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ISOLATION, IDENTIFICATION AND ANTIBIOGRAM STUDY OF C*lostridium Chauvoei* ISOLATED FROM FIELD CASES OF BLACK LEG IN CATTLE

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Clostridium chauvoei is the etiologic agent of Black quarter, a high mortality rate disease affecting mainly young cattle and sheep. Carcasses of animals affected by the disease are the chief source of soil infection and considered as an ever-present threat to livestock health. The present study was designed with a view to isolate and identifies *Clostridium chauvoei* from field cases. For this purpose, a total of 4 clinically suspected samples were collected during the period from January 2013 to November 2013 and cultured anaerobically in the Blood agar media. Gram's staining and hanging drop techniques were also performed. Biochemical properties of the isolates were studied and antibiotic sensitivity test was also performed. In Gram's staining technique, all isolates showed numerous short, thick, straight, round-ended, gram positive rod occurs singly or in short chains. The spores of the organism were elongated, oval, sub terminal or terminal and wider than the cell, giving a typical pear-shaped appearance. All the *Clostridium chauvoei* isolates fermented dextrose, maltose, lactose and sucrose and produce acid and gas. In case of mannitol production of acid and gas was absent. Catalase, oxidase, MR, VP and Indole tests were negative. All isolates were resistant to oxytetracycline, amoxicillin and ciprofloxacin, sensitive to penicillin and gentamicin and intermediate to neomycin. Penicillin or gentamicin can be a suitable drug of choice for the effective treatment of black quarter in cattle of Bangladesh.
with increase in their performance.

Key word: *Clostridium chauvoei*, Black quarter, Cattle, Penicillin.

Clostridium chauvoei is responsible for causing an economically important disease Black quarter/Blackleg in young cattle and sheep. This disease is characterized by the appearance of crepitating sounds with fluctuating swelling of one of the quarters, followed by rapid death (Naz *et al.*, 2005). In endemic areas *Cl. chauvoei* may be present in soil and feces (Hang'ombe *et al*., 2000). Once pasture has become heavily contaminated, cases of the disease usually occur year after year in susceptible animals (Quinn *et al*., 1994). In cattle, Blackleg appears to be a non-traumatic, endogenous infection. Since infection of muscle tissue occurs in the absence of a wound or a break in the skin (Smith and Williams, 1984) and a considerable proportion of bovines may harbor *Cl. chauvoei* in their liver. Affected animals are depressed, febrile and lame (one side limb), presenting a hot, painful swelling that becomes cold, edematous with crepitating sound. Death is seen within 12 to 48 hours. This agent seems to have a preferences for big muscles (thigh, diaphragm and heart), which at necropsy are dark red, dry and spongy (Sippel, 1982). Young growing ruminants on pasture are especially sensitive to *Cl. chauvoei* in soil may be a significant factor, since the disease occurs year after year on the same premises, usually in well-fed cattle less than 3 years old . Work on the present nature of black quarter is very scanty in Bangladesh.Therefore, the present study was undertaken to understand the nature of the prevalent *Cl. chauvoei* strain in Bangladesh, to isolate and identify *Clostridium chauvoei* from field cases and to study the antibiogram profile of the isolated organism

to select the antibiotic in the treatment of the disease.

MATERIALS AND METHODS

Sample collection

A total of four samples (blood from affected site) were collected from Monohardi, Narshindi; Sujanagar, Pabna and Veterinary Clinic of Bangladesh Agricultural University, Mymensingh. Blood from the affected site were collected by sterile syringe and carried in cool box to the Bacteriology Laboratory of the Department of Microbiology and Hygiene, Bangladesh Agricultural University (BAU).

Bacteriological analysis

Collected serosanguineous fluid with blood was transferred to Meat chop broth with mineral oil in anaerobic condition and kept in anaerobic candle jar, Incubated at 37^0 C for 36-48 hours. After growth, the bacteria were inoculated on to Blood agar and incubated at 37º C for 36-48 hours in anaerobic jar. Bacteria showed white raised colony. In Gram's staining the organisms appeared as bipolar small Gram positive rod. The carbohydrate fermentation test was performed by inoculating a loopful of thick test bacterial culture into the individual tubes containing sugars like dextrose, maltose, lactose, sucrose, mannitol and incubated at 37^0 C for 48 hours. Acid production was indicated by the change of media from pink to yellow color while gas production was indicated by the appearance of gas bubbles in the inverted Durham's tubes. Methyl-Red (MR) test was performed by inoculating a colony of the test organism in 0.5 ml sterile glucose phosphate broth. After overnight incubation at 37^0C, a drop of methyl red solution was added. A positive methyl red test was shown by the appearance of bright red colour indicated acidity while a yellow or orange colour was considered as negative. To perform Voges-Proskauer (VP) test five ml of sterile glucose phosphate peptone broth were inoculated with a pure colony of test organisms and incubated at 37^0C for 24 hours. A very small amount (knife point) of creatine was added and mixed and 3 ml of sodium hydroxide were added and shacked well. The bottle cap was removed and left for an hour at room temperature. It was observed closely for the slow development of a pink colour for positive cases. To perform Indole test two ml of peptone broth was inoculated with a pure colony of bacterial culture under observation and incubated at 37^0C for 24 hours after which 0.5 ml Kovac's reagent was added, shaked well and examined after 1 minute. A red colour in the reagent indicated positive test. The motility test was performed to differentiate motile bacteria from non-motile one. This test was performed in Hanging drop slide. One drop of distilled water was taken in the glass slide and with bateriological loop a single colony was taken from the Blood agar, mixed it very well. Then it was observed under the microscope after cover slip was added. All isolated organisms were found motile.

Antibiogram profile of the organisms

Isolated colonies of the same morphological type are selected from agar plate culture. The top of the colony is touched with a loop and the growth is transferred into a tube containing 4-5 ml of a suitable broth. The broth culture is incubated at 37 ^{0}C for overnight. The growth of bacteria was oberved and the turbidity were compared to Mecferland standard 0.5. Sterile cotton swab is dipped into the broth suspension. The swab should be rotated several times and pressed firmly on the inside wall of the tube above the fluid level. This will removes excess inoculums from the swab.The dried surface of a Mueller- Hinton agar plate is inoculated by streaking the swab over the entire sterile agar surface. This procedure is repeated by streaking two more times, rotating the plate approximately 6 each time to ensure an even distribution of inoculums. As a final step, the rim of the agar is swabbed. The plate was kept for 3 to 5 minutes, but no more than 15 minutes, to allow for any excess surface moisture to be absorbed before applying the drug impregnated disks. The predetermined battery of antimicrobial discs is dispensed onto the surface of the inoculated agar plate. Each disc must be pressed down to ensure complete contact with the agar surface. Whether the discs are placed down individually or with a dispensing apparatus, they must be distributed evenly so that they

are no closer than 24 mm from centre to centre. The plates are inverted and placed in an incubator set to 37^0C within 15 minutes after the disc are applied. After 16 to18 hours of incubation each plate is examined. If the plate was satisfactorily streaked, and the inoculum was correct, the resulting zones of inhibition will be uniformly circular and there will be a confluent lawn of growth. If individual colonies are apparent, the inoculum was too light and the test must be repeated. The diameters of the inhibition zones are measured to the nearest whole millimeter, using sliding calipers or a ruler, which is held on the back of the inverted petri plate. The petri plate is held a few inches above a nonreflecting background and illuminated with reflected light. The results were recorded at 16-18 hours post incubation. Transmitted light is used to examine the zone of inhibition. The zone margin should be taken as the area showing no obvious, visible growth that can be detected with the unaided eye. Faint growth of tiny colonies, which can be detected only with a magnifying lens at the edge of the zone of inhibited growth, is ignored. The sizes of the zones of inhibition are interpreted according to Zone of Diameter Interpretative Standards of CLSI (2007).

RESULTS

All the 4 samples showed numerous short, thick, straight, round-ended, gram positive rod occurs singly or in short chains. The spores of the organism are elongated, oval, sub terminal or terminal and wider than the cell, giving a typical pear-shaped appearance.

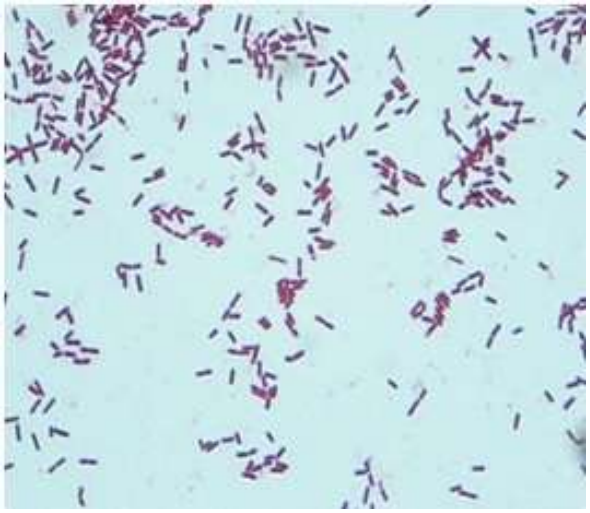

Figure -1 : Gram positive rod shaped *Clostridium Chauvoei*

In blood agar, the round colonies were tentatively identified as *Clostridium* Sp., which were used for sub culture. In the pure culture, the colonies were small, irregular, whitish pale colored, finely granular in the center but almost invisible toward the periphery. The edges of the colony resemble wisps of hair and surrounded by a typical zone of hemolysis which resembled to the colony characteristics of *Clostridium chauvoei*.

All of the four isolates fermented dextrose, lactose, sucrose, maltose and produced acid and gas but did not ferment mannitol. All suspected samples were catalase and oxidase negative. All the four isolates of clostridium were Indole, VP and MR negative. So, all were finally identified as *Clostridium*

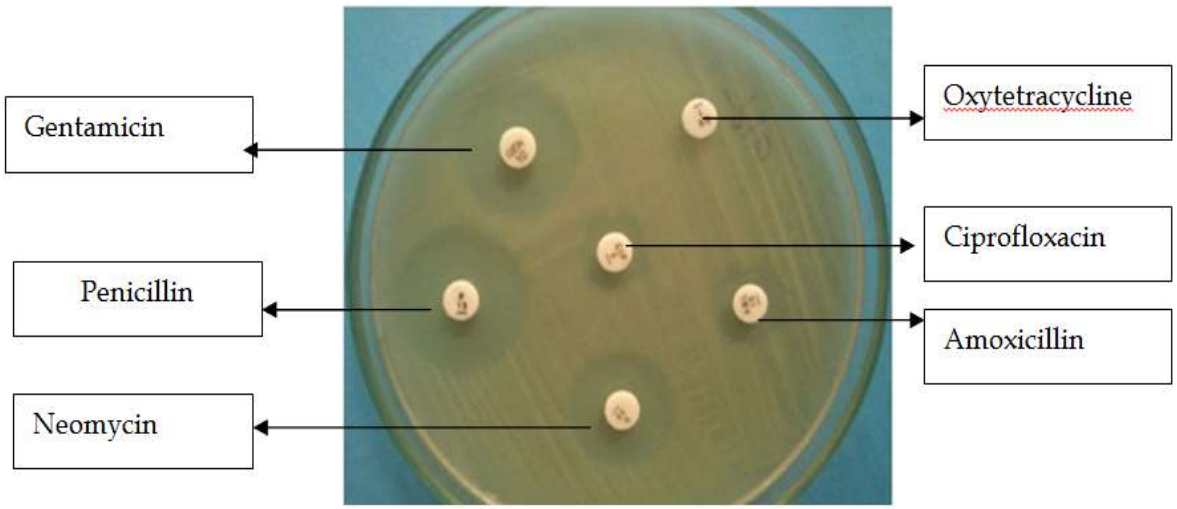

Figure - 4 : Antibiotic sensitivity test

chauvoei. The isolated organisms were sensitive to penicillin and gentamycin, intermediate to neomycin while resistant to oxytetracycline, amoxicillin and ciprofloxacin.

Figure – 2 : Characteristics growth of *Clostridium chauvoei* on blood agar

Figure – 3 : Characteristics hemolysis of *Clostridium chauvoei* on blood agar

DISCUSSION

Inam-ul-Haq *et al*. (2011) found efficiency of treatment trials as two broad spectrum antibiotics e.g. Amoxicillin and Oxytetracycline against black leg under field conditions. The isolated organisms showed resistant to Oxytetracycline, it may be due to frequent use of these antibiotics without maintaining proper dose. In our study, all isolates (100%) were sensitive to penicillin and Gentamicin whether all were resistant to Amoxicillin, Ciprofloxacin and Oxytetracycline. So, penicillin is the first choice of antibiotic to treat black leg in Bangladesh.

CONCLUSION

Clostridium chauvoei were isolated and identified as the causal agent of black quarter. Muscle samples could be the samples of choice. However, for usual application of the present research findings further studies should be focused on the detailed typification of *Clostridium chauvoei* from field cases. Development of different ideal PCR method for their relative sensitivity on different types of samples for the diagnosis of black quarter. Development of effective vaccine on the basis of molecular characterization. Researchers can use our findings as a reference during molecular research on Black leg in Bangladesh.

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