Original Research Article

In Vitro Detection Studies And *In Silico* Approaches To Evaluate Interactions Of *Clostridium perfringens* Enterotoxin (CPE) To Human Claudin – 4

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Abstract: Indicator bacteria are used to estimate the levels of faecal contamination in water. They are not dangerous but are used to indicate presence of a health risk. In the current study, efforts has been made to determine the presence of faecal indicator bacteria, like *Clostridium perfringens*, in two water samples of Nirjuli P-sector, District-Papum Pare, Arunachal Pradesh. Work has been restricted to simply check the presence or absence of faecal indicator group of microbes in the sample, rather going to the details of detection of particular microbe or studying the water quality. Results were found to be positive, indicating an evidence for the presence of faecal contaminants and thus has also cleared the path for further investigation on this topic by specialized methods. *Clostridium perfringens* Enterotoxin (CPE) is responsible for causing human food poisoning. Recent studies revealed that CPE specifically destroy those cancer cells which consist a protein named Claudin (Cld). In this study, also an attempt has been made to find out a proper binding mode of CPE with human Claudin 4/Cld-4. Studies were conducted on docking interactions followed by Molecular Dynamics (MD) simulations for 10ns between the homology modeled Cld-4 and CPE. Results showed that both the complex was well comparable, but for methods like MD, 10ns was insufficient for observing conformational changes. So, we have concluded the results with basic comparisons to have future exploration on this topic using computational methods.

Keywords: *Clostridium perfringens,* Enterotoxin, Claudin 4, Homology modeling, Docking, Molecular dynamics simulations

Introduction

Clostridium perfringens (formerly known as *C. welchii*), is a gram positive, anaerobic, spore forming bacilli. Traditionally, from decades, bacteria are in use as indicators of the sanitary quality of water. This has most probably started in 1880s when Von Fritsch described *Klebsiella pneumonia* and *K. rhinoscleromatis* as microorganisms characteristically found in human faeces (Ashbolt *et al.*, 2001). In the year 1885, Percy and Grace Franklands started the first routine bacteriological examination of water and in 1891, the idea that microorganisms of sewage characteristic must be identified as evidence of potentially dangerous pollutions, was evolved (Anon, 1996). From earlier studies, we came to know that the significance of coliforms like *Streptococci* and *C. perfringens* was recognized by the start of the twentieth century. In the current study, an attempt has been made to simply detect the presence of any faecal indicator bacteria like *C. perfringens* in the sample water collected. As from earlier reports it was clear that *C. perfringens* form colonies in a wide range of media like Nutrient agar, Blood agar, Robertson's cooked media, TCS Agar etc., (Fujita *et al.,* 2000; Koua et al., 2011)

samples were simply allowed to grow in the Nutrient Agar media, as it is still a widely used general purpose medium for supporting growth of a wide range of non-fastidious microbes and then examined by Gram staining. *C. perfringens* ranks as one of the leading causes of human food poisoning and gas gangrene. Since, these bacteria can form spores that can withstand cooking temperatures, if, cooked food is left to stand for long enough, germination can ensue and infective bacterial colonies may develop (Mc Clane et al., 2007). The virulence of *C. perfringens* is largely attributable to its ability to produce an arsenal of potent protein toxins such as alpha, beta, epsilon and iota which are used to classify *C. perfringens* strains into one of the five types (A-E).

Clostridium perfringens Enterotoxin (CPE) (PDB ID: 3AM2) is a non-secretory toxin, produced by type A strains which is made up of a single polypeptide chain consisting of 319 residues and a molecular mass of about 35 kDa (Mc Clane *et al.,* 2006). . Among the various toxins produced by the bacteria, CPE is a major toxin responsible for human food poisoning. The overall structure of CPE is composed of three distinct domains (Robertson *et al.,* 2010) namely, Domain-I, II and III. Domain I or C-terminal domain (cCPE) is not cytotoxic and is responsible for receptor binding. Domain II or the N-terminal domain is responsible for oligomerization and membrane insertion to form active pores. Domain III mainly participates in physical changes when the CPE protein inserts into membrane. More recently, from the site directed mutagenesis experiment, it has been found that the C-terminal domain of CPE (cCPE), residues from 194-319 has the full receptor binding affinity and specially the three Tyrosine residues, present in positions 306, 310, 312 and Leo 315 of the native toxin are important for receptor binding (Gao *et al.,* 2011).

Claudin, a tetra trans membrane (TJ) protein, regulating the paracellular permeability of epithelia, act as CPE receptors (Koua *et al.*, 2011). From the earlier studies we came to know that second extracellular loop of claudin takes part in the interaction. The structure of CPE composed of three domains, Domain I, II and III. Domain I, also known as the C- terminal domain (cCPE) is not cytotoxic in nature and performs receptor binding. Domain II, also known as N-terminal domain undergoes oligomerization and membrane insertion. Domain III carries out the physical changes that occur when the CPE protein inserts into membrane (Furuse *et al.,* 1998; Kondoh *et al.,* 2007). Claudins are a total of 24 member protein family (molecular mass ranging from 20-27 kDa) of TJ strands, which performs cell to cell junction in the apical part of plasma membrane (Mc Clane *et al.*, 2007).

Recently, from the various experimental investigations by Abuazza *et al.,* (2006), it was shown that CPE, very quickly and specifically destroys those cancer cells who express Claudin 3 (Cld-3) and Claudin 4 (Cld- 4). Further, earlier reports have indicated that Claudins are up regulated in different types of cancers and also can be used as a biomarker in cancer therapy (Gao *et al.*, 2011).

In the present study, some of the possible *in silico* approaches were carried out to determine the binding affinities of CPE to Cld-4.

Materials and methods

For faecal indicator test

Collection of Samples: Water samples were collected from two sources: (1) Central Poultry Farm, Nirjuli and (2) Drain Water, Nirjuli P-sector

Preparation of Nutrient Agar Medium

The nutrient Agar medium was prepared with the ingredients of Peptone (0.5%) , Beef Extract (0.3%), Agar(1.5%), NaCl (0.5%) and Distilled water. pH was adjusted to neutral at 37°C and autoclaving was done at 15psi pressure for 15 mins. The media was allowed to cool and then transferred to the Laminar Air Flow for solidification (Aneja, 2005).

Preparation of the culture

Samples collected from the two sources were poured in petri plates and were placed for incubation at 37 °C for 48 hours. It was observed that both the samples formed colonies but, the colonies obtained from the 'Drain Water' sample was having more variation indicating the chance of presence of a wide range of microbes. Colonies were very carefully picked and

inoculated in two new plates for pure culture. The same process was repeated for 3 times (for the sample collected from Central Poultry Farm) and 5 times (for the Drain Water sample) to attain pure culture. Bacterial smears are being prepared from both the samples and Gram Staining was done for identification. (Fig. 1 and 2) (Chakraborty, 2009; Dubey and Maheshwari., 2010).

Fig. 1. The smear of bacterial culture of the sample collected from drain water.

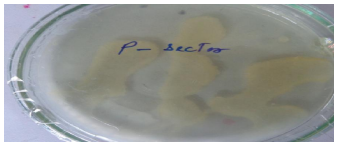

Fig. 2. The smear of bacterial culture of the sample collected from the Poultry Farm.

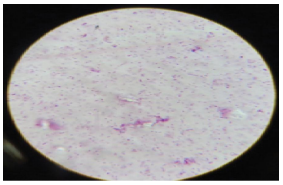

Fig. 3. The gram staining of the colonies of the drain water sample seen at 100x with oil immersion. Colonies are violet in colour depicting gram positive.

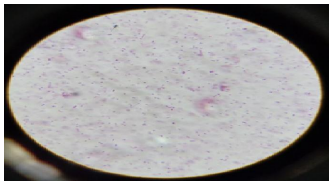

Fig. 4. The colonies viewed after gram staining at 100x from the water sample collected from poultry farm.

CPE-Cld-4 binding assay

A Hybrid Approach to *Ab initio*, Fold Recognition and Homology Modeling

Since, a three dimensional crystal structure of Cld-4 (UniPort ID: 014493) is not available till present, we attempted to predict the structure by homology modeling with the help of MODELLER 9.10 (Sali *et al.,* 1993).

SEQUENCE of CLAUDIN 4

MASMGLQVMGIALAVLGWLAVMLCCALPMWRVTAFIGSNIVTS
QTIWEGLWMNCVVQSTGQMQCKVYDSLLALPQDLQAARAL
VIISIIVAALGVLLSVVGGKCTNCLEDESAKAKTMIVAGVVFLLAGLM
VIVPVSWTAHNIIQDFYNPLVASGQKREMGASLYVGWAASGLLLL
GGGLLCCNCPPRTDKPYSAKYSA ARSAAASNYV

(Source: NCBI BLAST) : >sp|O14493| CLD4_HUMAN Claudin-4 OS=Homo sapiens GN=CLDN4 PE=1 SV=1

NCBI BLAST results of human Cld- 4 against PDB shows only 31% sequence identity and 27.7 maximum score. Due to the fact that low percentage of sequence identity is not favourable for modeling (Mizrachi, 2002), the sequence was then submitted to the I-TASSER web server (http:// zhanglab.ccmb. mcd.umich.edu/I-TASSER/) which is an online automated server for homology modeling. The server gave us five models (Fig. 5), each of whose C-score, Exp. TM-score, Exp. RMSD's

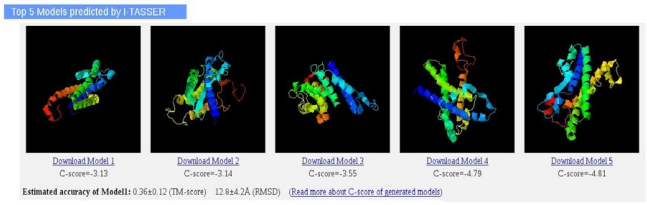

Fig. 5. Five models generated from the I-TASSER web server which is implemented with *Ab Initio* and Fold Recognition methods for protein structure prediction.

and No. of decoys were estimated. Out of the five models generated, model1 was the best, as it represents the SwissProt sequence annotation satisfactorily and the four trans membrane helices are formed. Ramachandran plots generated by PROCHECK shows 82.4% residues in the most favoured region (Fig. 6) (Ramachandran *et al.,* 1963). Since, the extracellular and cytoplasmic domains

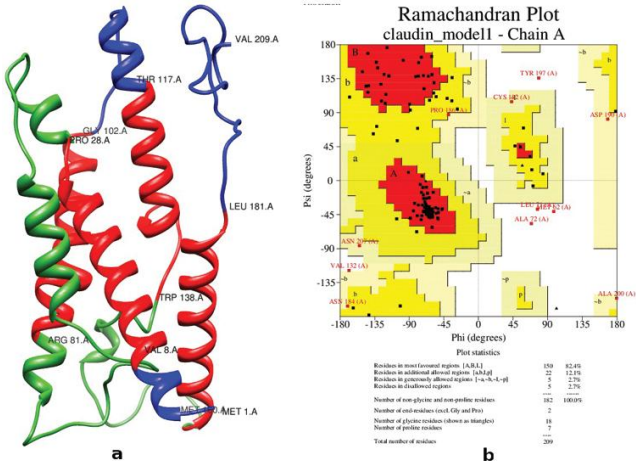

Fig. 6 (a). I-TASSER model 1. The transmembrane helices are colored red, cytoplasmic regions are blue and extracellular regions are green. Residue labeling shows to compare the Swissprot sequence annotation which is clearly conflicted with the cytoplasmic and extracellular regions. **(b).** Ramachandran plot generated by PROCHECK showing the residues distribution along the regions. 82.4% residues are observed in the most favored region.

are in conflicts, therefore, in order to position the domains appropriately, this model was taken as a template (Fig. 6a) after truncating it, taking only the residues that falls under

```
>P1;TMHallA_newA
structureX:TMHallA_new.pdb:1    :A:21+  :A:::-1.00:-1.00
-------VMGIALAVLGWLAVMLCCALP-----------------------------------------------
------ALVIISIIVAALGVLLSVVGG--------------MIVAGVVFLLAGLMVIVPVSW-----------
---------MGASLYVGWAASGLLLLGGGLL---------------------------*

>P1;EC1_R05_TA
structureX:EC1_R05_T.pdb:30    :A:80  :A:::-1.00:-1.00
----------------------------WRVTAFIGSNIVTSQTIWEGLWMNCVVQSTGQMQCKVYDSLLALPQ
DLQAA-----------------------------------------------------------------------
-------------------------------------------------------------*

>P1;EC2B
structureX:EC2.pdb:139    :B:160  :B:::-1.00:-1.00
-----------------------------------------------------------------------------
-------------------------------------------------------------TAHNIIQDFYNP
LVASGQKREM-----------------------------------------------*

>P1;CYT2A
structureX:CYT2.pdb:103    :A:117  :A:::-1.00:-1.00
-----------------------------------------------------------------------------
------------------------KCTNCLEDESAKAKT-------------------------------------
-------------------------------------------------------------*

>P1;CYT3C
structureX:CYT3.pdb:182    :C:209  :C:::-1.00:-1.00
-----------------------------------------------------------------------------
-----------------------------------------------------------------------------
------------------------------CCNCPPRTDKPYSAKYSAARSAAASNYV*

>P1;CLDN
sequence:CLDN:     : :     : ::: 0.00: 0.00
MASMGLQVMGIALAVLGWLAVMLCCALPMWRVTAFIGSNIVTSQTIWEGLWMNCVVQSTGQMQCKVYDSLLALPQ
DLQAARALVIISIIVAALGVLLSVVGGKCTNCLEDESAKAKTMIVAGVVFLLAGLMVIVPVSWTAHNIIQDFYNP
LVASGQKREMGASLYVGWAASGLLLLGGGLLCCNCPPRTDKPYSAKYSAARSAAASNYV*
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Fig. 7. The final alignment file of MODELLER. TMHallA_newA is the model prepared by UCSF Chimera consisting of four transmembrane helices, EC1_R05_TA is the first extracellular domain, EC2B is the second extracellular domain, CYT2A and CYT3C are the cytoplasmic regions as modelled by the I-TASSER server.

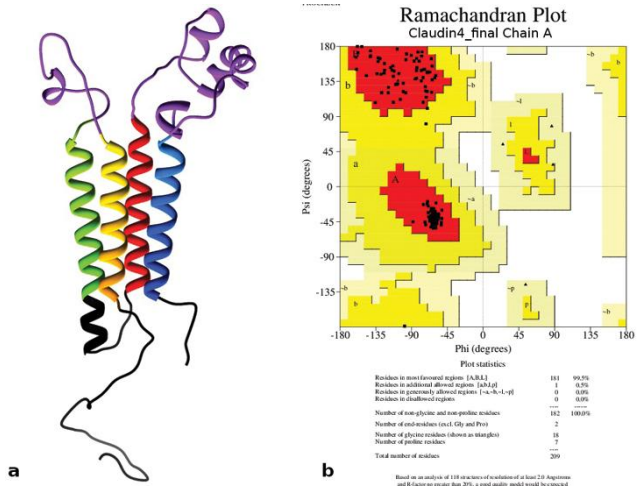

Fig. 8 (a). The final model of human Claudin 4 obtained by using MODELLER 9.10. Transmembrane helices are coloured differently with green, yellow, red and blue. Extracellular domains are coloured purple and cytoplasmic domains are coloured black.
(b). We have generated 500 models by homology modeling. PROCHECK was done every time and loops are refined until we get a Ramachandran Plot depicting 99.5% residues in the most favoured region.

the second extracellular loop (as we know by earlier reports that it only that portion interacts with CPE and not the whole sequence). Each of the helices of the model of I-TASSER was refined by UCSF Chimera tools (Yang *et al.,* 2012). The alignment file of MODELLER has been prepared manually by hand and subjected to homology modeling in order to generate about 500 homology models (Fig. 7). PROCHECK was done for all models every time and loops were refined rigorously, until a final model showing 99.5% residues in the most favoured region was achieved. (Fig. 8).

Protein-Protein Docking

Two rounds of protein-protein docking were performed taking CPE as the 'receptor' and Cld-4 as the 'ligand', with the help

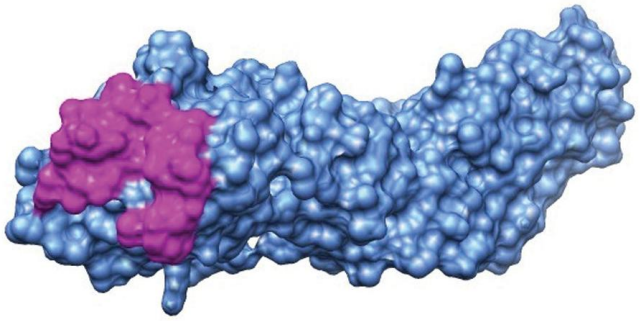

Fig. 9. The molecular surface of the binding pocket of CPE generated by UCSF Chimera which is determined after docking.

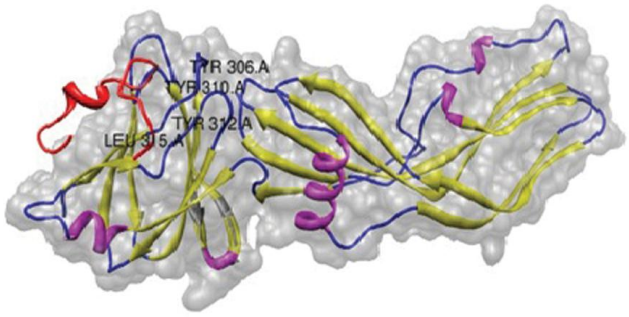

Fig. 10. Best selected model obtained from the first round of docking. This figure was generated with the help of UCSF Chimera, showing clearly the Beta-sheets and Helices in a semi-transparent surface mode and depicting the most important interacting residues (i.e., Tyr306, 310, 312 and Leu315).

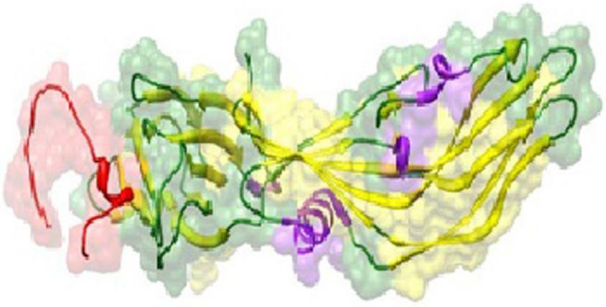

Fig. 11. Best selected model from the second round of docking. This model has also been generated like that of the first model. However as the interacting residues are same, therfore it is not shown here.

of ClusPro 2.0 which is an online automated server for protein-protein docking. The first step to use this server is to create an account by providing an academic email ID. Once the account was created, the PDB files of both the receptors and the ligand are uploaded with chain identifiers. In the first round, a truncated Cld-4 containing residue 139-160 (that belongs to the second extracellular loop) was used and full sequence length of Cld-4 was used in second round. Among the various models generated, a few showed similarities with that of modeled Cld-4. The best complex obtained from the two rounds (Fig. 10 and 11), which almost mimic the modeled structure were selected for further refinement. The binding pocket of CPE was also identified after docking (Fig. 9).

Molecular Dynamics (MD) Simulations

Two rounds of MD simulations were performed with a united-atom force- field using GROMOS 96 43Al on the Linux OS for the two models selected on the basis of the dockings and hot-spots generated. The two simulations performed are represented as: 1. Native CPE $_{36\text{-}321}$ with (homology modeled) truncated Cld-4 $_{139\text{-}160}$ for 10ns. 2. Native CPE $_{36\text{-}321}$ with (homology modeled) full length Cld-4 $_{1\text{-}209}$ for 10ns.

GROMACS is a high – end, high performance research tool designed for the study of protein dynamics using classical molecular dynamics theory (Van *et al.,* 1996). After completion of both the MD rounds, the two complexes were compared by checking two parameters such as: 1. g_rmsd (Root Mean square Deviation 2. g_dist (Guomacs Distances)

Results

Faecal indicator test

The Gram staining of both the samples shows positive results depicting violet bacilli colonies, which is an evidence for the presence of *C. perfringens* like faecal indicator bacteria. Despite of the fact that, *C. perfringens* strains donot forms spores readilly in artificial culture or animal tissue, spores were detected from the sample of Poultry Farm Water. (Fig. 3 and 4).

CPE-Cld Binding Assay

Homology Modeling Results

Table1: Scoring table generated by I-TASSER. 'C-score' is a confidence score for estimating the quality of predicted models by I-TASSER. C-score is typically in the range of [5, 2], where a C-score of higher value signifies a model with a high confidence and vice-versa. 'TM-score' and 'RMSD' are known standards for measuring structural similarity between two structures which are usually used to measure the accuracy of structure modeling when the native structure is known. 'No. of decoys' represents the total number of models generated in each iterations.

	C-score	Exp.TM-score	Exp.RMSD	No. of decoys
Model 1	-3.13	0.36 ± 0.12	12.8 ± 4.2	1346
Model 2	-3.14	-	-	1343
Model 3	-3.55	-	-	892
Model 4	-4.79	-	-	256
Model 5	-4.81	-	-	253

The five models generated by the I-TASSER web server are shown (Fig. 5) and the results are tabulated in Table 1.

Protein-Protein Docking Results

Receptor	Ligand
(i) CPE (PDB ID: 3AM2)	(i) Cld-4 (truncated)
(ii) CPE (PDB ID: 3AM2)	(ii) Cld-4 (whole sequence)
(iii) CHAIN A	(iii) CHAIN B

A total of 946 protein-protein complexes of CPE (PDB ID: 3AM2) and the second extracellular domain of Cld-4 in 22 clusters are generated by the ClusPro 2.0 serve. The domain that was cut from the model generated by the I-TASSER consists of residues from 139-160 (Thr, Ala, His, Asp, lle, lle, Gln, Asp, Phe, Tyr, Asp, Pro, Leu, Val, Ala, Ser, Gly, Gln, Lys, Arg, Glu and Met). Out of 946 only 94 complexes grouped as 27 Balanced, 28 Electrostatic Favoured, 17 Hydrophobic Favoured, 22 vdw+ Elec coefficients were shown in structure. From 94 complexes, one model, "Model 12" was found from the Balanced group to be a desired model showing maximum number of residues interacted with each other (Fig. 10).

Docking of CPE to the full cld-4 Modeled structure

In a similar way described above, full length Cld-4 (residues 1-209) obtained from a hybrid homology modeling protocol was docked to CPE. 568 complexes were generated in 30 clusters. In the server 120 filtered complexes were available in all four coefficients (Balanced, Electrostatic-Favoured, Hydrophobic Favoured, vdw+Elec), out of which one complex

"Model 26" from Balanced group, found to have desired residue information (Fig. 11).

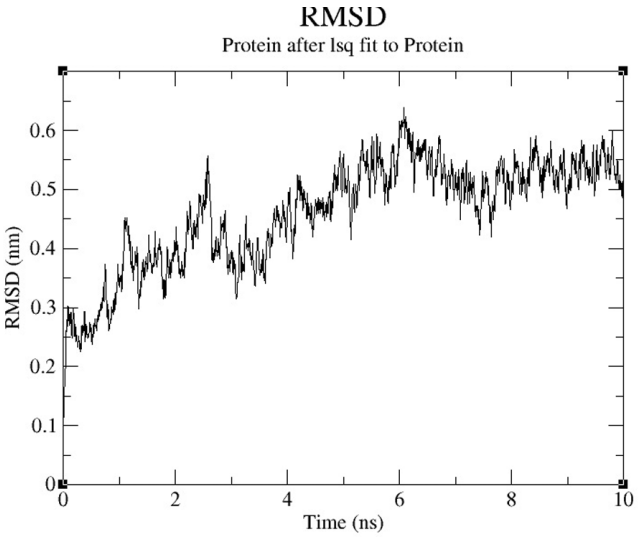

Fig.12. RMSD plot generated after the first MD run. It is seen that the graph shows an exponential growth till it reaches 0.6 nm forming a peak there itself and after that it decreases to 0.5 nm. However, after that, it almost shows a stable conformation.

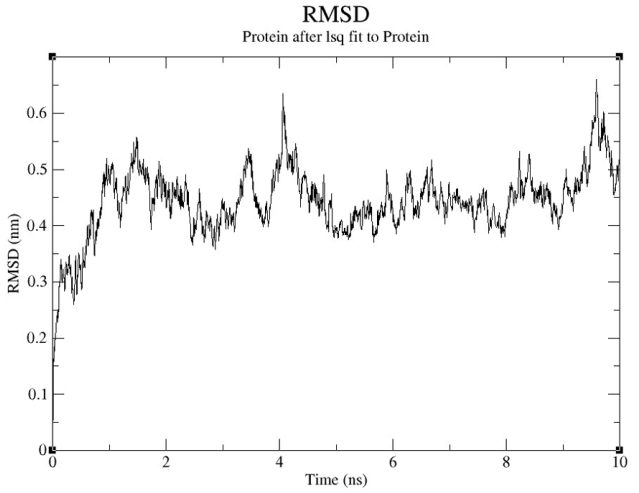

Fig. 13. RMSD plots generated after the second MD run. It is seen that there is a lot of fluctuations, ups and downs till it reaches 0.6 nm forming two higher peaks at that point. However, it is seen that it gradually decreases and tends to be stable from 0.5 nm. We can conclude that, if the time interval would have been increased a little, a nearly stable conformation of the graph can be expected.

Molecular Dynamics Results

Analysis of g_rmsd result

Analysis of g-dist result

(i) The graph depicts that the distance of Tyr 306 fluctuates at 1.4 nm. Although it is holding a stable conformation, it is showing more ups and downs than the other three i.e., Tyr 310, 312 and Leu 315 (Fig. 14).

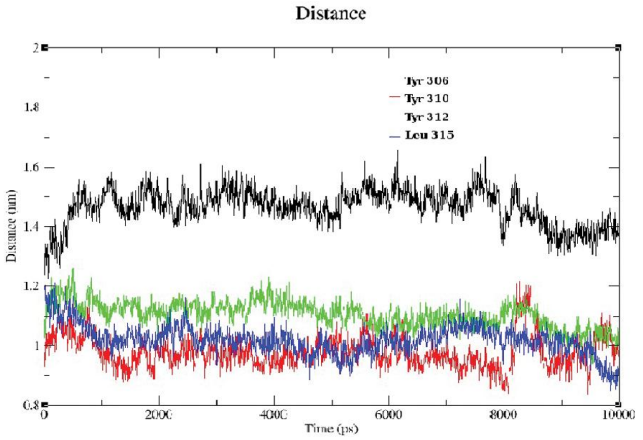

Fig. 14. Distance of atoms in each residue of the first complex. Distance of the Tyr306 fluctuates at 1.4 nm forming peaks at 1.6 nm. Tyr310 fluctuates a little throughout the graph at 1nm and forms a peak at 1.2nm. Tyr312 and Leu315 were seen to be almost stable.

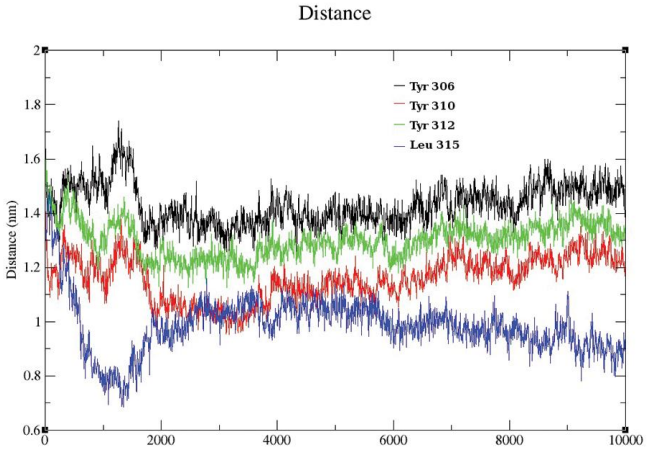

Fig.15. Distance graph of the second complex formed. Distance of the Tyr306 fluctuates at 1.4nm in the beginning showing a peak at 1.65nm. Tyr310 almost remained constant from 1.2nm except showing a downward peak initially at 1nm. Tyr312 showed almost a same type of graph as that of Tyr310 at 1.4nm and Leu315 showed a higher fluctuation forming a downward peak at 0.8nm.

(ii) The graph depicts that Tyrosine fluctuates at 1.4nm showing a peak at 1.65. Tyr310 remains constant at 1.2 nm except showing a downward peak initiating at 1nm. Tyr 312 showed similar type of graph that of Tyr 310 at 1.4 nm and Leu 315 showed a downward peak at 0.8 nm. (Fig. 15).

Discussion

The results of Faecal Indicator Test of both the samples were found to be positive. Appearance of gram positive bacilli and formation of spores gives a clear picture of the presence of *C. perfringens* in the water collected from both the sources. As the aim of this investigation was not to go into the detail

detection of particular type or strain, but, just to check the presence or absence of the faecal indicator group bacteria like *C. perfringens,* we have restricted our findings to simple detection techniques which were more or less like a presence or absence confirmatory test. Hence, we conclude our results on the detection of the presence of faecal coliform as completely valid with a hope that this approach will surely help in any kind of future study regarding detection of particular strains by specialized methods such as Membrane Filtration, using Chromogenic substances or by any Gene Sequence based methods or any other epidemiological studies. From decades, *Clostridium perfringens* enterotoxin (CPE) is of great importance for causing human food poisoning (Morita *et al.,* 1999). Tremendous progress has been made in the past few years in the characterization of the structure and function of CPE and its receptors, the claudins (Veshnyakova *et al.*, 2012). Firstly, in the homology modeling (Olson *et al.,* 1989), although the I-TASSER server gave one model accurately matching with that of the sequence annotation of cld-4 available in SwissProt. But, it was not perfect due to the conflict between the extracellular and cytoplasmic domains. So, considering this as a template, we performed the homology modeling. Secondly, in the two dockings, the two models selected represent the interaction of the cld-4 almost in the pocket region of CPE. Nevertheless, we should mention here that the first model seemed to be more fit from the sense that it contains all the interacting residues exactly in the binding site (Berman *et al.,* 2008). Thirdly, the parameters checked after two rounds of MD simulation gives a clear comparison between the two complexes:

(a) The RMSD results showed that the root mean square deviation graph of the first complex generated tends to be more stable than the second complex. However, we have to mention that both the models get fluctuate at some points but this fluctuation is seen to be more in the second model.

(b) The two graphs obtained after calculating the g_dist showed that the Tyrosine residue 306 of the first complex fluctuates more at 1.4nm compared to the other residues present (Tyrosine 310, 312 and Leucine 315). Nevertheless, the graph of the second complex showed that Leucine 315 fluctuates more compared to the other residues present (Tyrosine 306, 310 and 312).

From all the results mentioned and explained above, it was clear that the two complexes formed was well comparable. After completing the two docking rounds successfully, we had used MD in order to observe stability, dynamics and conformational change of the interacting claudin binding pockets of CPE upon claudin binding. However, it is necessary to mention here that, keeping in view of the limited capacities of the systems and processes, we had performed the MD simulation process for 10ns time scale. It is clear from the MD results that, the time interval was not enough to check which complex would show a more efficient binding of CPE and CLD -4. Nevertheless, we can definitely say that, if the time interval would have been increased i.e. up to 15ns or 20ns, we might get a clear view to analyze as well as interpret both the data sets in order to reach a conclusive observation that which binding was efficient. So, we tried to validate both binding modes in a way that with an extension of another 15 to 20ns time, would give a clear notion about how CPE binds to Claudins. Therefore, we have concluded the results with basic comparisons in a view to have future exploration on this topic using computational methods, which will lead to generate the hypothesis of designing peptido-mimetics drug against bacterial toxins.

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