

Isolation and molecular detection of *Pasteurella multocida* Type A from naturally infected chickens, and their histopathological evaluation in artificially infected chickens in Bangladesh

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ABSTRACT

Pasteurella multocida type A is the etiologic agent of fowl cholera, a highly contagious and fatal disease of chickens. The present research work was performed for the isolation, identification and molecular detection of *P. multocida* Type A from chickens. Liver, heart and spleen of suspected dead chicken (n=35) were collected from Gazipur and Pabna districts in Bangladesh. The targeted bacteria from the samples were isolated, identified and characterized based on their morphology, staining, cultural, biochemical characters, pathogenicity test, histopathological study and Polymerase Chain Reaction (PCR). The *P. multocida* organism was isolated from 11.42% (n=4/35) samples. The organisms were gram negative, non-spore forming rod, non-motile, occurring singly or pairs in Gram staining, whereas in Leishman's stain, bipolar shaped organisms were observed. All the isolates were found positive for oxidase and catalase tests, produced indole, and fermented glucose, mannitol and sucrose. Necrotic foci in liver and congestion with hemorrhages in heart were found on necropsy. After pathogenicity test, the pathological changes were reconfirmed by histopathology depicting congestion, hemorrhage and lymphocyte infiltration in heart, liver and spleen tissues. In type specific PCR reaction, the organisms were confirmed as *P. multocida* Type A. In conclusion, *P. multocida* type A is prevalent among poultry in the studied regions; thus, care must be taken to control of the disease.

Keywords

Chicken, Fowl cholera, Histopathology, *Pasteurella multocida* type A, PCR

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INTRODUCTION

In developing country like Bangladesh, poultry production in rural areas is considered as a primary source of eggs and meat. In Bangladesh, poultry industry started during 1980s (Huque, 2001). Since last decades, there was a tremendous development of this sector in Bangladesh. About 76.3% people of this country are directly or indirectly involved with poultry industry (Rahman, 2003). Besides, poultry has emerged as a potential sector for income generation and poverty alleviation, as well as improving human nutrition through the supply of meat and eggs. In Bangladesh, commercial poultry sector directly and indirectly supports the livelihoods of about 6 million people through 100,000 commercial farms, with an estimated total investment of 1875 million USD (Chowdhury, 2013). Currently, there are six grandparent stock farms producing about 60-70 thousand day-old-chicks (DOCs) per week and 140 parent stock farms producing 10.1 million commercial DOCs per week, resulting in production by the commercial farms of over 15,000 tonnes of broiler meat and 2.4 million eggs

per week (Khaled, 2015). Poultry sector contributes 2.79% to GDP in Bangladesh (Economic Survey, 2008).

Infectious diseases are considered as one of the most important causes of economic loss in Bangladesh (Singh et al., 2014). Among the bacterial diseases, fowl cholera (FC) is a major threat to the poultry industry that hampers the profitable poultry production (OIE, 2008). FC occurs sporadically or enzootically in most countries of the world (Heddleston and Rhoades, 1978). In Bangladesh, about 25-35% mortality in chicken is caused by FC (Ievy et al., 2013). In backyard poultry of this country, prevalence of FC was 59.72% (Belal, 2013), whereas in layer its prevalence was 12.50%, and in broiler it was 4.25% (Hasan et al., 2010). *Pasteurella multocida* was isolated from apparently healthy ducks (25.9%) and chickens (6.2%) (Mbuthia et al., 2008).

P. multocida is a Gram-negative, non-motile, coccobacillus, capsulated, non-spore forming bacterium occurring singly, in pairs or occasionally as chains or filaments belonging to the Pasteurellaceae family (Ashraf et al., 2011; Ievy et al., 2013; Akhtar 2013). *P. multocida* strains are classified into serogroups A, B, D, E and F based on capsular antigens, and further classified into 16 serotypes (1 to 16) primarily based on lipopolysaccharide antigens (OIE, 2008; Kwaga et al., 2013). FC is caused by *P. multocida* type A:1, A:3 and type D in Asian countries (Ranjan et al., 2011). Among the different serogroups, serotype A:1 strains causes 80% mortality, in contrast 20% mortality caused by type D strains of FC in chickens (Mohamed et al., 2012).

Pathogenicity or virulence of *P. multocida* is variable and complex, depending on the host species, strain, variation within the strain or host, and conditions of contact between two birds (Richard and Rimlen, 2001). The gross lesions were extensive congestion, enlarge and necrotic foci on spleen and liver, petechial hemorrhage in cardiac muscle, necrotic parenchymatous hepatitis, congestion and hemorrhages in the intestinal mucosa (Mohamed et al., 2012). The histopathological signs of FC were hemorrhage, congestion and lymphoid cell infiltration in liver, heart and spleen. (Shilpa et al., 2006).

So far in Bangladesh, several studies have been reported on isolation and identification of FC based on conventional bacteriological methods (Rahman, 2003; Bag, 2010; Ievy et al., 2013). However, there were very limited studies focused on molecular detection of FC organism in Bangladesh. To our knowledge, there is no report on identification of *P. multocida* type A by

molecular approach in Bangladesh. Thus, the present study was designed for isolation, identification and molecular detection of *P. multocida* Type A from naturally infected chicken. Besides, histopathological examinations have been conducted on artificially infected chickens with the *P. multocida* Type A.

MATERIALS AND METHODS

Study area and sample collection: This study was conducted in two different areas namely Gazipur and Pabna districts of Bangladesh. The study areas were selected based on the report of FC outbreak during the study period. Clinical specimens such as heart, liver and spleen of FC suspected chickens (n=35) were randomly collected. The samples were collected as per the standard guidelines (Roy et al., 2012), with prior permission from the owners. After collection, the samples were transported to Bacteriology Laboratory at the Department of Microbiology and Hygiene, Bangladesh Agricultural University (BAU).

Isolation and identification of *Pasteurella multocida*: *P. multocida* organisms were cultured according to the standard method described by Cowan (1985). In brief, the samples were inoculated separately into different culture media such as Blood agar (BA), Nutrient agar (NA) and Nutrient broth (NB) for screening of the samples. The inoculated media were incubated at 37°C for the appearance of characteristic colony. Based on the colony characteristics, subsequent selective subculture was done to obtain pure culture of *P. multocida*. The isolated pure culture was subjected for Gram staining and Leishman's staining for morphological identification of the bacteria. For biochemical characteristics, different tests such as Methyl red (MR), Voges-Proskauer (VP), Indole production, catalase, oxidase, and sugar fermentation tests were done. NA slants were used to maintain stock culture. For the maintenance, the *P. multocida* bacteria were inoculated in slant by streaking and were incubated at 37°C overnight. Finally, sterile mineral oil was overlaid and kept the slant at room temperature for future use.

DNA extraction: Crude DNA was obtained from the isolates using boiling method mentioned by Queipo-Ortun et al. (2008) with slight modification. Briefly, the organisms were cultured onto NA at 37°C for overnight aerobically. Then, a loopful of bacteria was picked up by swiping and mixed in 200 µL of deionized water. The mixture was then heated in boiling water for 10 min followed by dipping into ice for 10 min and

Table 1: List of primers used for the detection of *Pasteurella* spp.

Specificity	Primers	Sequence (5'-3')	Amplicon size (bp)	Reference
<i>P. multocida</i>	KMT1T7	ATCCGCTATTTACCCAGTGG	460	OIE (2008)
	KMT1SP6	GCTGTAACGAACTCGCCAC		
<i>P. multocida</i> Type A	PMcapEF	TCCGCAGAATGGAATTATTGACTC	511	
	PMcapER	GTCGTGCTTGATTTTGTC		
<i>P. multocida</i> Type B	KTT72	AGGCTCGTTGGATTATGAAG	620	

centrifugation was done at 13,000 rpm for 10 min. The supernatant was collected and stored at -20°C until used.

Polymerase chain reaction (PCR) and agar gel electrophoresis: The primers used for the detection of *P. multocida* at species and Type level are listed in **Table 1** (OIE, 2008). All the PCR reactions were carried out in a final 25 µL reaction volume using 2xPCR master mix (Promega, USA) 12.5 µL, DNA template 2 µL, 10 pmol of primer 1 µL each, and dH₂O 8.5 µL. The thermocycle profiles used for the *P. multocida* specific PCR consisted of initial denaturation at 94°C for 5 min, followed by 35 cycles of reaction consisting denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 1 min, with a final extension at 72°C for 5 min. Similarly, *P. multocida* Type A specific PCR consisted of initial denaturation at 95°C for 5 min, followed by 35 cycles of reaction consisting denaturation at 95°C for 0.5 min, annealing at 49°C for 0.5 min and extension at 72°C for 1.5 min, with a final extension at 72°C for 5 min. As negative control, DNA sample from *P. multocida* Type B was used. Electrophoresis of the PCR products was done using 2.0% (w/v) agarose gel. After electrophoresis, the DNA was stained for 10 min in Ethidium bromide (at 0.5 µg/mL) and visualized using UVsolo TS Imaging System (Biometra, Germany).

Pathogenicity test in chicken: Pathogenicity was observed by giving challenge infection with virulent *P. multocida* Type A. The challenge was done by following the guidelines set by the ethics committee of the Department of Microbiology and Hygiene, BAU. Unvaccinated chickens (n=3) were challenged with virulent *P. multocida* Type A isolate following the procedure of [Ievy et al. \(2013\)](#). The challenge dose containing 2.93x10⁸ CFU/0.5 mL was administered through intramuscular route ([Akhtar, 2013](#)). Isolation of bacteria following challenge was performed according to the procedures suggested by [Matsumoto and Helfer \(1977\)](#). After 24 h of challenge, the chickens were sacrificed, and swab or tissue materials were taken from liver, lungs and spleen. Immediately after collection, the samples were streaked onto BA and NA

plates, and were incubated at 37°C overnight. The positive cases were reconfirmed by re-isolation of *P. multocida* Type A by following the standard procedures described earlier ([Matsumoto and Helfer, 1977](#)). The collected samples (heart, liver and spleen) from the artificially infected chickens were collected and were sent to the Department of Pathology, BAU for histopathological study.

Histopathology: The formalin-fixed liver, heart and spleen samples from chickens were trimmed, processed, sectioned and stained following the standard hematoxylin and eosin (H&E) staining techniques ([Luna, 1968](#)).

RESULTS AND DISCUSSION

The prevalence of FC (11.42%; n=4/35) in Gazipur and Pabna districts found in this study was lower than the value reported in the earlier studies ([Hasan et al., 2010](#); [Hossain et al., 2013](#); [Belal, 2013](#)). [Hasan et al. \(2010\)](#) found 12.05% prevalence in layer chicken and 4.25% in broiler chicken, whereas [Hossain et al. \(2013\)](#) found 13.04% prevalence in chicken and [Belal \(2013\)](#) found 59.72% in backyard poultry. The lower rate of *P. multocida* found in this study might be due to differences in age and breeds of the chickens as also for the resistance power of the commercial chicken due to improved management, vaccine and nutrition.

Small, glistening, mucoid, dew drop like colonies were found on BA and NA plates after overnight incubation at 37°C. The bacteria were found to be non-hemolytic on BA, and did not grow on McConkey's agar, Eosine methylene blue agar and Salmonella-shigella agar. These findings were in agreement with several other studies ([Shivachandra et al., 2005](#); [Ashraf et al., 2011](#); [Manasa, 2012](#); [Ievy et al., 2013](#); [Akhtar, 2013](#)). Some strains of fowl cholera organisms might not grow in media without blood or blood serum, as reported by [Carter \(1972\)](#). In the present study, the selected isolates were found to grow well in bovine blood agar media, as described by [Kardos and Kiss \(2005\)](#) and [Mbutia et al. \(2008\)](#). All the isolated organism in this study were found to be gram-negative cocco-bacillary shape in

Gram staining method (Figure 1), and bipolar characteristics (Figure 2) in Leishman's staining method, which was in support of the findings of Ashraf et al. (2011), Levy et al. (2013), and Akhtar (2013). All the organisms were found to be non-motile when examined under microscope by hanging drop technique.

The *P. multocida* organism gave positive result for catalase test (Figure 3) by producing bubbles and oxidase test (Figure 4) by changing its color (Levy et al., 2013). On the other hand, no reaction was seen with MR and VP tests. *P. multocida* Type A organism fermented dextrose, sucrose and mannitol but not maltose and lactose. These fermented sugars producing acid without gas formation. These findings of biochemical reactions were consistent with the findings of Shivachandra et al. (2006), Tabatabai (2008), Levy et al. (2013), and Christensen et al. (2014) for *P. multocida*.

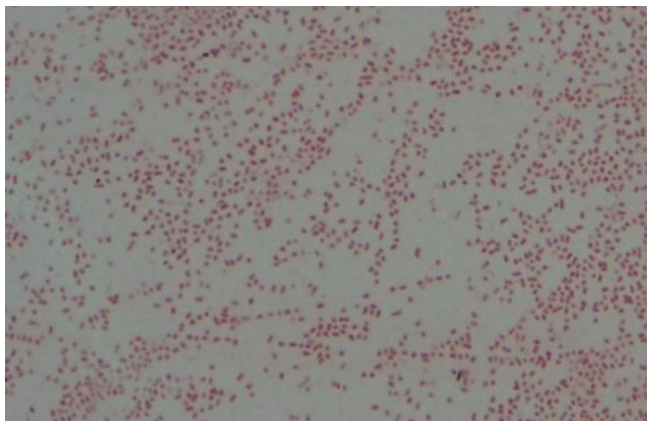


Figure 1: Gram negative, cocco-bacillary or rod shaped *P. multocida* in Gram staining (100x objects).

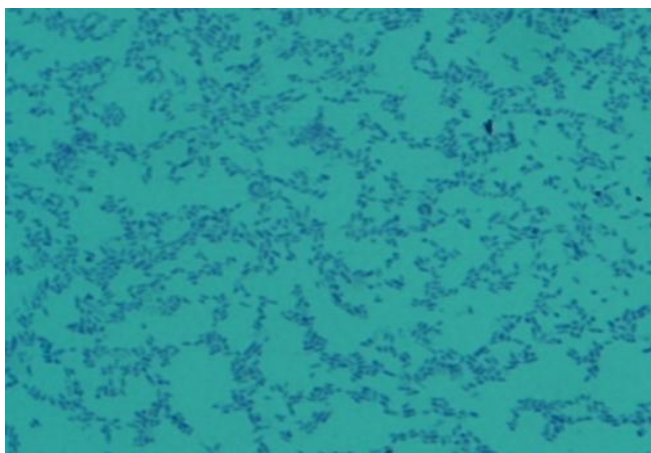


Figure 2: Bipolar appearance of *P. multocida* in Leishman's staining (100x objects).



Figure 3: Catalase test of *Pasteurella multocida* showing positive reaction.



Figure 4: Oxidase test of *Pasteurella multocida* showing positive reaction.

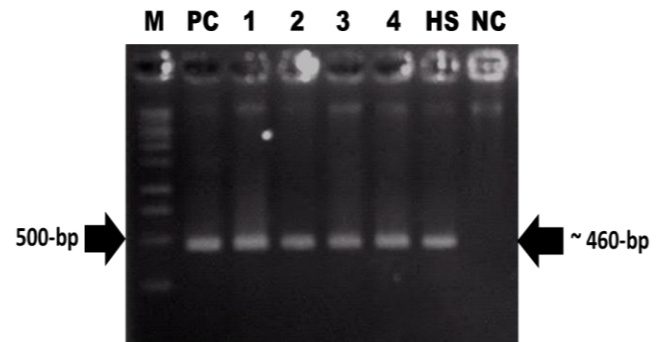


Figure 5: Amplification of *P. multocida* specific gene (*KMT1*). As negative control, H₂O and HS for DNA sample of *P. multocida* type B (for hemorrhagic septicemia) was used. As positive control, DNA sample of *P. multocida* type A was used. Lane M: 1-kb DNA ladder, Lane 1 to 4: Test samples, Lane PC: Positive control, Lane HS: *P. multocida* type B, Lane NC: Negative control.

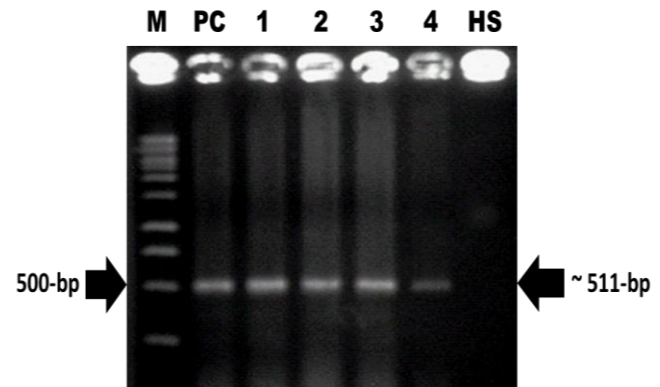


Figure 6: Amplification of *P. multocida* type A specific gene (*capE*). As negative control, DNA sample of *P. multocida* type B (for hemorrhagic septicemia) was used. As positive control, DNA sample of *P. multocida* type A was used. Lane M: 1-kb DNA ladder, Lane 1 to 4: Test samples, Lane PC: Positive control, Lane HS: *P. multocida* type B.

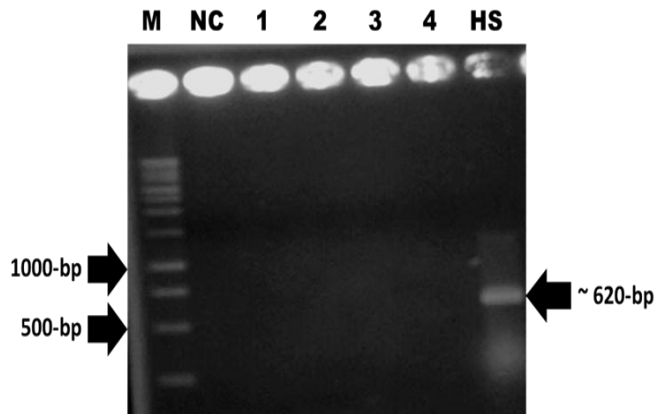


Figure 7: Amplification of *P. multocida* type B specific gene (*KTT*). As negative control, DNA sample of *P. multocida* type A (for Fowl cholera) was used. As positive control, DNA sample of *P. multocida* type B (for Hemorrhagic septicemia) was used. Lane M: 1-kb DNA ladder, Lane 1 to 4: Test samples, Lane NC: Negative control, Lane HS: *P. multocida* type B.

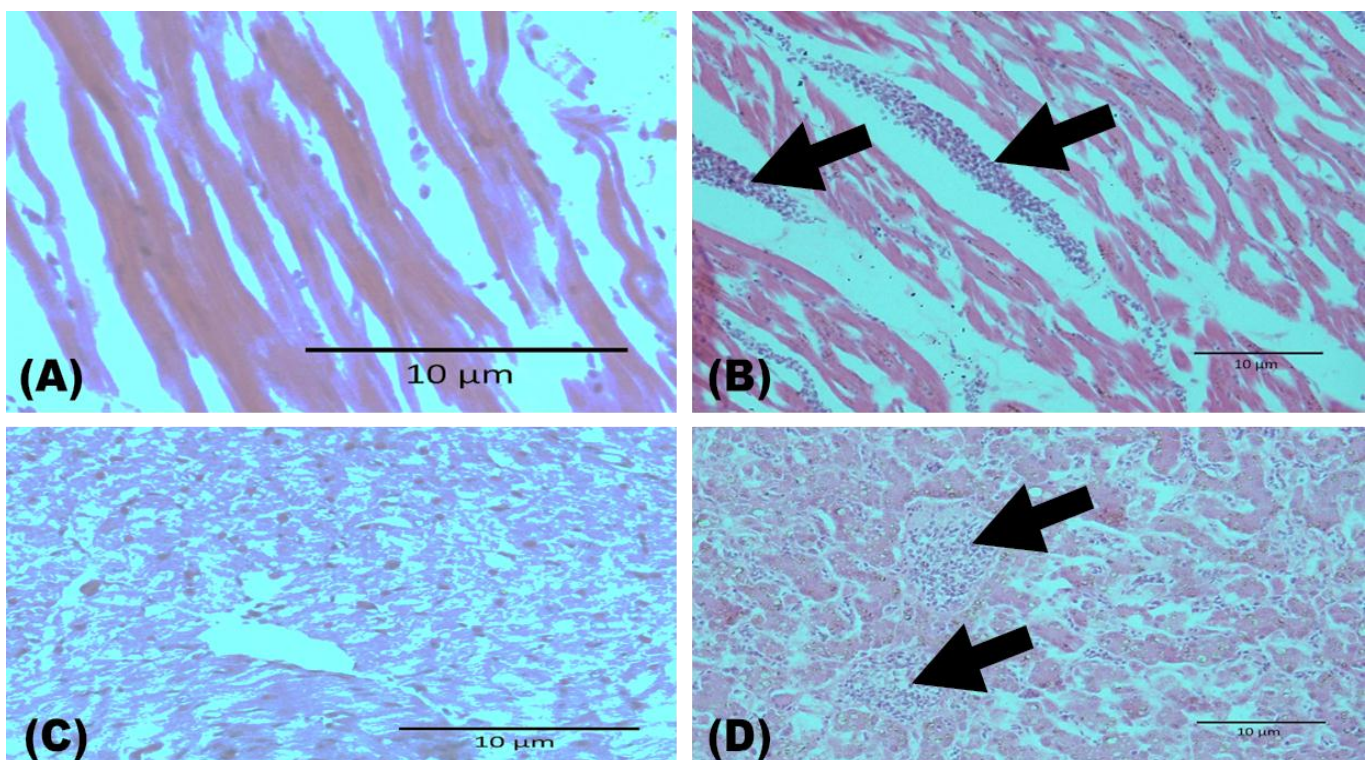


Figure 8: Histopathological examination of heart and liver of chicken that was artificially infected with *P. multocida* Type A. (A) Normal heart of chicken (40 x objects). (B) Experimentally *P. multocida* Type A infected heart muscle of chicken (40x object). Arrow: Lymphocyte infiltration, (C) Normal liver of chicken (40x object), (D) Experimentally *P. multocida* type A infected liver muscle of chicken (40x object). Arrow: Lymphocyte infiltration in central vein.

The isolated *Pasteurella* spp. was subjected to PCR using specific primers for molecular characterization and identification. Polymerase chain reaction with species specific KMT1SP6 and KMT1T7 primers identified six isolates as positive for *Pasteurella* showing amplification of 460-bp (Figure 5). The PCR done by using PMcapEF and PMcapER primers could amplify amplicons of 511-bp (Figure 6). PCR with KTSP61 and KTT72 primers identified one isolate as positive for *P. multocida* Type B (type specific) showing amplification of 620-bp (Figure 7).

The most common histopathological findings were congestion and hemorrhage in heart, liver and spleen. Inflammatory cells were found in the pericardium of heart, lymphocytic infiltration in central vein in liver, as compared to the normal heart and liver (Figure 8).

Experimental inoculation of *P. multocida* isolates in chickens produced characteristics changes in heart, liver and spleen. The chickens were died within 24 h after challenging with *P. multocida* Type A. In post-mortem examination, marked septicemic lesions

consisting of white necrotic foci and hemorrhages in heart, liver and spleen were found (Figure 9), as reported by Zahoor and Siddique (2006).

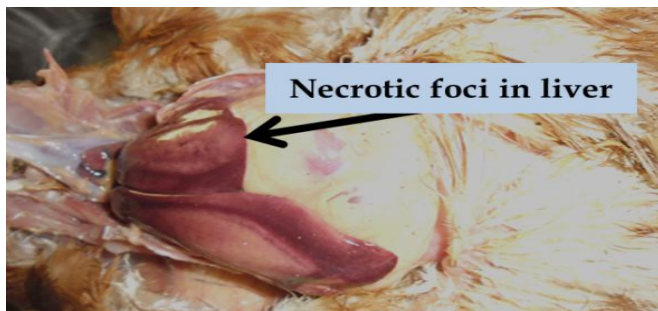


Figure 9: Necrotic foci in liver and hemorrhage in heart as post mortem lesion in chicken after artificial infection by *P. multocida* Type A.

Histopathological study confirmed the occurrence of FC in the experimental chickens. Huge lymphocytic infiltration in central vein of liver was found. Inflammatory cells were found in the pericardium of heart, and lymphocytic infiltration was noticed in the red areas of spleen, as described by Shilpa et al. (2006).

PCR with specific primers (Table 1) was used for the identification of *P. multocida* (OIE, 2008). The isolates of *P. multocida* exhibited the amplification of PCR amplicons of 460-bp (Figure 5), as reported by Townsend et al. (2000), Kumar et al. (2009), Ranjan (2011), Manasa (2012), and Akhtar (2013). All the 4 isolates *P. multocida* were confirmed to be Type A, indicated by the amplification of 511-bp size (Figure 6); similar results were reported by other authors (Shayegh et al., 2010; Kwaga, 2013; Akhtar, 2013).

The genomic DNA of *P. multocida* Type B, the causal agent of hemorrhagic septicemia in cattle (Townsend et al., 1997; OIE, 2008), was used as a negative control to validate the *P. multocida* Type A. The PCR amplification could amplify 620-bp size (Figure 7); however, no amplification was found in the cases of *P. multocida* Type A. These findings are in support of Ranjan (2011). As the sample size in this study is small, further research with representative and large sample size should be performed to establish accurate data of FC in Bangladesh.

CONCLUSION

Isolation and identification of *Pasteurella multocida* was successfully done from naturally infected chickens. The bacteria were subsequently confirmed to be *P. multocida* Type A, for the first time in Bangladesh. Histological

examination of livers and hearts of chickens revealed the changes consistent with this bacterium. Thus, the bacteria are present in chickens in the study areas of Bangladesh, which needs necessary attention for effective control of this disease.

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