



RESEARCH ARTICLE

MOLECULAR CONFORMATION OF BOVINE BOTULISM BY POLYMERASE CHAIN REACTION

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Received 17th February, 2018; Accepted 22nd March, 2018; Published 30th April, 2018

ABSTRACT

Botulism intoxication in bovine caused by exposure to botulinum neurotoxins (BoNTs), one of the most potent toxins known, produced by the bacteria *Clostridium botulinum*. It can affect all mammals. Recent years, an increased frequency of new form of bovine botulism has been observed. A total of 121 Cattles and 10 sheep and goats irrespective of breed, age and sex included in this study. Cultural examination of rumen fluid, dung samples and poultry manure samples, only three cases showed photolytic changes in cooked meat medium and lecithinase activity in *Clostridium botulinum* isolation agar. The positive bacterial culture showed sub terminal spore in gram staining, acid- gas production in carbohydrate fermentation in lactose and sucrose medium, liquefaction of gelatin and stormy clot fermentation in milk. In case PCR reaction, two samples showed 800bp product size in 2% agarose gel.

Key words: Bovine *Clostridium botulinum* - BONT gene- PCR reaction.

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Citation: Kalaiselvi, G., Gunaseelan, L., Sukumar, K., Vijayakumar, G. and Suresh, P., 2018. "Molecular conformation bovine botulism by polymerase chain reaction" *International Journal of Current Research in Life Sciences*, 7, (04), 1794-1796.

INTRODUCTION

Botulism is a rapid onset, usually fatal disease caused by the botulinum toxin produced by the bacterium *Clostridium botulinum*. This spore-forming anaerobic organism proliferates in decomposing animal tissue and sometimes in plant material. Botulism is in most cases an intoxication, not an infection, and results from ingestion of toxin in food (Martin2003). There are seven types of *Clostridium botulinum*, differentiated on the antigenic specificity of the toxins: A, B, C₁, D, E, F, and G. Types A, B, and E are most important in people. C in most animal species, notably wild ducks, pheasants, chickens, mink, cattle and horses; and D in cattle. Botulism is the cause of acute recumbency in 50.68 per cent of cattle. About 94.59 per cent of the incidences were directly associated with poultry layer farms (Otter et al., 2006) The significant clinical findings of botulism in cattle were acute recumbency, abdominal breathing, tripping gait, restlessness, scanty - unformed dung, elevation of tail, rumen atony and flaccid paralysis progressing to lateral recumbency and death (Pandian et al., 2015) Common sources of toxin include animal carcasses, rotting organic material and poorly prepared silage.

Treatment is rarely attempted but vaccines are available in foreign countries for disease prevention in cattle. A recent study states that fatal botulism in cattle could be managed if treated at the early stage. The researchers of this study themselves accepts that even though modified therapy is successful in treating the affected animals but it cannot be claimed superior to antitoxin administration.

MATERIALS AND METHODS

A total of 121 Cattles and 10 sheep and goats irrespective of breed, age and sex brought to the Teaching Veterinary Clinical Complex, Namakkalor telephonically reported to the same with the history and clinical signs of acute recumbency, respiratory distress and anorexia will be included for the study in and around Namakkal District of Tamil Nadu, India, included for this study. The selected cases were evaluated as per standard procedure. History of using poultry slurry and farmyard manure will be collected from the animal owners. In field visits, details of the grazing area management, fodder and water samples will be collected. Rumen liquor, dung, serum and soil samples will be collected from ailing animals. Rumen liquor, dung, serum and soil samples collected from the farm premises were subjected to bacteriological anaerobic culture in

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Robertson cooked meat medium and thioglycolate broth after treating with equal volume of absolute ethanol for one hour. After an incubation of 96 hours at 37 ° C, broth cultures will be inoculated on *Clostridium botulinum* selective agar. The selective agar will be incubated for six to eight days under anaerobic gas pack system at 37 ° C. The colony morphology was studied and smear of the colony will be examined under oil immersion objective (100X) after Gram's staining was performed as per method prescribed by Nakamura *et al.*(2010) The bacterial DNA will be extracted from culture by phenol chloroform method. PCR was performed as per method of Nakamura *et al.*(2010) for identification of *Clostridium botulinum* type D. This method consists of a first PCR amplification with a degenerate primer pair encoding open reading frame (forward primer 5' TTGTGGATATTAGAAAGTTAGGAG-3': reverse primer 5' TTTAACATAATTTATACTATTACTTATTAT -3'). PCR amplification was carried out in the reaction mixture containing 25-50ng of 4µl of template DNA and 10µM of each of the two primers (0.5 µl), 2.5mM of polymerase and 10X buffer (1µl). The PCR cycle condition includes initial denaturation 95°C for 5 min, amplification at 95°C for 30 s, 53°C for 30s and 72°C for 1 min was performed for 30 cycles. The extension reaction was further continued at 72°C for 10 min. The amplified PCR products were analyzed by 2% agarose gel electrophoresis staining with ethidium bromide.

RESULTS AND DISCUSSION

Cultural examination of rumen fluid, dung samples and poultry manure samples, only three cases showed photolytic changes in cooked meat medium and lecithinase activity in *Clostridium botulinum* isolation agar. The positive bacterial culture showed sub terminal spore in gram staining, acid- gas production in carbohydrate fermentation in lactose and sucrose medium, liquefaction of gelatin and stormy clot fermentation in milk. In case PCR reaction, two samples showed 800bp product size in 2% agarose gel. Bulk PCR was performed with positive samples and purified with gel extraction kit (Qiagen) submitted for eurofin sequence Bangalore India. BLAST analysis of sequence showed that it matches with *Clostridium botulinum* (99% query coverage and 78% sequence identity, Accession no. CP009267) and also other *Clostridium* sps. Botulism is a fatal neurological disease caused by ingestion of preformed exotoxin produced by *Clostridium botulinum*. Domestic and wild animals are susceptible to botulism.

The organisms grow well in the decaying carcasses of mice, cat and birds and cause outbreak with high mortality (Smith and Turner, 1989 and Smart *et al.* 1987) reported botulism in cattle associated with poultry litter consisting of poultry excreta, wood shavings and occasional broiler carcasses that was spread onto the pastures which contaminated the big bale silage being fed to the cattle. Gray (1982) reported an outbreak of botulism in dairy cattle fed with high moisture whole corn, alfalfa and rye haylage from pit silos. Ingestion of contaminated dead carcass and bone predisposed the cattle for botulism (Jones, 1996). Carrion-mediated botulism (Radostits *et al.*, 2009), outbreak of botulism due to ingestion of carcasses / broiler litter (Otter *et al.*, 2006; Kennedy and Ball, 2011; Payne *et al.*, 2011; Kummel *et al.*, 2012) or silage contaminated with poultry litter including carcasses (Hogg *et al.*, 1990) could occur. Molecular detection of type C and type D and their mosaic BONT genes were utilized by PCR (Nakamura *et al.*, 2010).

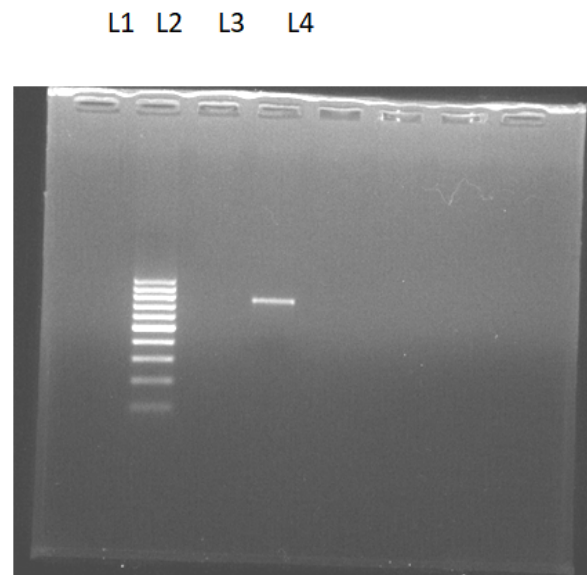


Figure 1.

L2 – 100 bp Ladder

L3 – Negative Control

L4- Qiagen kit purified 2.5 µl PCR product shows amplicons size of 800 bp in 2% agarose gel.

L1 L2 L3

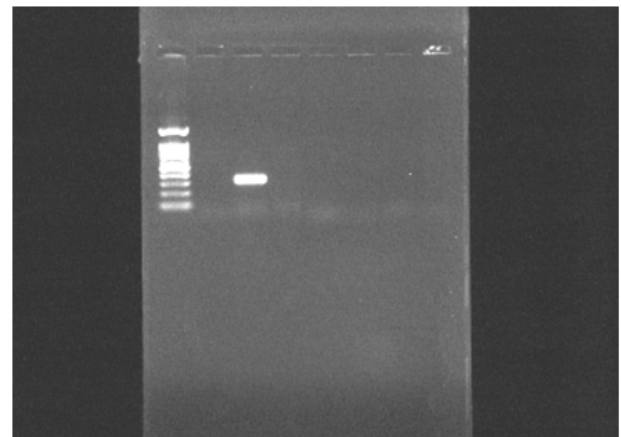


Figure 2.

L1 – 200 bp ladder

L2 – Negative Control

L3- Unpurified 8 µl PCR product shows amplicons size of 800 bp in 2% agarose gel.

Non toxic component of genes in type C and type D strains will be useful for epidemiological studies of botulism. Very difficult to identify the bacterium correctly among many other anaerobic bacteria on an agar medium in most of the infected animals. However bovine botulism might be horizontally transmitted from affected bovine dung samples. The prevalence of non proteolytic strains in Swedish cattle showed the possibility that the spore were confined to environment of the bran increasing of recontamination (Dahlenborg *et al.*,2003). Since *Clostridium botulinum* excreted in dung even if cattle have been immunized by Toxoid, it is necessarily to investigate the extent of contamination in pasture using a rapid inspection system for the disease to prevent its spread and several researcher developed an immunological detection methods from dung and rumen liquor samples for the diagnosis of type C and D botulism (Brook *et al.*, 2001).

In this short preliminary study, bovine *Clostridium botulinum* characterized by polymerase chain reaction but in depth detailed further studies are need.

Acknowledgement

The authors are very much thankful to the Dean, Veterinary College and Research Institute, Namakkal, India for providing fund and necessary facilities to carry out the research.

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