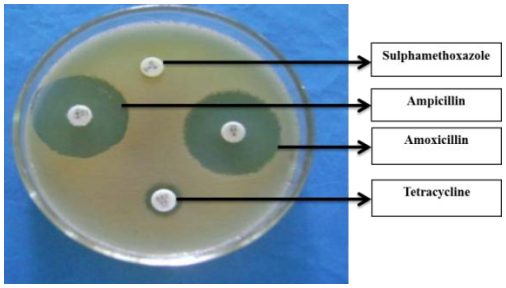


**Figure 8. Antibiotic sensitivity pattern of *S. aureus.***

The aims of the present research work were to isolate and identify the bacterial etiological agents using cultural, biochemical and molecular techniques and to study the antibiogram profiles of the isolated bacterial species from respiratory diseases of buffalo in Bangladesh.

Colony characteristics of *P. multocida* from buffalo 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 in this study 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, as stated 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 characteristics of the isolated *E. coli* observed on NA, EMB and MacConkey agar were similar 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 620-bp band was seen in each lane with the product of the PCR for *P. multocida* type B. The isolates of *P. multocida* type B in this study were similar to the findings of the researcher Townsend *et al.*, (2001).

From the antibiogram study, it was revealed that *P. multocida* were sensitive to ampicillin, amoxicillin, ciprofloxacin, gentamicin, intermediate to kanamycin and resistant to erythromycin, tetracycline and sulphamethoxazole. For *E. coli*, ampicillin, ciprofloxacin, gentamicin, tetracycline and sulphamethoxazole were sensitive, intermediate to amoxicillin but resistant to kanamycin, 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, erythromycin & gentamicin were sensitive, intermediate to kanamycin but resistant to sulphamethoxazole, tetracycline. Farzana *et al., (*2004), found almost similar sensitivity to these antibiotics.

## 4. Conclusions

With the findings of the present research work the field veterinarians will certainly be able to select the correct antibiotics against respiratory diseases of buffalo throughout the country and to overcome the multi-drug resistant problem of the bacteria. Finally it would be said that, the respiratory disease in buffalo and their drug resistance are obviously of great concern. Therefore, steps should be taken by government to maintain strict hygienic measurement and proper use of antibiotics.

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## Conflict of interest

None to declare.

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