ISOLATION, IDENTIFICATION AND CHARACTERIZATION OF *CLOSTRIDIUM PERFRINGENS* FROM COOKED MEAT-POULTRY SAMPLES *AND IN SILICO* BIOMODELING OF ITS DELTA ENTEROTOXIN

[1]Sinosh Skariyachan*, [1]Arpitha B Mahajanakatti,[1] Usha B Biradar,[1] Narasimha Sharma, [2]Abhilash M
1. Department of Biotechnology, Dayananda Sagar College of Engineering, Bangalore.
2. UST Global, Technopark, Trivandrum.
***Email:** sinoshskariya@gmail.com

ABSTRACT

Improper cooking of meat and poultry products may cause food borne gastroenteritis by *Clostridium perfringens* due to its enterotoxin. In this study we isolated *Clostridium perfringens* from cooked meat, pork and poultry samples from different areas of Coimbatore District, Tamilnadu, India. The presumptive, confirmed and completed characterization showed that isolated organism was *Clostridium perfringens* and it produces various toxins during sporulation. The toxin detected by simple immunodiffusion and various temperatures treatment showed its hyperthermophlicity. The antibiogram has indicated resistance of the organism to conventional antibiotics hence *in silico* study is significant. Comparative modeling of the δ-enterotoxin, most important toxin in gastroenteritis, was carried out with its protein sequence present in NCBI as no crystallographic structure of the same is available. PSI-BLAST analysis indicated the δ-toxin showed perfect homology (31% identity and 51 % similarity) with Staphylococcal β-hemolysin. This has confirmed by MSA and phylogentic analysis. The ORF has 1300 coding frames and predicted secondary structure consist of mainly randomcoil. The model refinement and validation is done by various empirical force fields and RMSD value, 1.43 A^{o}, showed that the model is good. Most residues of the toxin are located in allotted regions of Ramchandran plot. The modeled structure could give novel ideas for molecular docking, development of new agonist and potential target for studies of drug- receptor interaction.

Keywords: *Clostridium perfringens*, Delta toxin, *Insilico* biomodeling.

INTRODUCTION

Clostridium perfringens is one of the most common food-borne versatile pathogenic bacteria which have a predominant role and importance in medical and food microbiology. It is the major causative agent of many diseases including food poisoning (gastroenteritis), gas gangrene, skin and soft tissue infections, enterotoxemias in humans and domestic animals, acute and chronic diarrhea in dogs and necrotizing enteritis[1,2]. It is a Gram-positive, rod-shaped, anaerobic, spore-forming bacterium of the genus *Clostridium*. *C. perfringens* is ubiquitous in nature and can be found as a normal component of decaying vegetation, marine sediment, the intestinal tract of humans and other vertebrates, insects, and soil. [3-9]

Most probably the organism acquire through the consumption of contaminated meat and poultry products[10]. Food poisoning caused by *Clostridium perfringens* may occur when food such as meat or poultry are cooked and held without maintaining adequate heating before serving. In such cases the spores of some strains are resistant to temperature even at 100ºC for more than 1 hr, their presence in food may be unavoidable and the oxygen level may be sufficiently reduced during cooking to permit growth of Clostridia. Spores that survive cooking may germinate and grow rapidly in food that is inadequately refrigerated after cooking. [11]

The bacterial toxins are a major cause of diseases since they are responsible for the majority of symptoms and lesions during infection[12,13]. The elucidation of the cellular mechanism of bacterial toxins remains a complex problem, but they appear to share a common mechanism of action such as (i) binding to specific receptors on the plasma membranes of the sensitive cells, (ii) pore-formation, (iii) internalization or translocation across the membrane barrier and (iv) direct secretion. [14,15]

Clostridium perfringens produces numerous toxins and is responsible for severe diseases in humans and animals including intestinal or food borne diseases as well as gangrene. Individual strains produce only subsets of toxins and are classically divided into five toxin types (A–E) based on their ability to synthesize Alpha, Beta, Epsilon and Iota toxins[17] Delta toxin is one of the three hemolysins released by a number of *C. perfringens* type C and also possibly type B strains.

The bacteria and its toxins are involved in several disease like food toxinosis in men[18] septicemia after parturition and abortion, wound infection, pneumonia and empyema[19,20], meningitis[21], corneal wounds[22], mionecrosis and cystitis[23]. In animals, enterotoxemia was observed[24]. The purified alpha toxin can caused serious acute pulmonary disease, as well as vascular leak, haemolysis, thromocytopenia and liver damages. It is expected to be lethal by aerosol. Beta toxin is a lethal necrotizing toxin found in types B and C. The Theta toxin stimulates cytokine release and can cause shock. This toxin can damage blood vessels, resulting in leukostasis, thrombosis, decreased perfusion and tissue hypoxia. It was further showed that Delta toxin is cytotoxic for other cell types such as rabbit macrophages, human monocytes, and blood platelets from goat, rabbit, human and guinea pig[24] In addition, Delta toxin selectively lyses malignant cells expressing GM2, such as carcinoma Me180, melanoma, neuroblastoma, and *in vivo* administration of

Delta toxin to mice bearing these tumors significantly reduces tumor growth[25]. However, the mechanism of cytotoxicity remains unclear, since Delta toxin was reported to not insert into cell membrane and to induce membrane lysis by an unknown process. The molecular characterization and spore forming activity of the *C. perfringens* Delta toxin in lipid bilayer experiments in comparison with *C. perfringens* β- toxin and *Staphylococcus aureus* alpha toxin, two well established pore-forming toxins. Channel formation by Delta toxin was more frequent than by beta toxin.[26-29]

MATERIALS AND METHODS

Isolation and identification of *Clostridium perfringens* from meat and poultry samples

The cooked samples of meat, chicken and poultry were collected from different regions of Coimbatore district (Tamil Nadu) and the samples were homogenized and serially diluted under aseptic conditions. All the dilutions were plated on selective Tryptose-Sulfite Cycloserine (TSC) Agar and incubated at 37^0C for 24 hrs under strict anaerobic conditions (Macintosh Anaerobic Jar with a gas pack system).[30-34] The presumptive detection of isolated bacteria was carried out by Gram staining, capsule staining and cultural characteristics of bacteria in special media such as Fluid thyoglcolate broth, Robinson Cooked meat medium, Blood agar and Iron –Milk medium. The confirmed test is performed by the detection of motility in buffered motility-nitrated medium, gelatin – liquefaction in Lactose – Gelatin Medium[35,36], nitrate reduction in Nitrate Broth and spore formation in Modified Ducan-Strong Sporulation Medium. The completed tests of the isolated culture was done by performing IMViC reactions, Lecithinase activity of bacteria in Lactose –Egg yolk milk agar, production of H_2S in SIM Agar and fermentation of various carbohydrates.

Study of effect of temperature and antimicrobial susceptibility testing of isolated culture

The sporulated culture is prepared by inoculating the bacteria in to a Ducan Strong sporulation broth and boiled at 100^0C. The boiled sample is inoculated to reinforced Clostridial agar and incubated under anaerobic condition. The antimicrobial susceptibity of bacteria is determined by Kirby –Bauer disc diffusion method.

Detection of enterotoxin by immuno diffusion technique.

The isolated culture is inoculated to Ducan Strong Sporulation medium and incubated for overnight. The sporulated culture has centrifuged at 10,000 rpm for 10 minute and the pellets are collected. It was resuspended in buffer and treated with cell destructing enzyme. The culture is centrifuged and supernatant (toxin) was collected. The toxins (antigen) were detected by anti-gasgangrene serum obtained from Bharath Serum and Vaccines Ltd., Mumbai, India.

Sequence retrieval of *Clostridium perfringens* Delta toxin and determination of best homologues

The protein sequence of delta enterotoxin was retrieved from NCBI (Accession number: ACD67884; GI: 188998312) and the similarity searching was performed to detect the best homologues by P-BLAST (http://www.ncbi.nlm.nih.gov/BLAST) and PSI-BLAST.[37]

Multiple sequence analysis and Phylogenetic characterization

Multiple sequence analysis was performed with the target sequence and best homologues using CLUSTAL W2 (http://www.ebi.ac.uk/embl/clustalw2)[-38]. The phylogenetic characterization of the deltatoxin was carried out and the phylogram was generated by TREEVIEW (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html) and NJPLOT (http://pbil.univ-lyon1.fre/software/njplot.html)

Predictive Bioinformatics and Proteogenomic characterization of deltaenterotoxin

The functional sites of the toxin were predicted by GENSCAN (http://genes.mit.edu/GENSCAN.html). The proteomics studies have been conducted using ExPASy Server (http://us.expasy.org/) Such as PROTPARAM (primary structure analysis, http://us.expasy.org/tools/protparam.html), SOPMA (Secondary structure prediction, http://npsa-pbil.ibcp.fr/cgi bin/npsa_automat.pl?page=npsa_sopma.html) and HMMTOP (prediction of transmembrane domain and topology, http://www.enzim.hu/hmmtop/)[39]

Insilico comparative Modeling of Delta Enterotoxin

A BLAST searching was performed to detect the best structural templates in RCSB PDB for the comparative modeling. Since the X-ray crystallographic structure of β--hemolysin of *Staphylococcus aureus*, Chain B (PDB ID: 7ahlB) has shown highest identity and similarity with (31% identity and 51 % similarity) target protein it has been selected as the suitable template. The comparative modeling was performed by MODELLER 9v7[40] (http://www.salilab.org/modeller/) and the quality of the modeled protein is evaluated by SWISSMODEL[41-43,50]**.** The final model is visualized by pyMOL.

Refinement and Validation of Modeled structure

The modeled delta protein is further validated by performing energy minimization with the help of various force fields such as ANNOLEA[45], GROMOS[46] and VERIFY3D[47]. The parameters included the covalent bond distances and angles, steriochemical validation, atom nomenclature were validated using PROCHECK[48]. Target model is then superimposed with the template and RMSD was calculated. The modeled structure is visualized by pyMOL and MOLMOL.[49]

RESULTS AND DISCUSSIONS

Isolation and identification of *Clostridium perfringens* from meat and poultry samples

Isolation and identification of *Clostridium perfringens* from the collected samples were successfully done; all the tested samples contained the *Clostridium perfringens* in very less number. (Table-1) This organism produced typical black colonies on the selective TSC plate due to the reduction of sulfite. (Fig: 1) Large gram positive, straight parallel rods were observed under microscopes after Gram staining (Fig.2) and in blood agar, β-haemolytic colonies with double zone of haemolysis was observed (Fig.3) The bacteria showed high lecithinase activity which indicated by opaqueness and halo around the colonies, (Fig.4). This is because of breakdown of casein in the milk by the enzyme proteinase (Proteolysis) and breakdown of lecithin in the egg yolk (lecithinase activity). The isolated culture was characterized biochemically and confirmed that the black colonies formed on the TSC plate was *Clostridium perfringens* and all the results are tabulated (Table-2).

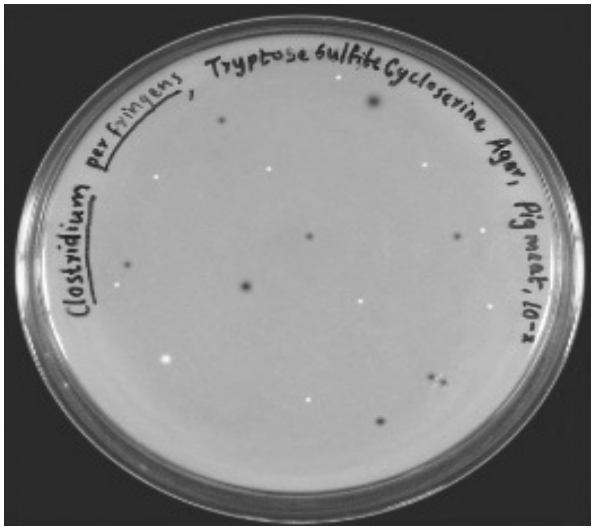

Figure 1: Growth of *Clostridium perfringens* in TSC Agar

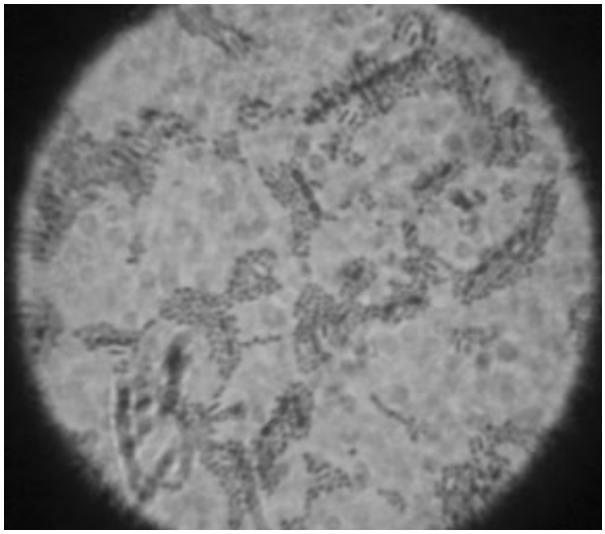

Figure 2: Gram staining of isolated bacteria

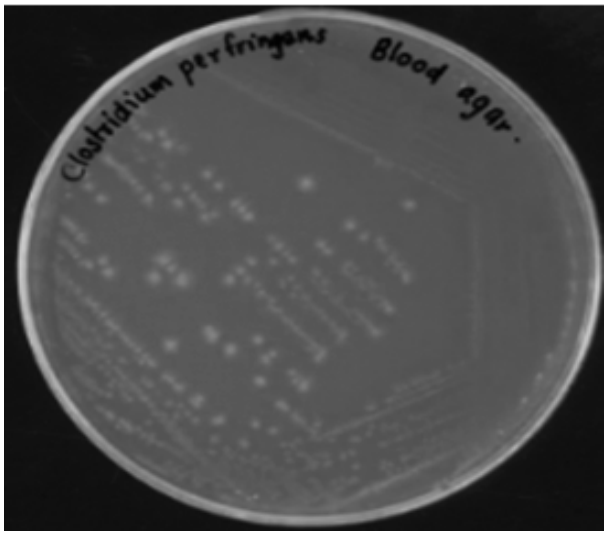

Figure 3: Isolated culture showing β- hemolysis with double zone of inhibition.

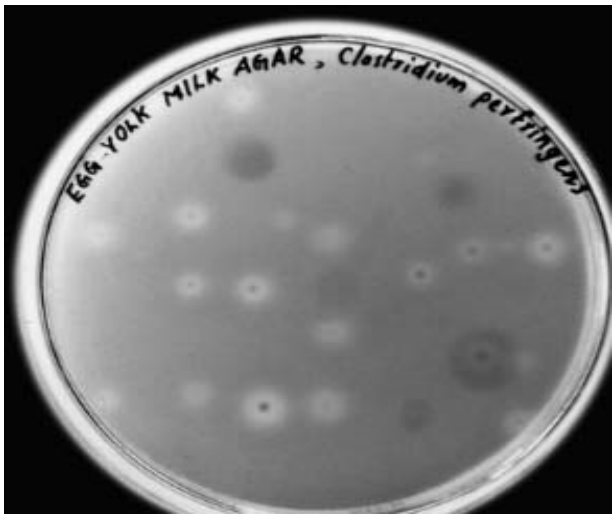

Figure 4: Lecithinase activity of isolated *C.perfringens*

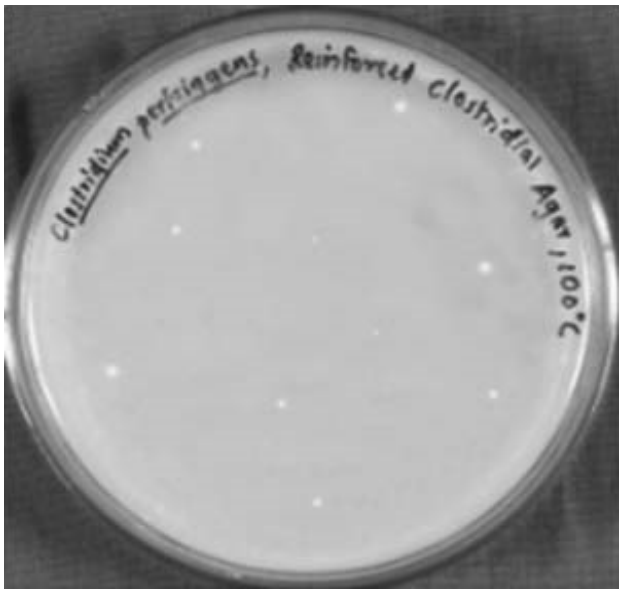

Figure 5: Heat resistant colonies produced by sporulated *C. perfringens* at 100^0C.

Table 1: Isolation of *Clostridium perfringens* from selected samples

S. No.	Cooked Samples collected	No. of sample tested	No. of infected Sample	No. of colonies on TSC plate
1.	Meat	10	02	03,05
2.	Chicken	12	01	02
3.	Pork	12	01	09

Table 2: Biochemical characterizations of isolated *Clostridium perfringens*

S. No.	Name of Test	Result obtained
1.	Growth in selective TSC agar	Black colour colonies
2.	Gram staining	Gram positive, large rods
3.	Motility	Non motile
4.	Spore staining	Presence of spores
5.	Capsule staining	Presence of capsule
6.	Blood agar	β-haemolytic, double zone of haemolysis
7.	Iron milk test	Presence of stormy fermentation
8.	Nitrate reduction	Reduced nitrate to nitrite
9.	Lactose gelatin medium	Lactose fermentation and produce acid
10.	Gelatin liquefaction	Liquefied gelatin after 48 hrs.
11.	Indole	Negative
12.	Methyl red	Positive
13.	Vogus proskauer	Negative
14.	Hydrogen sulphide	Positive
15.	Carbohydrate formation	Ferment glucose, sucrose, lactose, maltose produced acid and gas
16.	Egg-yolk milk agar	Lecithinase activity is present.

Table 3: Antibiotic sensitivity pattern of isolated *Clostridium perfringens*

S. No.	Antibiotic Disc tested	Disc Concentration	Zone diameter (in mm)
1.	Ampicilin	25 mcg/disc	39
2.	Amphotericin – B	20mcg/disc	No zone
3.	Bacitracin	10mcg/disc	25
4.	Chloramphenicol	25mcg/disc	30
5.	Erythromycin	30mcg/disc	21
6.	Polymyxin-B	50 mcg/disc	10
7.	Rifamysin	30mcg/disc	32
8.	Streptomycin	25mcg/disc	12
9.	Tetracycline	30mcg/disc	30
10.	Vancomycin	15mcg/disc	21

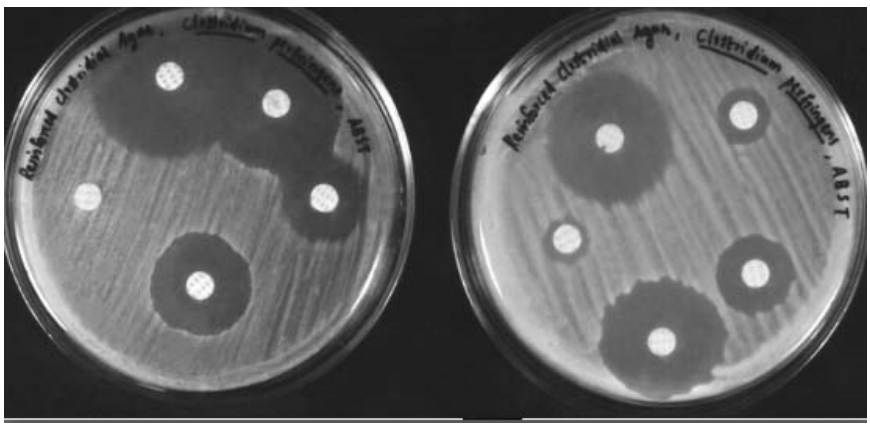

Figure 6: Antimicrobial testing of isolated culture

Table 4: BLAST results of Delta enterotoxin and selected homologous

NCBI Accession No.	Name of the protein	Name of the Organism	No. of Amino acids	E-value	Identity %	Similarity %	% of Gaps
ACD67884	Delta toxin	*Clostridium perfringens*	318	0.0	100	100	0
7AHL_B	Alpha Hemolysin, Chain B	*Staphylococcus aureus*	293	8e-25	31	52	7
BAG06216	Panton-Valentine Leucocidin S	*Pseudomonas putida*	312	4e-17	26	46	11
AAT54665	Hemolysin II	*Bacillus anthracis*	240	1e-14	30	51	7
ZP_00739479	Cytotoxin K	*Bacillus thuringiensis*	368	9e-17	26	43	13
CAA55783	Synergohymenotropic toxin	*Staphylococcus intermedius*	326	6e-23	32	50	5

Figure 8: MSA and phylogenetic analysis of best homologues

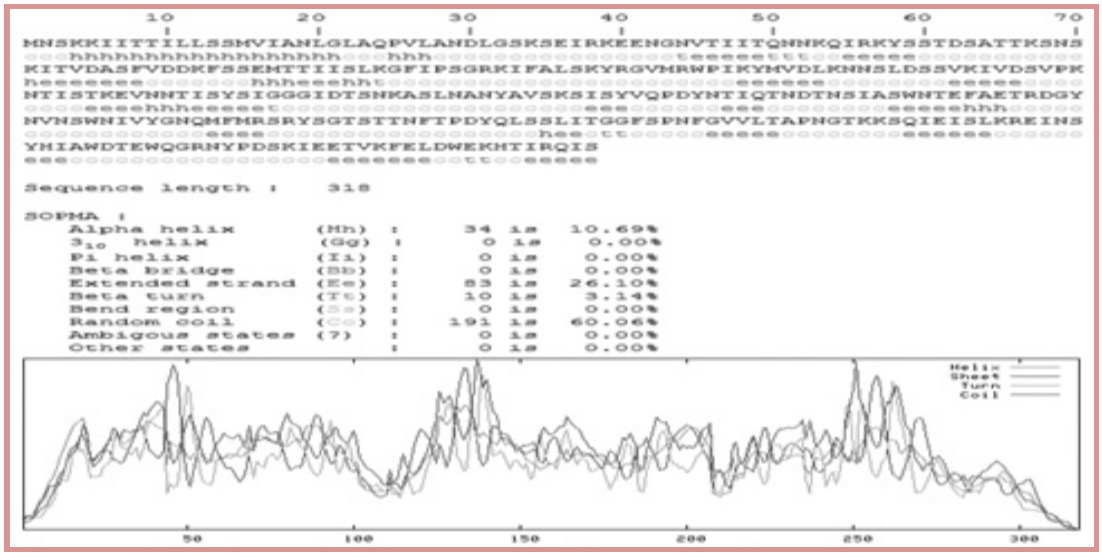

Figure 9: Predicted secondary structure of Delta enterotoxin

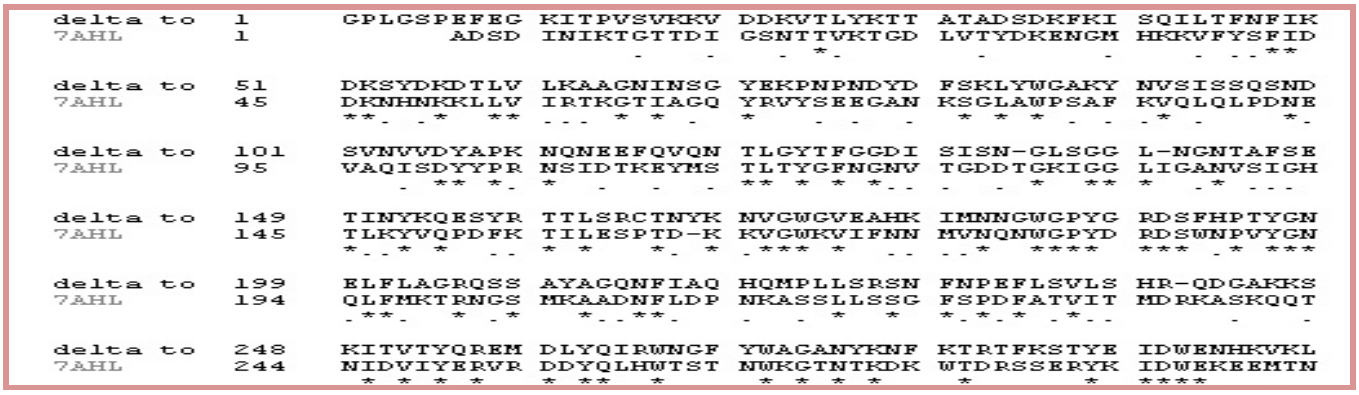

Figure 10: Alignment of the template and target

Study of the effect temperature and antimicrobial activity of the isolated culture

The effect of various temperature on *Clostridium perfringens* is studied, which clearly indicates that the spores are highly heat resistant and can survive even at 100^0C (Fig.5) So the thermal death point of the organism is above 100^0C, hence spores of the isolated culture can survive and germinate at higher temperature and can grow rapidly in food. After overnight incubation under anaerobic condition the zone of inhibition around the antibiotics discs were observed and the results are tabulated (Table-3) The isolated bacteria has susceptible to Rifamypsin (32mm), Ampicillin (39mm), Chloramphenicol (30mm), Tetracyclin (30mm) and Bacitracin (25mm). The organism is partially susceptible to Erythromycin (21mm), Vancomycin (18mm), Streptomycin (12mm) and Polymyxin-B (10mm). The organism is resistant to Amphotericin-B (Fig.6)

Identification of enterotoxin from *Clostridium perfringens* using immunodiffusion technique

Antibodies are very specific to particular antigen and it formed precipitation reaction (antigen-antibody complex appear as precipitin arc). The tested antiserum was α - antitoxin. So, the enterotoxin produced by the isolated *Clostridium perfringens* might be α- enterotoxin. The precipitation arc was formed when the toxin (antigen) is taken from Ducan Strong sporulation medium and not from fluid thioglycollate broth. This indicates the toxin is produced only in sporulation medium. (Fig.7)

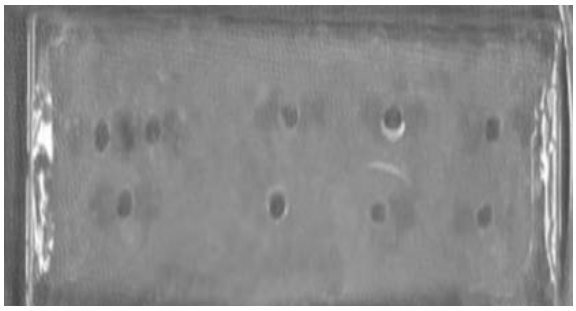

Figure 7: Detection of CPE delta immunodiffusion technique.

Sequence retrieval of *Clostridium perfringens* Delta toxin and determination of best homologues sequences by various biological databases and tools

The protein sequence of *Clostridium perfringens* Delta enetrotoxin (NCBI accession: ACD67884; GI: 188998312) was retrieved and the best homologous sequences were identified by NCBI-BLAST. The BLAST result clearly indicated that β- hemolysins of *Staphylococcus aureus* have significant similarity to delta enterotoxin. The toxin proteins of *Bacillus anthracis*, *Bacillus thuringensis* and *Pseudomonas putida* are also showing some percentage of identity and similarity to the query sequence. The best homologous sequence and its BLAST search information are tabulated (Table: 4)

Multiple sequence analysis and Phylogenetic characterization

Multiple sequence alignment between the Delta toxin and its homologous sequences was done using Clustal W and it has shown that the conservation between delta toxin and Staphylococcal hemolysin are high compared to other toxins. The evolutionary relationship between Delta toxin and other toxins were successfully analyzed by Clustal X and tree building tools like NJ plot and Tree View. The maximum parsimony distance and bootstrap values showed in phylogram express the evolutionary significance between *Clostridium* delta toxins and Staphylococcal β-hemolysins(Fig.8).This data is very crucial for the determination of templates for *in silico* modeling of delta toxin.

Predictive Bioinformatics and Proteogenomic characterization of delta-enterotoxin

The Delta toxin sequences are reverse translated to DNA sequences using ExPASy proteomic tool and the functional sites are predicted using GenScan. The result indicated that the GC content is about 48.2% .The length of exon sequence is 864 and it contains 1300 coding regions. Similarly the proteomic features of the toxin were identified by various tools and software. The secondary structure analysis shows delta toxin comprises high content of super secondary structure-random coil (60%), 30% of beta sheet and bends and only 10 % of alpha helical structures (Fig.9) The topology prediction explain that the deltatoxin contains one transmembrane helix which has oriented the amino acid between 6 and 28.

Homology Modeling, Model Refinement and Validation of Delta enterotoxin

Homology modeling of the delta enetrotoxin was done with MODELLER 9v7 using *Staphylococcus areus* β-hemolysin as template. Blast results, MSA and phylogenetic analysis shows that Staphylococcal β-hemolysins have perfect similarity to *Clostridium perfringens* Delta toxin. The Ex PDB templates were obtained by BLAST search and the sequence alignment between target and template were performed by MODELLER and 3 D model of the protein has been generated.(Fig.10)The RMSD value of the super imposed structure was calculated and it has shown as 1.4 A^0, which is accepted. Energy minimization and the steriochemical validity of modeled structure is so satisfactory. The validity of the model is further confirmed by Ramachandran plot generated by PROCHECK (Fig.11) Most of the residues are situated in allotted region and only limited residues are located in disallowed region. The modeled structure is displayed in pyMol and predominantly it consists of random coil than beta sheet and helices (Fig.12)

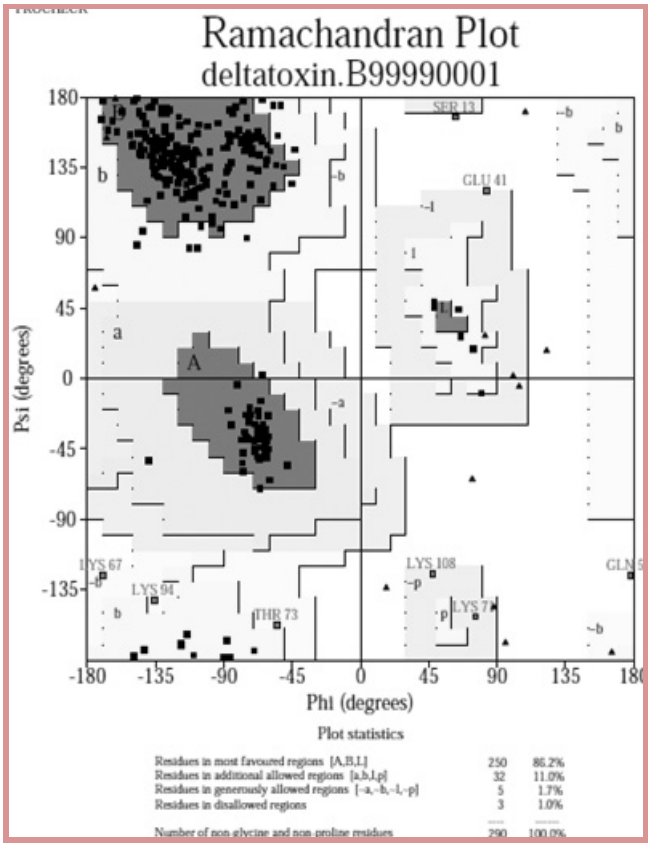

Figure 11: Ramachandran plot of modeled protein generated by PROCHECK.

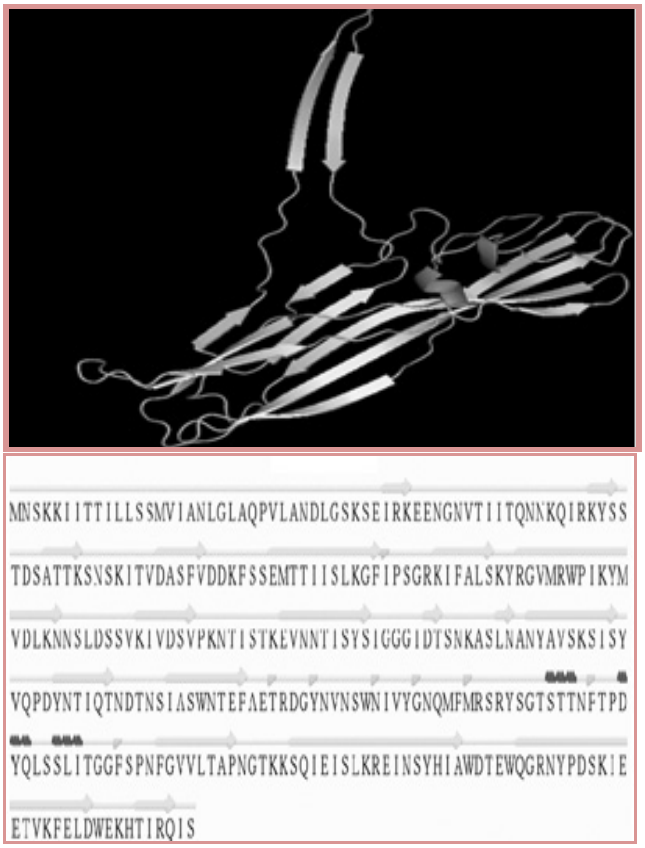

Figure 12: Visualization of Modelled Delta toxin by PyMOL and prediction of its secondary structure.

CONCLUSION

Isolation and identification of *Clostridium perfringens* was successfully done from the collected meat samples. The organism is gram-positive, anaerobic, nonmotile spore forming and encapsulated rods, which can shown typical β-haemolytic double zone in blood agar, lecithinase activity in egg yolk milk agar, reduce nitrate, producing H_2S, ferment almost all the sugars, stormy fermentation and other biochemical characters. *Clostridium perfringens* food poisoning is common in foods which are inadequately refrigerated. The spores can survive at 100°C and can germinate in food and produces toxin, a major reason for the food poisoning outbreaks. The toxin is an antigen that can precipitate with the α-antitoxin by immunoprecipitation. The organism shows high susceptibility to common antibiotics. *Clostridium perfringens* gastrointestinal infection occur mainly due to (1) the food contains or becomes contaminated with *Clostridium perfringens,* (2) Usually the food is cooked and reduced conditions develop, (3) the food is inadequately cooked and enough time are allowed for appreciable growth, (4) the food is consumed without reheating so that large numbers of viable cells are ingested and (5) the cells sporulate in vivo and elaborate the enterotoxin.

Delta toxin is one of the three hemolysins released by a number of *C. perfringens* type C and also possibly type B strains. Bioinformatics plays a key role in prediction and analysis of the deltatoxin. The protein sequence of the deltatoxin is available at NCBI GenBank and which can be used as a potential target for complete Bioinformtics studies. The BLAST result of delta toxin shows that it has significant similarity to β- toxin of *Clostridium perfringens*, *hemolysins* of *Staphylococcus aureus, Bacillus sps*. and *Pseudomaonas putida.* This is further confirmed by multiple sequence analysis and evolutionary studies using various Bioinformatics tools.

The sequence information obtained from NCBI plays a key role in the function prediction, structure prediction (primary, secondary and tertiary) and topology prediction. The ExPASy proteomic tools used as an effective, accurate tool for the primary structure analysis, secondary structure determination and transmembrane region prediction.

The structures of few betatoxin *of C.perfringens* are available in PDB. But no structural model is available for delta toxin. Based on the above studies and PDB Blast search it has identified that Staphylococcal β- hemolysin shows some structural homogeneity to delta enterotoxin. The structures of these toxin proteins are available in PDB which used as templates for homology modeling. The MODELLER server is an effective *insilico* modeling tools, which has modeled a detailed three dimensional of delta enterotoxin. The modeled structure has an acceptable RMSD values, Ramachandran plot and global energy minima, alignment and refinement parameters.

As a future perspective, the homology model of Delta enterotoxin may be used in Pharmacoinformatics and drug designing. Even though effective vaccines are available against CPE endotoxins, the structural information may useful in molecular docking and rational (structure based) drug designing, which has a significant value in various drug discovery projects. The models can be severed as a potential target for developing various drugs against CPE endotoxins.

REFERENCES

1. Beers, M.H., Berkow, R. (2002), Clostridial Infection., The Merck veterinary Manual, 17th Ed
2. OIE. (2004), epsilon toxin of Clostridium perfringens, the Center for Food Security and Public Health, Iowa state University
3. Granum, P.E. (1990), *Clostridium perfringens* toxins involved in food poisoning. Int.J. Food Microbiol, 10, 101-112.
4. Hatheway, C.L. (1990), Toxigenic Clostridia. Clin. Microbiol.Rev, 3, 66-98.
5. Hauschild, A.H.W and Hilsheimer, R. (1994), Enumeration of food borne *Clostridium perfringens* in egg-yolk free tryptose cycloserine agar.Appl.Microbiol, 27, 521-526.
6. Hauschild, A.H.W and Hilsheimer, R. (1997), Purification and characteristics of the enterotoxin from *Clostridium perfringens* type A.Can.J. Public Health, 17, 1425-1433.
7. PHLS. (2000), Advisory Committee for Food and Dairy products: Guidelines for the Microbiological Quality of some ready- to eat food samples at the point of sale, Communicable disease and public health, 3(3), 163-169.
8. Rhodehamel, E.J and Harmon, S.M. (1998), *Clostridium perfringens* .Ch.16. In Food and Drug Administration Bacteriological Analytical Manual, 8th Ed (revision A), R.L. Merker (Ed), AOA, International Gauithersbury, M.D.
9. Rood, J.I., Cole, S.T. (1991), Molecular Genetics and pathogensis of *Clostridium perfringens.* Microbiol. Rev., 55, 621-648
10. Mc Clane, B.A. (1992), *Clostridium perfringens* enterotoxin: Structure, action and detection. J. Food Safety, 12, 237-252
11. Asha, N. J., D. Tompkins, and M. H. Wilcox., (2006), Comparative analysis of prevalence, risk factors, and molecular epidemiology of antibiotic-associated diarrhea due to *Clostridium difficile*, *Clostridium perfringens*, and *Staphylococcus aureus*. J ClinMicrobiol .,44:2785-2791
12. Blackall, D. P. and Marques, M. B. (2004). Hemolytic uremic syndrome revisited: Shiga toxin, factor H, and fibrin generation. Am. J. Clin. Pathol., 121, S81-S88.
13. Böhnel, H. and Gessler, F. (2005), Botulinum toxins – cause of botulism and systemic diseases? Vet. Res. Commun. 29, 313-345.
14. Middlebrook, J. L. and Dorland, R. B. (1984), Bacterial toxins: cellular mechanisms of action. Microbiol Rev., 48, 199-221.
15. Popoff, M. R. (2005), Bacterial exotoxins. Contrib. Microbiol., 12, 28-54.
16. Petit L, Gibert M, Popoff, M R., (1999), *Clostridium perfringens*: toxinotype and genotype. Trends Microbiol .,7: 104–110
17. Jolivet-Reynaud C, Cavaillon J M., Alouf JE (1982), Selective cytotoxicity of *Clostridium perfringens* delta toxin on rabbit leukocytes. Infect Immun., 38: 860–864.
18. Bayer, A.S., Nelson, S.C., Galpin, J.E., Chow, A.W and Guze, L.B. (1975), Necrotizing pneumonia and empyema due to *Clostridium perfrigens,* Am.J. Med., 59, 851-9.
19. Maclennan, J.D. (1962), Histotoxic Clostridial infection of man Bacterial. Rev., 26, 177-276.
20. Mc Clane, B.A. (1992), *Clostridium perfringens* enterotoxin: Structure, action and detection. J. Food Safety, 12, 237-252
21. Stern, M. and Warrack, G.H. (1964), The types of *Clostridium perfringens* J.Pathol, Bacteriol, 88, 2079-83.
22. Maliwan, N. (1979), Emphysematous cystitis associated with *Clostridium perfringens* bacteriemia, J. Urol., 121, 819-820.
23. Sigurdarson, S., Thorsteinsson, T. (1990), Sudden death of Icelandic dairy cattle. Vet. Rec., 127, 410-415.
24. Cavaillon JM, Jolivet-Reynaud C, Fitting C, David B, Alouf JE (1986) Ganglioside identification on human monocyte membrane with *Clostridium perfringens* delta-toxin. J Leukocyte Biol ., 40: 65–72.
25. Jolivet-Reynaud C, Cavaillon J M., Alouf JE (1982), Selective cytotoxicity of *Clostridium perfringens* delta toxin on rabbit leukocytes. Infect Immun., 38: 860–864.
26. Keyburn AL, Boyce JD, Vaz P, Bannam TL, Ford ME, et al. (2008), NetB, a New Toxin That Is Associated with Avian Necrotic Enteritis Caused by *Clostridium perfringens*. PLoS Pathog., 4: 26
27. Manich M, Knapp O, Gibert M, Maier E, Jolivet-Reynaud C*, et al*. (2008), *Clostridium perfringens* Delta Toxin Is Sequence Related to Beta Toxin, NetB, and *Staphylococcus* Pore-Forming Toxins, but Shows Functional Differences. PLoS ONE 3(11): 3764.

28. Michelet N, Granum PE, Mahillon J (2006) ,*Bacillus cereus* enterotoxins, bi- and tri-component cytolysins, and other hemolysins. In: Alouf JE, Popoff MR, eds (2006) The Comprehensive Sourcebook of Bacterial Protein Toxins. 3u ed. Amsterdam: Elsevier, Academic Press. 779–790

29. Blackall, D. P. and Marques, M. B. (2004). Hemolytic uremic syndrome revisited: Shiga toxin, factor H, and fibrin generation. Am. J. Clin. Pathol., 121, S81-S88.

30. Ducan, C.L and Strong, D.H. (1968), Improved medium for sporulation of *Clostridium perfrigens*, Appl, Microbiol, 16, 82-89.

31. Ducan, C.L., Strong, D.H and Sebald, M. (1972), Sporulation and enterotoxin production by mutants of *Clostridium perfrigens.* J.Bacteriol. 110, 378-391.

32. Emswieler, B.S., Pierson, C.J and Kotala, A.W. (1976), Bacteriological quality and shelf life of ground beef, Appl. Environ. Microbiol, 31, 826-820.

33. Evans, D.G. (1945), the invivo production of α-toxin, haemolysis and hyaluronidase by strains of *Clostridium perfrigens* type A, and the relationship of in vitro properties to virulence in guinea pigs, J. pathol. Bacteriol, 57, 75-85.

34. Frieben, W.R and Ducan, C.L., (1973), Homology between enterotoxin protein and structural protein in *Clostridium perfrigens* type A. Eur.J. Biochem, 39, 393-401.

35. Ducan, C.L and Strong, D.H. (1968), Improved medium for sporulation of *Clostridium perfrigens*, Appl, Microbiol, 16, 82-89.

36. Ducan, C.L., Strong, D.H and Sebald, M. (1972), Sporulation and enterotoxin production by mutants of *Clostridium perfrigens.* J.Bacteriol. 110, 378-391

37. Alouf J E, Jolivet-Reynaud,C. (1981), Purification and characterization of *Clostridium perfringens* delta toxin. Infect Immun., 31: 536–546

38. Bairoch, A. (2005), the Universal Protein Resource (UniProt). Nucleic Acids Res, 33, (Database Issue) D154–D159

39. Kabsch, W. and Sander, C. (1983), Dictionary of protein secondary structure: pattern recognition of hydrogen-bonded and geometrical features. Biopolymers., 22:2577–2637

40. N. Eswar, M. A. Marti-Renom, B. Webb, M. S. Madhusudhan, D. Eramian, M. Shen, U. Pieper, A. Sali(2006.) Comparative Protein Structure Modeling With MODELLER. Current Protocols in Bioinformatics, John Wiley & Sons, Inc., Supplement 15, 5.6.1-5.6.30,

41. Kopp, J. and Schwede, T. (2004), The SWISS-MODEL Repository of annotated three-dimensional protein structure homology models. Nucleic Acids Res., 32: D230–D234

42. Krieger,E., Nabuurs,S.B., and Vriend, G. (2003),Homology modeling. Methods Biochem. Anal., 44:509-523

43. Laskowski, R.A., et al. (1993), PROCHECK: a program to check the stereiochemical quality of protein structures. J. Appl. Cryst., 26:283–291

44. Melo, F. and Feytmans, E. (1998), Assessing protein structures with a non-local atomic interaction energy. J. Mol. Biol, 277:1141–1152

45. Melo F, Devos D, Depiereux E, Feytmans E.(1997), ANOLEA: a www server to assess protein structures. Proc Int Conf Intell Syst Mol Biol.5:187-90.

46. Cao Z, Lin Z, Wang J, Liu H.(2009), Refining the description of peptide backbone conformations improves protein simulations using the GROMOS 53A6 force field.J Comput Chem. 31(1):1-23.

47. Eisenberg D, Lüthy R, Bowie JU.(1997),VERIFY3D: assessment of protein models with three-dimensional profiles.Methods Enzymol. 1997; 277:396-404.

48. Laskowski, R.A.,(1993), PROCHECK: a program to check the stereiochemical quality of protein structures. J. Appl. Cryst., 26:283–291

49. Koradi R, Billeter M, Wüthrich K.(1996) MOLMOL: a program for display and analysis of macromolecular structures.J Mol Graph.51-5, 29-32.

50. Arnold K., Bordoli L., Kopp J., and Schwede T. (2006). The SWISS-MODEL Workspace: A web-based environment for protein structure homology modelling. Bioinformatics, 22,195-201.

****