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Original Research

Study of Plasmid Profiling of Clostridium perfringens Isolated from Small Ruminants of Odisha

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Abstract

In the present study 209 intestinal and faecal samples of small ruminants were collected from different farms and localities in and around Bhubaneswar, Odisha. Out of which 159 samples were of goats and 50 samples of sheep, it includes both intestinal and faecal samples. The collected samples were processed for plasmid profiling of C. perfringens. A total of 19 samples were found positive for C. perfringens indicating the prevalence of 9.09% in small ruminant population of Odisha. In order to study the epidemiological strain variation, all the present isolates were subjected to plasmid profiling. Plasmid profiling of the local isolates is an important tool for strain related identification of disease in sheep and goat. In this study plasmid DNA yield was low, as for high yield large amount of fresh C. perfringens culture was needed, but an attempt has been carried out for plasmid curing. Effect of various parameters like different percentage of gel, running buffer and temperature were done and it was found that 0.7% agarose gel, TBE buffer at 30°C of temperature were optimum condition for plasmid profiling. The present study showed that DNase activity remains a block in the plasmid isolation and profiling of Clostridial species. Therefore, need for a DNase inhibitor that may increase the efficacy of plasmid curing.

Keywords: Clostridium perfringens, Plasmid Profiling, Phenol-Chloroform Method, Small Ruminant

Introduction

In Clostridial species, plasmid(s) have been shown to be responsible for several characters. The presence of plasmid(s) in *C. perfringens* has been documented and they have been shown to mediate bacteriocin production, antibiotic resistance and possibly Beta and Epsilon toxin synthesis. According to Mahony *et al*. (1986), study of plasmid can be useful in differentiating strains of *C. perfringens* and as reported plasmid typing is an alternative tool for serological typing of *C. perfringens* during epidemiological outbreaks like food poisoning, diarrhoeal diseases and other infections. The first studies on plasmid isolation technique were described by Marmur (1961). A wide range of methods for plasmid isolation are now available but their applicability differs with the nature of the organism. The advantage is taken of some features which are basic to these methods: (1) treatment of cells with Tris-sucrose buffer (2) treatment of the cells with lysozyme to make cells osmotically sensitive which affect the integrity of the outer cell wall. (3) Lysis of cell wall with ethylene diamine tetra-acetic acid (EDTA) and some detergent like sodium dodecyl sulphate (SDS), sarkosyl. This treatment results in release of total cellular DNA. The mixture of chromosomal and plasmid DNA is subsequently subjected to shearing force so that the chromosomal DNA will be fragmented whereas plasmid DNA, owing its smaller size and super coiled nature is left intact. If non-ionic detergent e.g. Brij-58 is used, the bulk of chromosomal DNA remain attached to cellular components and will sediment with the residual cell debris during centrifugation. The DNAs are freed from RNA and proteins by RNase and protease treatment respectively.

Some protocols use selective denaturation and renaturation step by sodium hydroxide and Tris-HCL. But the clostridial organism has been reported to pose problem when subjected to plasmid DNA isolation due to DNase activity in this genus. A good number of protocols have been devised and modified for the purpose of plasmid DNA isolation in clostridial species. Many methods have been used to isolate the plasmid DNA in *Clostridium perfringens*. Alkali lysis methods used by various workers with some modification in *C. perfringens* have been found to yield very good results (Heefner *et al.,* 1984; Magot, 1984; Robert *et al*., 1986 and Mahony *et al.,* 1988). Blaschek *et al.,* (1984) reported that recovery of plasmid DNA from *Clostridium perfringens* 10543A and 3626B cleared that lysates were significantly improved by the addition of 0.2% (v/v) diethylpyrocarbonate (DEP) before protoplast disruption in the cleared lysate protocol. Roberts *et al*. (1986) have compared various methods of plasmid DNA isolation for *C. absonum.* The authors found that treatment ratio (culture to lysis volume) was critical factor for good results. Mahony *et al*. (1986) has described two approaches (Method-I - phenol chloroform extraction method and Method-II - sodium dodecyl sulphate method) for rapid extraction of plasmid from *C. perfringens* without using density gradient centrifugation. Schijman *et al*. (1987) have developed a rapid method for screening of plasmid DNA form *C. acetobutylicum*. The protoplastization has been accomplished by lysozyme, nuclease inhibition by EDTA or DEP followed by lysis with high concentration of SDS. In this study the *C. perfringens* isolates collected from small ruminants were used to study the effects of various parameters like different percentage of gel running buffer and temperature.

Materials and Methods

In the present study 209 samples of sheep and goats were collected from different farms and localities in and around Bhubaneswar, Odisha. It includes intestinal (n = 84) and faecal samples (n = 125) of small ruminants *i.e.* 159 samples of goats and 50 samples of sheep. The collected samples were processed for *C. perfringens* and total of 19 samples i.e. 11 samples of sheep and 8 samples of goat were found positive indicating the prevalence of 9.09% in small ruminant population. A standard "Classical enterotoxaemia *Clostridium perfringens* Type D sheep strain" was obtained from Division of Biological Standardization, I.V.R.I., Izatnagar, U.P. as a reference strain for the present study. All strains were maintained in prewarmed Robertson's Cooked Meat (RCM) Broth and incubated at 37^{o}C for 12 hr. The culture was checked for aerobic contamination on blood agar slant incubated at 37^{o}C for 48 hr. The purity of the culture was determined by examination of smears stained by Gram's Method. The plasmid DNA isolation and effect of various parameters on plasmid profiling were carried out in the laboratory of Division of Biological Standardization, I.V.R.I., Izatnagar, U.P. The stock culture was revived in prewarmed RCM broth for about 12 hours at 37^{o}C having 1 % inoculum. The actively growing (1%) culture was inoculated in 100 ml of prewarmed BHI broth containing Gentamycin 10 µg/ml. This was incubated anaerobically till an OD of 1 at 600 nm was achieved. 2 to 4 hours of grown culture was taken and cells were collected by centrifugation at 5000 rpm for 30 minutes at 4^{o}C. The supernatant was aspirated completely. The pellet was weighed and processed for plasmid DNA isolation.

Phenol-chloroform method described by Mahony *et al*. (1986) was slightly modified and used for isolation of

plasmid DNA from various amount (1.5ml, 10ml, 30ml, 50ml, 100ml, 500ml) of culture. The organisms were grown in 100 ml BHI broth. 2 to 4 hours of grown culture was taken, cells were collected by centrifugation at 5000 rpm for 30 minutes at 4ºC. Pellet was resuspended in 9 ml of TES (0.05 TrisM HCL, 0.005M EDTA, 0.15M NaCl-pH 7.4). 1 ml of hyaluronidase (1mg/ml) was added and incubated at 37ºC for 1 hour. Samples were centrifuged at 10,000 rpm for 10 minutes at room temperature and pellet was suspended in Tris sucrose buffer (0.05M Tris HCL, 0.001M EDTA-pH 8.0, 25% sucrose). 440µl lysozyme (1mg/ml) was added and incubated for 10 minutes at 37ºC followed by incubation in ice bath (0ºC) for 20 minutes. 630µl of Tris-EDTA buffer (0.05M Tris HCL, 0.04M EDTA-pH 8.0) was added and incubated for 10 minutes at room temperature. 5ml of sarcosyl buffer (0.05M Tris HCL, 0.005 M EDTA, 5% sodium lauryl sarcosinate (sarcosyl) (pH 8.0) was added and incubated at 0ºC for 20 minutes. 10 ml of Phenol-Chloroform (1:1) was added, mixed and spun at 8000 rpm for 30 minutes. To the upper aqueous layer, equal volume of chilled isopropanol was added and kept at -20ºC overnight. Plasmid DNA was recovered by centrifugation at 10,000 rpm for 30 minutes at 4ºC. Pellet was washed once with 70% ethanol followed by washing with 100% ethanol and was vacuum dried and suspended in 100µl of TE buffer. 0.7% agarose gel was used for electrophoresis and gel was run at 40 volts for 5 to 8 hours in TBE (running buffer). The agarose gel was prepared by boiling agarose in 1x TBE buffers. The gels of different dimension were casted as per requirement. Samples were mixed with loading buffer (1x final conc.) and loaded in the slot with the help of a Gilson pipette. DNA marker was run along the side of the sample. The gel was run at a constant voltage of 40 volts at room temperature. For staining purpose ethidium bromide at final concentration of 0.5 µg/ml was added in the gel tank. Then the gel was destained in double distilled water for 30 minutes and visualized under UV transilluminator. Effect of various parameters like different temperature, percentage of gel and various running buffers were observed during plasmid profiling and results are recorded.

Result and Discussion

Various pathogens have been reported for having single or multiple plasmid profile including cryptic plasmids. Plasmid of various molecular weights ranging from 1.6 kb to 113 kb have been reported in *Clostridial* species and this had promoted us to investigate the plasmid profile of the local strain which is responsible for important disease of small ruminants such as sheep and goats. In this present study cells were harvested from BHI broth medium at an OD of 1 to 1.2. For this mid log phase cells were chosen for plasmid isolation keeping in view the reports that cells harvested from late log phase and stationary phase gave poor result due to problem of lysis of cells in *Clostridial* species (Weickert *et al*., 1986; Lee *et al*., 1987). Storm *et al*. (1984) reported that for isolation of plasmid DNA from certain *C. botulinum* strains, cells would not lyse by any of the given protocols, once they reached the stationary phase. The yield obtained was 15 mg/ml of the culture (wet wt.). As plasmid isolation from saccharolytic Clostridia proved to be difficult due to DNA degradation by high activity of endogenous nucleases (DNase), therefore in present study Phenol-chloroform method described by Mahony *et al*. (1986) was slightly modified and used for higher recovery of plasmid DNA. The cells were washed with TES buffer before processing for plasmid DNA isolation, may help in getting rid of extracellular DNase activity, if any. It was reported that 26.8% of DNase to be extracellular in *C. perfringens* 3626B (Blaschek and Klacik, 1984). And the ratio of volume of the cells to lysis buffer remains low. Chloroform mixed with phenol is more efficient at denaturing proteins than either reagent alone.

The phenol-chloroform method reduces the partitioning of poly(A)+mRNA into organic phase and decrease the formation of insoluble RNA-protein complexes at the interphase (Perry *et al*., 1972). Different volumes of culture 1.5ml, 10 ml, 30 ml, 50 ml, 100ml and 500ml, were used. Two to three trials were attempted for getting better results. When the gel was run at 40 volts, a total of five very faint and diffused bands were observed (Fig. 1 & 2). Three bands above and two bands below the chromosomal DNA in the three hours grown culture were observed. The bands which were visible above and below the chromosomal band in three hours grown culture were very faint due to diffusion of the bands. This is in agreement with the methods and findings of Roberts *et al*. (1986). The isolated plasmid DNA samples when stored at 4ºC overnight and run again, showed degradation. So, the present finding was in agreement with Lee *et al*. (1987) as reported that two to three days storage of plasmid DNA from *Clostridial* species resulted in complete degradation. Svaren *et al*. (1987) have reported that denaturation of DNA occur upon drying after ethanol precipitation. An attempt was also made to amplify the plasmid DNA by cultivating the cells in the presence of chloramphenicol. The gel was run at 40 volts for five hours and very diffuse bands were observed. Gels of different percentage 0.5%, 0.7% and 0.8% were used for running the plasmid DNA samples. 0.7% agarose gels were found more suitable although better result were obtained in 0.5% gels but 0.5 % gels were difficult to handle. Different running buffers (TBE, TAE and TEAS) were compared and better results were obtained in TBE.

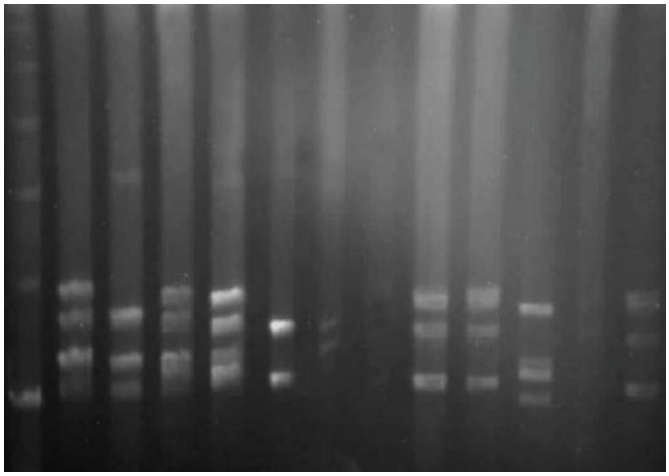

Figure 1: Plasmid DNA of *C. perfringens* showing faint bands on Agarose gel Electrophoresis

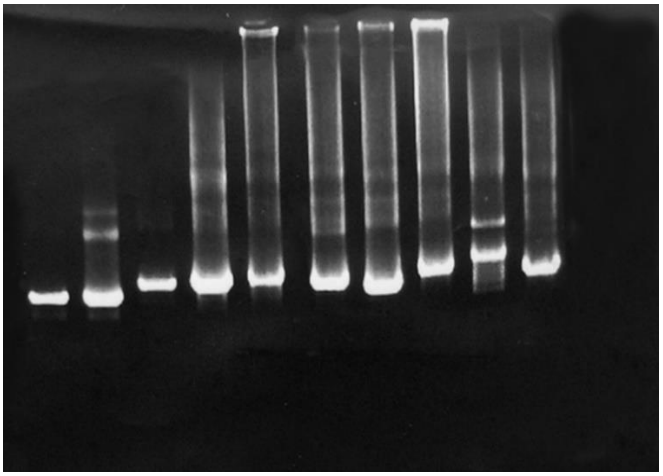

Figure 2: Plasmid DNA of *C. perfringens* showing defused bands on Agarose gel Electrophoresis

A lot of diffusion was observed when the gel was run in Tris acetate EDTA (TAE) or Tris EDTA acetate sodium chloride (TEAS) buffer. The effect of temperature on plasmid profile was studied by running the gel at ambient temperature of 30ºC to 35ºC, 25ºC, 15ºC, 10ºC, and 4ºC to 8ºC. This was done to check the DNase activity. When the gels were run at higher temperature of 30º C to 35º C more DNase activity was observed as smear type of picture was seen. Diffused type of bands were observed after one to two hours of run. When the gel was run at lower temperature better results were observed.

Conclusion

In the present study plasmid DNA was isolated and aimed to detect the potential of plasmid profiling as a measure for the distribution of epidemiologically related and unrelated strains of *C. perfringens* isolated from different parts of Odisha. Phenol-chloroform Method described by Mahony *et al*. (1986) was slightly modified and used for isolation of plasmid DNA from *C. perfringens*. The molecular weight of the isolated plasmid(s) was compared with the reference strain obtained. Molecular weight of plasmid isolated from intestinal isolates of *C. perfringens* ranges from 45 Kb to 195 Kb and showed five bands whereas there was variation of 2 or more bands in faecal isolates. In the study plasmid cured from the local isolates of *C. perfringens* from Odisha showed slow growth and less number of bands in comparison to reference strain obtained from Division of Biological Standardization, I.V.R.I., Izatnagar, U.P. A great difficulty was felt during isolation and profiling of plasmids due to high DNase activity and poor stability of the plasmid(s). So, the method of Mahony *et al*. (1986) was attempted to isolate the plasmid DNA for better results. The study showed that apparent DNase activity remains a stumbling block in the way of an investigator dealing with Clostridial species and there is need for a DNase inhibitor which would work to the absolute satisfaction. Hence, molecular characterisation of the local isolates is needed for the study to develop a locally isolated strain specific vaccine against enterotoxaemia in small ruminants.

Conflict of Interests

There is no conflict of interest.

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