



Veterinary Science
ORIGINAL ARTICLE

Isolation and identification of *Clostridium chauvoei* from cattle in Mymensingh city of Bangladesh

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ARTICLE INFORMATION

Article History

Submitted: 02 Aug 2018

Revised: 29 Sep 2018

Accepted: 14 Oct 2018

First online: 25 Oct 2018

Academic Editor

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ABSTRACT

Black quarter (BQ), caused by *Clostridium chauvoei*, is an economically important, highly infectious bacterial disease of cattle, goat and sheep. The present study was conducted to isolate and identify the causal agent of BQ from field cases. A total of 32 samples from necropsied areas of suspected cattle brought to the Veterinary Teaching Hospital, Bangladesh Agricultural University, Mymensingh were collected. The samples were subjected for bacteriological culture followed by identification through a series of conventional bacteriological techniques, staining properties and biochemical tests. The bacteria were cultured in anaerobic condition using candle jar. Morphological characters of bacteria were observed after Gram stain. The suspected samples were subjected for several passages in liver infusion broth to get pure culture. On neomycin blood agar, the bacteria showed hemolysis. Based on the cultural and staining characteristics and biochemical tests, 4 (12.5%) out of 32 samples were detected as *Clostridium chauvoei*. It is concluded that *Cl. chauvoei* has been successfully isolated and identified from BQ affected cattle.

Keywords: Black quarter, cattle, *Clostridium chauvoei*, liver infusion broth, neomycin blood agar

Cite this article: Alam MN, Mahmud MM, Nime J, Khan MR, Khan MS, Rahman MB, Nazir KHMNH. 2019. Isolation and identification of *Clostridium chauvoei* from cattle in Mymensingh city of Bangladesh. Fundamental and Applied Agriculture 4(1): 649–654. doi: 10.5455/faa.2733

1 Introduction

Black quarter (BQ) (popularly known as Blackleg) is a fatal disease of cattle, goats and sheep caused by *Clostridium chauvoei* which was first reported in 1870 (Armstrong and McNamee, 1950). *Cl. chauvoei* is an anaerobic, endospore-forming Gram-positive bacterium known to be the causative agent of BQ (Hirsh et al., 2004). In endemic areas, spores of *Cl. chauvoei* may be present in soil and feces (Hang'ombe et al., 2000).

The virulence of *Cl. chauvoei* is caused by rapid spread of the activated, vegetative form of the bacterium in the infected tissue, followed by the production of potent toxins (Hatheway, 1990; Useh et al., 2003). BQ appears to be a nontraumatic endogenous infection in cattle, since infection of muscle tissue occurs in the absence of a wound or a break in the skin (Smith and Holdeman, 1984). Affected animals are anorectic, depressed, febrile, and lame (one side limb), presenting a hot, painful swelling that becomes cold, edematous with crepitation. Death is seen within 12

to 48 h. In sheep, blackleg is frequently associated with wounding, such as at shearing cuts, tail docking and castration site (Songer, 1998). Once these stimulating events occur, the spores in the infected tissue germinate and multiply into the disease-causing *Cl. chauvoei* bacteria. Then, the disease progresses rapidly and the infected animal dye in 12 to 36 hours after the appearance of the first symptoms (Hatheway, 1990). The affected muscles are dark in color, and edema is restricted to the local area (Heller, 1920).

Up to 2008, scientists thought that *Cl. chauvoei* has no zoonotic importance because *Cl. chauvoei* infections are specifically found in ruminants but a case of human fulminant gas gangrene caused by *Cl. chauvoei* was described and confirmed by PCR (Nagano et al., 2008). Livestock rearing has become seriously impaired due to high mortality with BQ like symptoms (Jowel et al., 2016). The marginal and landless farmers most easily live on rearing livestock in Bangladesh. Cattle, goats and sheep are the most important livestock in Bangladesh which play an important role in the rural economy and earn substantial amount of foreign currency by exporting skins and other by-products. Only few studies have been conducted earlier on isolation and identification of *Cl. chauvoei* in Bangladesh (Jowel et al., 2016). The present study was undertaken with the objective of isolation and identification of the BQ causing bacterium, *Cl. chauvoei* from field cases.

2 Materials and Methods

2.1 Samples collection

A total of 32 suspected samples were collected directly from the affected area of cattle that were brought to the Veterinary Teaching Hospital, Bangladesh Agricultural University (BAU), Mymensingh with a history of suffering from depression, anorexia, rumen stasis, high fever (41–42 °C), characteristic edematous and crepitant swellings develop in the hip, shoulder, chest, back, neck or elsewhere (Fig. 1). Serosanguineous rancid fluid was also collected from discolored skin in affected areas by using cotton bud or needle and syringe. The samples were transported to the Department of Microbiology and Hygiene, BAU for isolation and identification.

2.2 Culture and Gram staining

The collected serosanguineous samples were transferred to neomycin blood agar media (7% cattle blood) and were incubated anaerobically for 24 h at 37 °C. The suspected colonies were sub-cultured on blood agar (Hi Media, India) plates and were incubated in anaerobic jar (Quinn et al., 2004) for 24 h at 37 °C. Gram-stain and spore staining were done as per the

method described by (Jowel et al., 2016). Morphological characteristics and biochemical tests were also performed from the culture as described by Pires et al. (2012). Passages were repeated on blood agar plates until the culture was considered as pure. Then it was transferred in liver infusion broth (Hi Media, India) and incubated anaerobically for 48 h at 37 °C. Passages were repeated until the culture was considered as pure.

2.3 Biochemical tests

The carbohydrate fermentation test was performed by inoculating 5 mL of nutrient broth (Hi Media, India) culture of the organisms into the tubes containing different sugar media and incubated for 72 h at 37 °C. Acid production was indicated by the color change from red to yellow of the medium and the gas production was noted by the appearance of gas bubbles in the inverted Durham's tube. Methyl red (MR) test, Voges-Proskauer (V-P) test, Indole test, Dulcitol fermentation test and Catalase test were performed as per the methods described by Jowel et al. (2016).

3 Results

Among the 32 samples, 4 showed typical staining characteristics on Gram stain which is 12.5% of total samples. Numerous short, thick, straight, rounded, gram positive rod arranged in singly or in short chains or long filaments were observed under microscope. The spores of the organism were elongated, oval, sub-terminal or terminal, and wider than the cell, giving a typical pear-shaped appearance (Fig. 2). 20 samples showed mixed organism both Gram positive and Gram negative bacteria which was 62.5% of total sample and 8 samples did not grow on neomycin blood agar or liver infusion broth which was 25% of total samples.

Neomycin blood agar plates were inoculated with suspected samples and incubated anaerobically at 37 °C for 24–48 h, which showed the growth of bacterial colonies. The colonies tentatively identified as *Clostridium* sp. were subcultured, revealing small, irregular, whitish pale colored, finely granular in the center but almost invisible toward the periphery (Fig. 3). The edges of the colony resemble wisps of hair and surrounded by a typical zone of haemolysis which resembled to the colony characteristics of *Cl. chauvoei*. Huge number of short, thick, singly-arranged or short chained, Gram positive rod shaped bacteria was observed under microscope. In liver infusion broth, a typical opalescent growth with slight gray whitish sediment was observed (Fig. 4).

All the suspected isolates were found to be catalase negative (Table 1). They fermented all the sugars and produced acid and gas except mannitol and dulcitol. All isolates of clostridia were indole, MR and

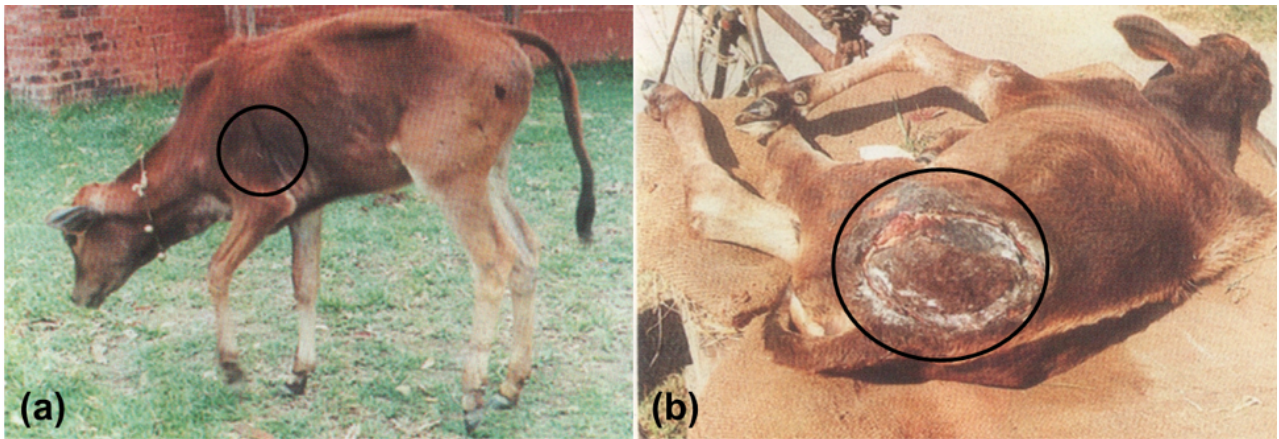


Figure 1. Black Quarter affected cattle. (a) A heifer affected with black quarter showing lameness on the left foreleg, (b) A heifer affected with BQ showing myonecrosis of the gluteal muscles. Black circle indicates necrosied area

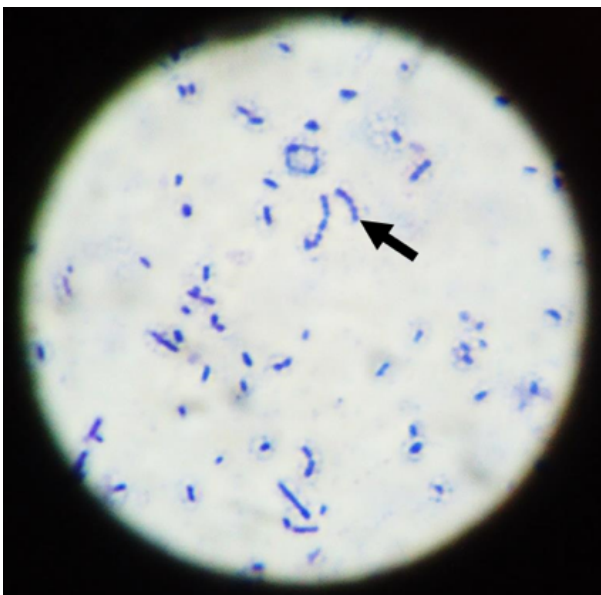


Figure 2. Gram positive *Clostridium chauvoei* under microscope, ×100 (black arrow)



Figure 3. Characteristics single colony on neomycin blood agar. Black arrow indicates small, irregular, whitish pale colonies

Table 1. Results of carbohydrate fermentation and biochemical tests

SL	GS	G	L	S	F	Mal	Man	D	I	MR	VP	C	Bacteria
1	+	AG	AG	AG	AG	AG	–	–	–	–	–	–	<i>Cl. chauvoei</i>
17	+	AG	AG	AG	AG	AG	–	–	–	–	–	–	<i>Cl. chauvoei</i>
23	+	AG	AG	AG	AG	AG	–	–	–	–	–	–	<i>Cl. chauvoei</i>
25	+	AG	AG	AG	AG	AG	–	–	–	–	–	–	<i>Cl. chauvoei</i>

GS = Gram stain, G = Glucose, L = Lactose, S = Sucrose, F = Fructose, Mal = Maltose, Man = Mannitol, D = Dulcitol, I = Indole, MR = Methyl Red, VP = Voges Proskauer, C = Catalase, + = Gram positive, AG = Acid and Gas

VP negative (Table 1). The results of culture, staining and biochemical tests indicated that the suspected isolates were *Clostridium chauvoei*.

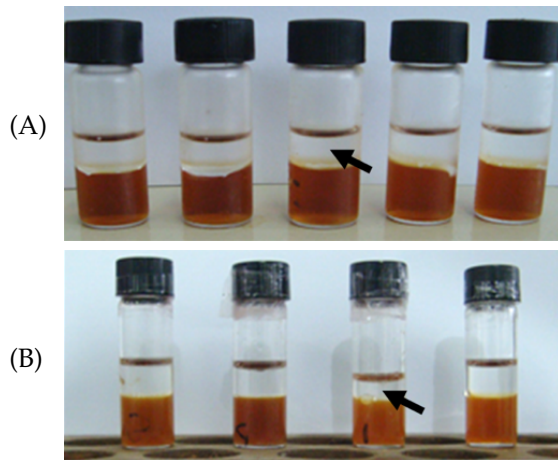


Figure 4. Culture of *Cl. chauvoei* in liver infusion broth. (A) Negative control, (B) Culture of *Cl. chauvoei* showing opalescent growth with slight gray whitish sediment

4 Discussion

BQ has been hindering the cattle development in Bangladesh and causing economic losses. Investigation of BQ and its suitable diagnosis are prerequisite for effective control of the disease. As there is no work on isolation and identification of causal agent of BQ in Bangladesh, early identification of disease is important for appropriate treatment and prophylactic measures. Routine methods of bacterial cultures in different media, specific colony characters, microscopic examination, Gram's staining techniques and different types of biochemical tests were used for the isolation and identification of *Cl. chauvoei*.

The cattle that were suspected of having BQ showed clinical signs resembled to those were reported by Uzal et al. (2003), Blood et al. (1983) and Heller (1920). Based on the colony characteristics mentioned in neomycin blood agar media and liver infusion broth indicated the presence of *Cl. chauvoei* which corresponded to the results of Hirsh et al. (2004) and Jewel et al. (2016). The organism identified as *Cl. chauvoei* on the basis of characteristics mentioned in biochemical and sugar fermentation test. These results correlated to the results of Chattopadhyay and Harbola (1988).

Characteristic lesions and Conditions favoring spore germination, bacterial growth, and toxin production cause formation of local lesions marked by edema, hemorrhage and, myofibrillar necrosis. The centers of lesions become dry, dark emphysematous due to bacterial fermentation, while the periphery was edematous and hemorrhagic. A rancid butter odor is typical which is corresponding to some au-

thors (Hirsh et al., 2004). Symptoms resembling blackleg can also be caused by *Cl. septicum*, *Cl. novyi*, *Cl. perfringens* or *Cl. sordellii*. In a recent study on the phylogenetic position of *Cl. chauvoei* and *Cl. septicum* on their 16S rRNA gene (rrs) sequences, a similarity of 99.3% between the rrs genes of *Cl. chauvoei* and *Cl. septicum* that is also reflected at the phenotypic level, was determined reported by Kuhnert et al. (1997). BQ in cattle were reported in Nigeria (Osiyemi, 1975), Iran (Ardehali and Darakhshan, 1975) and India (Dutta, 2003). Blood et al. (1989) isolated *Cl. chauvoei* from the intestinal tract of normal animals. Hatheway (1990) and Useh et al. (2003) diagnosed BQ on the basis of epidemiology and clinical manifestations and also isolated the vegetative form of the bacterium in the infected tissue. Hang'ombe et al. (2000) diagnosed *Cl. chauvoei* from soil and feces.

In the present experiment, *Cl. chauvoei* has been isolated and identified from the infected areas of cattle. This identification of bacteria corresponded to the results of Sathish and Swaminathan (2008). A stab-culture was prepared from 32 samples which was corresponded by Eyre (2009). Smears prepared from 32 samples were stained with Gram's staining method. The stained slides were examined according to the procedure described by Rahman (1995). The collected 32 samples in liver infusion broth were kept in a candle jar and incubated for 48 h at 37 °C. For maintenance of anaerobic condition olive oil (2–3 cm) were poured on surface of culture broth in test tube and this technique was reported by Eyre (2009). In Gram's staining, the morphology of the isolated bacteria was Gram positive, rod shaped, anaerobic, spore forming single or paired in arrangement which was supported by Shamimuzzaman (1999).

Based on the colony characteristics mentioned in neomycin blood agar media, colonies showed a typical zone of haemolysis formed in each plate indicating the presence of *Cl. chauvoei*, as described by Ellner (1956). All of the 4 isolates fermented glucose, sucrose, lactose and maltose but did not ferment dulcitol and mannitol, and all of the isolates were Indole, MR and VP negative except one which were previously suggested by a number of scientists (Sacks and Olson, 1979; Chattopadhyay and Harbola, 1988). The occurrence of BQ in the present study was 12.5% which were relatively higher than the reports by Islam et al. (2003), Hossain et al. (2002), Talha et al. (2001) and Islam et al. (1998) and Alam et al. (2018). Kuhnert et al. (1997) supported that in case of blackleg outbreak, culture and detection by PCR gave better results than biochemical analysis. However, in our study, we could not do PCR for molecular identification as we could not identify the bacterium by conventional bacteriological techniques and biochemical characteristics. These findings provide support for the requirement of continuous monitoring of the cattle suspected to BQ and the development of techniques that may allow simple

and reliable identification of different field isolates.

5 Conclusions

It is concluded that the suspected isolates contain *Cl. chauvoei*, the causal agent of BQ with an incidence of 12.5%. Tissue samples can be the sample of choice. However, for useful application of the present research findings, further studies are suggested for typing and PCR identification of *Cl. chauvoei* from field cases.

Acknowledgements

The work was conducted with the financial support from the Ministry of Science and Technology, People's Republic of Bangladesh.

Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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