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Molecular detection of *Pasteurella multocida* Type B causing haemorrhagic septicemia in cattle and buffaloes of Bangladesh

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Abstract

Hemorrhagic septicemia (HS) is an acute septicemic disease that primarily affects cattle and buffaloes. The disease is caused by *Pasteurella multocida* sero types B:2 and E:2. The objective of this study was to isolate *P. multocida* from clinical cases and to confirm its identity using polymerase chain reaction (PCR) based approach. Clinical samples of two suspected cases of haemorrhagic septicemia of cattle and buffalo from Mymensingh and Rajshahi districts respectively were collected. Two isolates were isolated from these suspected cases and primarily identified as *P. multocida* based on morphological study, staining properties, and cultural and biochemical characteristics. The isolates were confirmed initially as *P. multocida* at genus level by PCR using genus specific primers. Later, the isolates were confirmed as *P. multocida* type B, the causal agent of haemorrhagic septicemia, by PCR with primers specific for *P. multocida* type B. These isolated organisms can be used as vaccine candidate for the production of effective vaccine against haemorrhagic septicemia.

Key words: Haemorrhagic Septicemia, cattle, buffaloes, *Pasteurella multocida* type B, PCR

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Introduction

Haemorrhagic septicaemia (HS) is an acute and highly fatal septicemic disease of cattle and buffaloes caused by *Pasteurella multocida*. The disease has a high morbidity and mortality in cattle, particularly in buffaloes. *P. multocida* are classified into several serotypes based on their capsular and somatic antigens. According to the capsular polysaccharides *P. multocida* according are classified into five serotypes designated A, B, D, E and F, while according to the cell wall lipopolysaccharides typing they are classified into 16 somatic serotypes (Cartet, GR., 1955; Carter and Alwis, 1989). The general and biochemical properties of the various strains are very similar, and from this point of view these organisms all belong to the single species, but different serotypes show different pathogenicity when tested in various hosts. The etiology of HS is *P. multocida* serotype B: 2 and E: 2 (Annas *et al*., 2014; Chung *et al*., 2015; Marza et al., 2015).

P. multocida, based on pathogenicity could be either those causing a HS or those causing non-haemorrhagic septicaemia. In addition, pasteurellosis causes great economic losses in sheep, goats, pigs, dogs, poultry, quails, ducks and sometimes in wild animals such as tiger, lion, leopard, panther, etc (De Alwis, 1996). In cattle, the is characterized by a rapid course, fever, oedematous swelling in the head-throat-brisket region, swollen and haemorrhagic lymph nodes and presence of numerous subserous petechial haemorrhages (Carter and De Alwis, 1989). *P. multocida* is a small, non-motile, Gram negative coccobacillary rod, inhabiting the nasopharynx and gastro-intestinal tract of many wild and domestic

animals and produces disease when the animals are under stress (De Alwis, 1996). High ambient temperature, overcrowding, inadequate ventilation, transportation and malnutrition are factors, linked with the onset of the disease (Stahel *et al*., 2009).

Although there are reports on the occurrence of HS in Bangladesh (Hafiz, 2012; Akhtar, 2013), however, as far as we know, none of the studies have identified the etiological agent of HS using molecular based approach. The aim of the present study was to isolate and identify accurately *P. multocida* from suspected cases of HS by molecular technique *i.e.,* PCR, so that the isolates could later be used for the development of effective vaccine to control the diseases in Bangladesh.

Materials and Methods

Isolation and identification of Pasteurella

For the isolation of *P. multocida* edematous fluid was collected from the throat of buffaloes of Rajshahi (N=1) and cattle of Mymensingh (N=1) regions that showed characteristics signs of HS. The sampleswere immediately transported to the Bacteriology laboratory of the Department of Microbiology and Hygiene, Bangladesh Agricultural University, Mymensingh. Isolation and identification of *P. multocida* was carried out based on morphology, staining, cultural and biochemical characteristic, as described by Cheesbrough (2006).

***Polymerase Chain Reaction (PCR) for Pasteurella multocida* type B**

Conformation of the isolated organisms as *P. multocida* type B, the causal agent of haemorrhagic septicemia in buffaloes and cattle were done based on PCR as described by Townsend *et al.* (1998), Panna *et al*. (2015) and Akhtar *et al*. (2016). Initially PCR was carried out to confirm the isolate as *Pasteurella* spp. using the primer KMT1T7 5′-ATC-CGC-TAT-TTA-CCC-AGT-GG-3′ and KMT1SP6 5′-GCT-GTA-AAC-GAA-CTC-GCC-AC-3′**.** This was then followed by conformation of the isolates as *P. multocida* type B using the specific primers pairs KTT72 5′-AGG-CTC-GTT-TGG-ATT-ATG-AAG-3′ and KTSP61 5′-ATC-CGC-TAA-CAC-ACT-CTC-3′**.** Briefly, for PCR bacterial DNA was first extracted using Wizard genomic DNA Purification Kit (Promega, USA) according to the instruction of the manufacturers to use as PCR template. Extraction of DNA and its quality was checked by running 5 µL suspension of the extracted DNA in a 1% (w/v) agarose gel. All the PCR was done in a final 25 µL volume containing 12.5 µL PCR mastermix (Promega, USA) , 1 µL of each primer (10 pmol), PCR grade water 8.5 µL and DNA template 2 µL. The thermal profile used for the *Pasteurella* genus specific PCR was performed as follows with slight modification from Townsend *et al.* (1998): initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturant at 94°C for 1 min, annealing at 49°C for 1 min, and elongation at 72°C for 1 min and finally a final extension at 72°C for 9 min. The thermal profile used for the *P. multocida* type B specific PCR was also done as per protocol described by Townsend *et al.* (1998) with slight modification: initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturant at 95°C for 1 min, annealing at 49°C for 1 min, and elongation at 72°C for 7 min and finally a final extension at 72°C for 9 min. Following the completion of PCR, 5 µL PCR products were loaded into 2% agarose gel (w/v) along with 1 µL 6X loading dye for electrophoresis in 1X TBE buffer at 100 V for 35 min. A standard 100 bp DNA ladder (Promega, USA) was also loaded in the same gel to check the size of the amplified PCR products. Prior to casting the gel, ethidium bromide (0.5 µg/mL) was added to the gel. The PCR products were visualized under UV light in an image documentation system (Bio Rad, USA).

Results and Discussion

Haemorrhagic septicemia caused by *P. multocida* is a devastating disease of domestic and wild animals. The disease is often associated with severe economic loss in the livestock industries. HS, together with anthrax and black quarter, are responsible for an estimated economic loss of US$148.6 million each year (Ahmed, 1996; Mondal and Yamage, 2014). In this study, *P. multocida* was isolated and identified from suspected cases of HS of cattle and buffalo by conventional bacteriological method. On blood agar media, the isolated bacteria produced small, round, whitish colonies with no hemolysis. Gram's staining revealed presence of Gram negative small rod shaped

bacteria (Figure 1). The isolated organisms fermented dextrose, sucrose and mannitol but not maltose and lactose. These fermented sugars produced acid without gas. The organisms also gave positive indole test and negative methyl red (MR), voges-proskauer (VP) test. All these findings are similar to those reported by Cheesbrough (2006) as specific for *Pasteurella* spp.

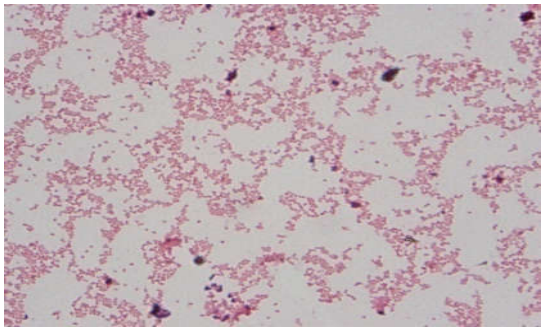

Figure 1. Gram's staining showing small Gram negative rod bacteria (100X)

In the past, several studies have been carried out in Bangladesh on the isolation and identification of *P. multocida* by conventional microbiological methods from filed cases, vaccine development and its efficacy determination (Choudhury *et al*., 1985; Hafez, 2012). However, as far as we know, none of these studies were carried out at molecular level. To the best of our knowledge, this is probably the first study in Bangladesh describing the PCR based detection of *P. multocida* from suspected clinical cases of HS in Bangladesh.

The isolated organisms were found Gram negative and morphologically they were coccobacillary in shape. On Blood agar the isolated organisms produced whitish, opaque, circular, translucent colonies and with no hemolysis that resembles the characteristics colonies of *P. multocida*, as described by Choudhury *et al*. (1985), Cheesbrough (2006) and Rahman *et al*. (2016). Biochemically the isolated organisms were found positive for oxidate, catalase, indole tests, negative from MR and VP test. The organisms were found positive for sucrose, dextrose, mannitol and negative for lactose and maltose. Results of these biochemical tests suggested that the isolated organisms could be considered as *P. multocida.* Shivachandra *et al*. (2011) also reported similar biochemical characteristics for *P. multocida* type B.

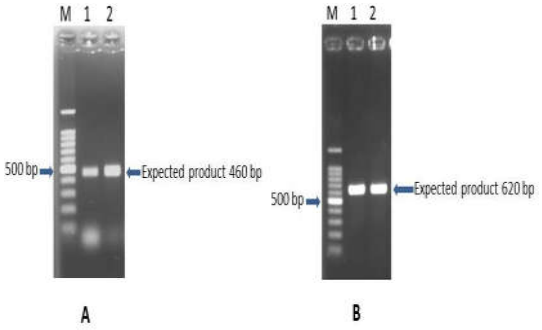

Figure 2. PCR amplification of *Pasteurella multocida*.A. Amplification using the primers KMT1SP6 and KMT1T7 specific for the genus *Pasteurella*. B. Amplification using the primers KTSP61 and KTT72specific for the *Pasteurella multocida*. Lane 1= Isolate from cattle, Lane 2= Isolate from buffalo, M=100 bp DNA marker

PCR is a rapid, robust and highly specific molecular technique for confirmatory detection of many species of bacteria including *P. multocida* (OIE Manual, 2008).The development of molecular tools has paved the way for rapid and specific identification of infectious agents, thus PCR was used for identification of *P. multocida.* During the present study, both the buffalo and cattle isolates of *P. multocida* exhibited species specific amplification of approximately 460-bp size using primers KMT1SP6 and KMT1T7 (Townsend *et al*., 1998). These findings confirmed the results obtained by Townsend *et al*. (2000), Javia (2004) and Patel (2004), who reported the specificity of this primer pair for all *P. multocida* isolates. Townsend *et al.* (1998) reported that the primer pairs gave amplification from all strains of *P. multocida* (all serotypes and capsular serogroups), the three subspecies *i.e.*, *P. multocida* subsp. *multocida*, *P. multocida* subspp. *gallicida*, *P. multocida* subspp. *septica* and also *P. canis* biotype 2. Dutta *et al*. (2001) also carried out PM-PCR using various serotypes of *P. multocida*.

Presumptive identification of HS caused by type-B-specific *P. multocida* is also possible by PCR amplification (Townsend *et al*., 1998). To date, *P. multocida* type-B-specific PCR performed by Townsend *et al*. (1998) remains 100% specific for the detection of this organism. In the present study *P. multocida* type B cultures both from buffalo and cattle with the predominant somatic antigen being either type 2 or 5 are identified by the amplification of a 620-bp fragment with the KTSP61 and KTT72 primers. Results of the PCR are presented in Figure 2. A standard PCR protocol which is slightly modified from Townsend *et al.* (1998) successfully was optimized to detect *P. multocida* type B circulating in Bangladesh.

Conclusion

P. multocida has been isolated from the filed cases of haemorrhagic septicemia of cattle and buffalo in Bangladesh. Besides, molecular identification of the isolated organism as *P. multocida* type B is confirmed by PCR. The isolate can be used in effective vaccine development.

Conflict of interest

The authors declare that they have no competing interest.

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