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Influence of Growth Stages and Nutrient Media on Production of Membrane Vesicles of *Clostridium perfringens* Type A and their Molecular Characterization

Deuri Nirab Kumar[1*], Sharma Kr. Rajiv[2], Borah Probodh[1], Hazarika Parishmita[2], Sonowal Joyshikh[1], Borguhain Indrani[1] and Pathak Bedanta[1]

[1]Department of Animal Biotechnology, [2]Department of Veterinary Microbiology, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati-781022, India

**Corresponding author*

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A B S T R A C T

All Gram-negative bacteria and a few gram-positive bacteria shed membrane vesicles in suitable medium and environmental condition. The present study was undertaken to determine the influence of stages of growth and nutrient media on production of membrane vesicles (MVs) of *Clostridium perfringens* type A (*C. perfringens* type A), and molecular characterization of the same. An isolate of *C. perfringens* belonging to type A was selected and grown in Trypticase Peptone Glucose broth (TPGB) and Robertson's Cooked Meat broth (RCMB) media. The MVs were extracted at 4, 8, 12 and 24 hours of growth. The protein profile based on SDS-PAGE revealed the appearance of six bands at 4 and 8 hrs of growth, with one prominent band and nine bands at 12 hrs of growth on TPGB. No bands were observed in MVs extracted at 24 hrs of growth in TPGB. The molecular weight of the protein bands ranged from 43.3 kDa to 75.2 kDa. The MVs extracted from RCMB medium revealed the appearance of six protein bands at 4 and 8 hrs and nine bands at12 hrs of growth with six prominent bands. The 24 hrs growth culture in RCMB also revealed no bands in SDS-PAGE. The RCMB protein bands also ranged from 43.3 kDa to 75.2 kDa. The DNA content of MVs was demonstrated by PCR and presence of DNA 16S rRNA and *cpa* (alpha toxin) genes in MVs was confirmed. Agarose gel electrophoresis revealed two distinct bands of 795bps and 324 bps of 16S rRNA and *cpa* (alpha toxin) genes, respectively.

Introduction

The clostridia are saprophytes and ubiquitous, large gram-positive bacteria, which are anaerobic, fermentative, catalase-negative, spore-forming, rod-shaped and require enriched media for growth. They are found mainly in the habitat of organic compounds, including soil sewage, sediments, intestinal tract of humans and animals[1]. *Clostridium perfringens* produces over 15 different toxins, out of which only four are identified as major toxins, *viz.* alpha (α), beta (β), epsilon (ε) and iota (ι), while the others are considered to be

minor toxins[2]. On the basis of the major toxin production, either alone or in combination, *C. perfringens* is classified into five toxin types, *viz.* A, B, C, D and E[3, 4]. The organism is associated with diseases like haemorrhagic abomastitis and haemorrhagic diarrhoea in calves and foals[5], necrotic enteritis and necrotic enterotoxaemia in pigs[6], pulpy kidney disease in sheep of all ages[7], gas gangrene in animals and human beings[8], necrotic enteritis and cholangio-hepatitis in poultry[9], and necrotic enteritis and food poisoning in children[10].

Membrane vesicles (MVs) are spherical, bilayered structure, 20-250nm in size, and produced by the gram-negative as well as gram-positive bacteria in non-replicative form. They are complex, chemically heterogeneous bilayered structure[11]. Secretion of membrane vesicles is an evolutionary conserved and universal process that occurs from simple to multicellular organisms. The MVs are produced by the organisms*in-vitro*[12] or *in-vivo*[13]condition during their cell growth[14]. Membrane vesicles contain important surface antigens, such as peptidoglycan, lipopolysaccharide, lipoprotein, membrane protein, cytoplasmic content, periplasmic content, DNA, RNA (tRNA, rRNA), cytokines and enzymes[15]. Production of the bacterial MVs is reported to be dependent on different factors, e.g. cultivation time, growth media, physical and chemical stress[16].

The aim of this study was to determine the molecular characteristics of the membrane vesicles of *C. perfringens* type A and the effect of nutrient media and stages of growth on production of membrane vesicles in *in-vitro* condition.

Materials and Methods

Bacterial strain: Five isolates of *C. perfringens* were obtained from the repository of Department of Veterinary Microbiology, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati-22. All the five isolates were reconfirmed by inoculating onto 5 percent (v/v) sheep blood agar to observe the typical smooth, round and glistening colonies surrounded by inner zone of complete haemolysis and outer zone of partial haemolysis as described by Baldassi *et al.*, (2002). All experiments were performed anaerobically in the anaerobic jar with anaerogas pack system (Hi-Media, Mumbai, India) at 37°C for 24 hrs. Further confirmation of the isolates was done based on cell morphology and staining characteristics by staining the smears with Gram's strain.

Molecular confirmation by PCR: All five isolates were screened for the virulent genes, *cpa* (alpha toxin), *cpb* (beta toxin), *etx* (epsilon toxin) and *iA* (iota toxin) by simplex PCR as per the method described by Titball *et al.*, (1989) and Hunter *et al.*, (1992).The template DNA was extracted by inoculating the *C. perfringens* into 5ml of Brain Heart Infusion (BHI) broth (Hi-Media, Mumbai, India) by hot cold lysis procedure as per the method described by Titball *et al.*, (1989). The supernatant was directly used as template DNA for PCR for screening of virulence genes using specific primers (Table 1). PCR was carried out with 25 µl reaction volume containing 12.5 µl of 2X PCR master mix (Thermo Scientific), 0.05 units/µl Taq DNA Polymerase, reaction buffer, 4 mM magnesium chloride, 0.4 mM of each dNTP (dATP, dCTP, dGTP and dTTP); 0.5 µl each of forward and reverse primers (10 picomol each) and 2 µl of template DNA. Sufficient amount of nuclease-free water was added to make the final volume of 25 µl. The cyclic condition used was 94°C for 4 min, 35 cycles of 94°C for 1 min, 55°C for 1min, 72°C for 1 min followed by 72°C for 10 min in a thermal cycler (Bio-Rad, USA).

Extraction and purification of membrane vesicles: Membrane vesicles of *C. perfringens* type A were extracted from the inoculated broth cultures of Trypticase Peptone Glucose Broth (TPGB) and Robertson's Cooked Meat Broth (RCMB) grown for different periods, as per the methods described by Gurung *et al.*(2011) and Jiang *et al.*(2014) with minor modifications. Briefly, a pure colony of *C. pefringens* type A was taken and grown overnight in two tubes of 20 ml BHI broth each. The samples were then centrifuged at 5000 rpm for 5 minutes. After centrifugation, the supernatants were discarded and cell pellets were resuspended in 5 ml of PBS solution. The resuspended cell pellet from the respective tube was transferred to 50 ml each of TPGB (Hi media, Mumbai, India) and RCMB (Hi media, Mumbai, India). The tubes were incubated anaerobically at 37°C for different periods, *i.e.* 4, 8, 12, and 24hrs. Bacterial cells were removed from the culture supernatants of each growth condition by centrifugation at 16,000 × g for 20 min. The supernatants were passed through a polyvinylidene difluoride filter (Milipore) of 0.45 µm pore size to ensure complete removal of bacterial cells. Vesicles were pelleted by initial centrifugation at 40,000 × g, 2 h, 4°C and washed twice with Phosphate-Buffered Saline (PBS). The washed pellets were resuspended in sufficient PBS and repelleted with final centrifugation at 110,000 × g for 4 hours at 4°C and the pellets were collected as MVs. The MVs pellet was resuspended in 1000 µl of sterile PBS (pH 7.4).

Extracted MVs were subjected to Tri-chloro Acetic Acid precipitation for partial purification. Precipitation was done in 1:4 ratio (tri-chloro-acetic acid: samples) as per the methods described by Jiang *et al.,* (2014) in standing position for 10 minutes in 4°C. The suspensions were centrifuged at 10,000 rpm for 5 minutes and the sediments were washed twice with 200 µl ice cold acetone. The washed samples were air-dried and dissolved in 500 µl of PBS and stored at -20 ^{o} C as MVs samples. Each extracted MVs of *C. perfringens* type A under the influence of different growth conditions was subjected toestimation of total protein as per the method of Lowry *et al.,* (1951).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE): The MVs extracted from cultures of *C. perfringens* type A was carried out for protein profiling by SDS-PAGE as per the method of Laemmli (1970) using 12.0 percent acrylamide gel. The resolving and stacking gel concentrations used for the present study were 12.0 and 4.0 percent, respectively.

The samples along with the pre-stained protein ladder were electrophoresed at 15 mA (constant) current, until the bromophenol blue dye reached the bottom of the resolving gel (~120 mins). After completion, the gel was stained with Coomassie brilliant blue stain overnight in dancing shaker, followed by de-staining in shaker, till the background of the gel became clear leaving the blue coloured protein bands. The SDS-PAGE gel was visualized in gel documentation system (Gel Doc, BIO-RAD, XR+) for detection of number of protein bands with their molecular weights.

DNA extraction from membrane vesicles: DNA was extracted from the MVs of *C. perfringens* type A using the method described by Pitcher *et al.,* (1989) with slight modification. The MVs were treated by GES (5M Guanidium thiocyanate, 100mM EDTA, and 0.5% sarkosyl) reagent (Lysis Buffer) to release the DNA associated with MVs. The MVs were washed with phosphate buffered saline (PBS) and centrifuged again at 110,000 × g for 2 hrs at 4°C. The pelleted MVs were suspended in 500 µl of GES reagent in a 1.5 ml micro-centrifuge tube and kept standing at

room temperature for 5 min. The lysates were cooled on ice for 10 minutes with 0.25 ml of cold 7.5M ammonium acetate. After 10 min of incubation on ice, 0.5 ml of Phenol: Chloroform: Isoamyl alcohol (25:24:1) was added, mixed and centrifuged for 15 min (16,000 × g). The supernatant (500µl) was transferred to a fresh micro-centrifuge tube and nucleic acids were precipitated overnight with 50 µl of 3M sodium acetate and 1ml of ice-cold ethanol (96.0%). After overnight incubation, the suspension was further centrifuged at 16,000 x g for 15 min and the DNA pellets were washed with 70 percent ethanol by centrifugation at 16,000 × g for 15 minutes. The final pellets were dried and resuspended in 50 µl nuclease-free water.

Polymerase Chain Reaction (PCR): The DNA extracted from MVs was used as a template for PCR for amplification of 16S rRNA and *cpa* (Alpha toxin) genes using primers (Table 2) as per the methods described by Titball *et al.,* (1989) and Kikuchi *et al.,* (2002).The amplification was carried out in 25 µl reaction volume containing 12.5 µl of 2X PCR master mixes (Thermo fisher scientific, India), with 0.5 µl of each primer (10 pico-mol each), 3µl of template DNA and nuclease-free water to make volume up to 25 µl.

The PCR reaction was performed with the PCR condition of 94°C for 2 min, 35 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 45 s followed by 72°C for 2 min in a thermal cycler (Bio-Rad, USA). The PCR amplified products were separated by electrophoresis at 80 V for 1 hr with 1.5% (w/v) agarose gel containing ethidium bromide (10 mg/ml) in ×1X Tris-Acetate -EDTA along with gene ruler 100 bp DNA ladder (Thermo scientific) as a molecular weight marker, and visualized as a single compact band of expected size under ultraviolet light in gel documentary system (BIO-RAD, XR+).

Results and Discussion

Based on the morphological and staining characteristics, all the five isolates were tentatively identified as *C. perfringens*, while the molecular confirmation was done by simplex PCR, which revealed the presence of alpha toxin associated *cpa* gene (324 bp) in the isolates (Fig. 3). Among the five *cpa* bearing isolates, *cpb* gene (180 bp) coding beta toxin could be detected in two isolates (Fig. 4). None of the isolates was found to possess *etx* (epsilon toxin) and *iA* (iota toxin) genes. Based on the presence of virulence genes, either alone or in combination, three of the five *C. perfringens* were identified to be of toxin type A (with *cpa* gene alone). The other two isolates were confirmed as *C. perfringens* type C with the presence of *cpa* and *cpb* genes. Thus, the *C. perfringens* bearing only *cpa* gene was selected for further study.

The protein concentration in the MVs extracted from the growth in TPG broth after4 hrs of incubation was found to be 5.54 mg/ml, while MVs released from growth of RCMB at the same incubation period revealed 4.98 mg/ml. The increasing trend in protein concentration was observed till 12 hrs of incubation in both TPG broth (10.84 mg/ml) and RCMB (10.02 mg/ml). However, the concentration decreased on 24 hrs of incubation (2.43 mg/ml) in TPG broth. Similar decreasing trend was also observed in MVs extracted from the growth in RCMB (0.89 mg/ml).

The protein profiling of the MVs extracted from *C. perfringens* growth in both TPG broth and RCMB could reveal almost similar pattern of protein profile in respect to the number of protein bands with their molecular weight (Fig. 1 and 2). Irrespective of type of nutrient medium, the MVs extracted from the growth with 4 hrs and 12 hrs of incubation could reveal six protein bands with mol. wt. ranging

from 49.0 to 100.0 kDa, only one band among which was prominent with mol. size of 51.0 kDa. On the other hand, the protein profile of MVs extracted from growth of both the nutrient mediaafter12 hrs of incubation exhibited nine different bands with mol. wt. ranging from 49.0 to 130.0kDa. Among these bands, seven with mol. wt. of 43.3 kDa, 44.1 kDa, 45.5 kDa, 58.3 kDa, 60.8 kDa, 70.9 kDa, and 75.2 kDa were found to be prominent. The SDS-PAGE study could not show any band pattern in the MVs extracted from both the nutrient media after incubation for 24 hrs. The Membrane vesicles (MVs) extracted from the *C. perfringens* type A were subjected to molecular characterization in respect to the escape of chromosomal DNA, either whole or partial. The escape of chromosomal DNA during release of MVs from *C. perfringens* type A was established by the detection of 16s rRNA and *cpa* genes by simplex PCR. The present study showed presence of 16s rRNA (795 bp) and *cpa* (324 bp) genes in the amplified product (Fig. 5).

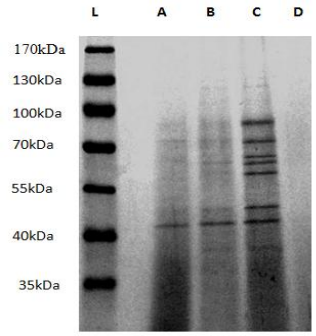
Table.1 Details of primers used for PCR reaction

Primers	Sequence (5'-3')	Product size	Reference
cpa(F)	5-GCTAATGTTACTGCCGTTGA-3	324bp	Titball *et al.*, (1989)
cpa(R)	5-CCTCTGATACATCGTGTAAG-3		
cpb(F)	5-GCGAATATGCTGAATCATCTA-3	180bp	Hunter *et al.*, (1993)
cpb(R)	5-GCAGGAACATTAGTATATCTTC-3		
etx(F)	5-GCGGTGATATCCATCTATTC-3	655bp	Hunter *et al.*, (1992)
etx(R)	5-CCACTTACTTGTCCTACTAAC-3		
iA (F)	5-ACTACTCTCAGACAAGACAG-3	446bp	Perelle *et al.*, (1993)
iA (R)	5-CTTTCCTTCTATTACTATACG-3		

Table.2 Details of primers used for PCR reaction of MVs

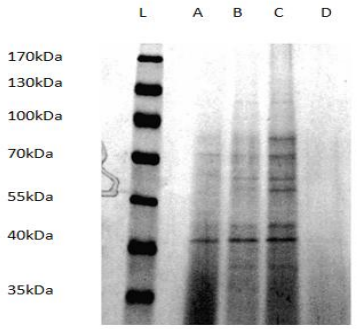
Primers	**Sequence (5'-3')**	**Product size**	**Reference**
***16SrRNA*(F)**	5'-AGATGGCATCATCATTCAsAC3'	795 bp	Kikuchi *et al.*, (2002)
16SrRNA(R)	5'-GCAAGGGATGTCAAGTGT-3'		
cpa(F)	5'-GCTAATGTTACTGCCGTTGA-3'	324 bp	Titball *et al.*, (1989)
cpa(R)	5'-CCTCTGATACATCGTGTAAG-3'		

Fig.1 SDS-PAGE protein profile of MVs of *C. perfringens* type A grown in T.P.G.B



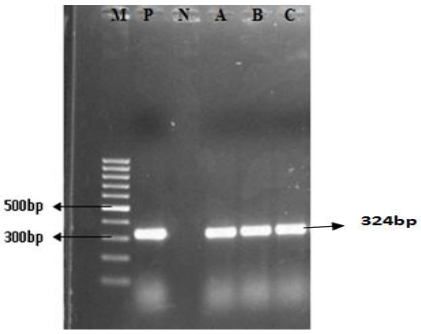
Lane L = Protein ml. wt. marker
Lane A, B, C, D = MVs extracted from growth of 4, 8, 12 and 24 hrs. incubation

Fig.2 SDS-PAGE protein profile of MVs of *C. perfringens* type A grown in R.C.M.B



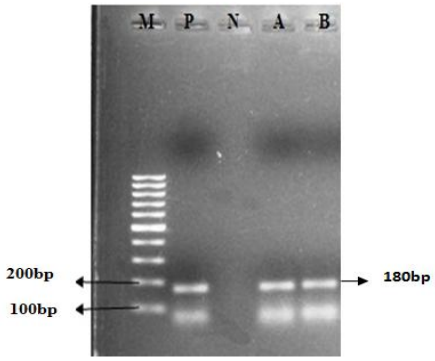
Lane L=Protein ml. wt. marker
Lane A, B, C, D = MVs extracted from growth of 4, 8, 12 and 24 hrs

Fig.3 Gel electrophoresis of *cpa* gene encoding alpha toxin (324bp) in *C. perfringens*amplified by simplex PCR



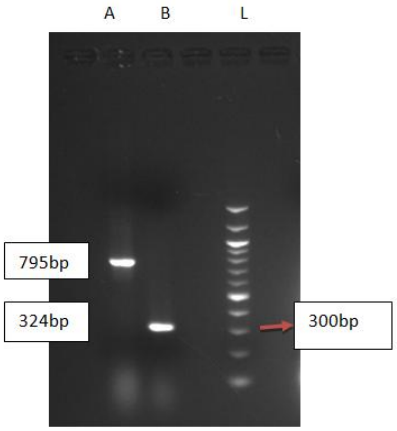
Lane A, B, C : Samples positive for *cpa* gene
Lane M : 100 bp DNA ladder (thermo scientific)
Lane N : Negative control
Lane P : Positive control

Fig.4 Gel electrophoresis of *cpb* gene encoding alpha toxin (180bp) in *C. perfringens* amplified by simplex PCR



Lane A,B : Isolates from repository
Lane M : 100bp DNA ladder
Lane N : Negative control
Lane P : Positive control

Fig.5 Detection of 16SrRNA and *cpa* genes in extracted MVs of *C. perfringens* type A by agarose gel electrophoresis



Lane A = 16SrRNA (795bp)
Lane B = *cpa* (alpha toxin) gene
Lane L = 100bp DNA Ladder

During the present study, the protein concentration as well as the number of bands was observed to be maximum at 12 hours, followed by 4 and 8 hours, respectively. The present observations on trend of protein concentration was similar to the observations reported by Brown *et al.,* (2014) on MVs of *Bacillus subtilis* strain 168, in which increased release of MVs from the bacterial strain was observed with an increase in time of incubation till 12 hrs. They reported the production of MVs to be leveled off after 12 h of growth. Similar to the present observations on influence of nutrient media on spontaneous release of MVs, Jiang *et al.,* (2014) could not observe any influence of different nutrient medium on the release of MVs of *C. perfringens.* In a similar study, Haas *et al.,* (2015) on *Streptococcus suis* reported an average recovery of MVs per liter of overnight culture, which corresponded to approximately 300µg of vesicular protein content. Based on their study, they opined that maximum recovery of MVs could be possible only from live cultures.

Based on the present findings, it can be opined that maximum release of MVs could be possible during the mid or late exponential growth of *C. perfringens* type A. However, before giving a conclusive remark in this aspect, further study has to be carried out involving large number of different strains under different nutritional environments.

The membrane vesicles (MVs) extracted from the *C. perfringens* type A were characterized in respect to the release of chromosomal DNA, either whole or in partial along with the release of MVs. The escape of chromosomal DNA during release of MVs from *C. perfringens* type A was established by detection of 16s rRNA and *cpa* gene in the MVs by simplex PCR DNA. The study could reveal clear distinct bands of 795bp and 324bp size in the PCR product. This was an indication of release of 16S rRNA (795bp) and the alpha toxin associated *cpa* gene (324bp). Contrary to the present observation on release of chromosomal DNA along with MVs of *C. perfringens* type A, Dorward *et al.,* (1990) reported that the extracellular vesicles produced by certain gram-positive bacteria like *Bacillus*, *Streptococcus* and *Staphylococcus* were devoid of DNA content, while the Outer Membrane Vesicles (OMVs)

of gram-negative bacteria, *viz*. *Pseudomonas* and *Salmonella* showed the presence of chromosomal DNA. On the other hand, Liao *et al.,* (2014) reported the presence of DNA in the extracellular vesicles of gram-positive bacteria.

Gram-negative bacteria MVs were reported to be present of either chromosomal origin or plasmid origin DNA. The present observation on the release of chromosomal DNA, either complete or partial in the MVs of *C. perfringens* type A was in agreement with the findings of Renelli *et al.,* (2004). They could detect both externally associated chromosomal DNA and internally or externally associated plasmid DNA in the MVs of *Pseudomonas aeruginosa*. In a similar study, Jiang *et al.,* (2014) also reportedthe partial release of chromosomal DNA in the MVs.

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