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Identification of Drug Target Site on Citrate Synthase of Food Pathogen-Campylobacter Jejuni

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ABSTRACT

Campylobacter jejuni [C.jejuni] is commonly associated with poultry, and it naturally colonises the digestive tract of many bird species. Food poisoning caused by Campylobacter species can be severely debilitating, but is rarely life-threatening. In this study, C. jejuni was isolated and identified from spoiled food source from a restaurant in Chennai, Tamilnadu, India. To identify potential drug target in this pathogen, we targeted intermediate enzymes in carbohydrate metabolic pathway. In our studies, the nucleic acid and amino acid sequences of Citrate synthase were extracted from KEGG database and Non par log sequences were obtained using CD-Hit [Cluster Database at High Identity with Tolerance]. The solved gene from the KEGG pathways was retrieved and the output of CD-HIT was the base results for drug target identification. We used GOLD program for calculating the docking modes of small molecules inhibitor in protein binding sites. In Docking studies, our results showed better interaction of Citrate synthase with inhibitor namely Nitazoxanide and the modeled proteins can be used as target sites for disease eradication.

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INTRODUCTION

The presence of microorganisms in food is a natural and unavoidable occurrence. Cooking generally destroys most harmful bacteria, but undercooked foods, processed ready-to-eat foods, and minimally processed foods can contain harmful bacteria that are serious health threats. *Campylobacter jejuni* is one of the most common causes of human gastroenteritis in the world [1-3]. Food poisoning caused by *Campylobacter* species can be severely debilitating, but is rarely life-threatening. Several environmental reservoirs can lead to human infection by *C. jejuni,* it can infect humans directly through the drinking water or through the consumption of contaminated animal products, such as unpasteurized milk or meat, particularly poultry.

Campylobacter demonstrate considerable ecological diversity. Most taxa appear to be pathogenic and may be associated with a wide variety of diseases in animals and humans [4,5]. Accurate identification of these organisms is required to provide important clinical and epidemiological information. A variety of approaches have been used to distinguish between species [6,7]. Subtyping of *Campylobacter* spp. is an important aspect of epidemiological studies. Criteria for subtyping include the following: cost, ease of use, and discriminatory power. Phenotypic methods such as serotyping and phage typing are cheap and relatively easy to perform [8]. In the present study, we used bioinformatics tools to identify drug target in carbohydrate metabolic pathway of C.jejuni and evaluated the interaction of drug target with its inhibitor.

MATERIALS AND METHODS

Sampling

Contaminated snack food samples were obtained from a restaurant in Chennai. 10gm of the sample was retrieved aseptically into sterile vials, using sterilized spatulas. The samples were transported to the laboratory within 1hour in a clean thermal container filled with freezing mixture. The media required for the enumeration of organism i.e. Nutrient agar – 2.8g/100ml and Mac conkey – 5.15g/ml were prepared in conical flasks and autoclaved [121oC/15min]. Sterile water [9ml and 10ml] was prepared in test tubes [7,9]. Glass rods, spreaders, pipette tips were also sterilized.

Serial dilution of the sample was done inside a laminar airflow unit that is properly sterilized by UV irradiation method for 20minutes. After irradiation, the air blower was switched on. 1 gm of the food sample was retrieved from the collection vial, using sterile [with 70% ethanol] spatulas/pipettes. It was then added to 10ml of sterile saline/distilled water in test tubes and macerated thoroughly using a glass rod. The sample was allowed to settle down for a few minutes so that the microbial flora will come to the aqueous phase. This forms the 'Master stock'. The sample was then serially diluted by transferring 1ml of the master stock into 9 ml of sterile water. The procedure was repeated till the 10th tube such that the dilution will gradually increase from 10-1 to 10-10. Using sterile tips, 0.1ml of the sample from the 3rd, 5th and 7th dilutions were plated in Nutrient agar and Mac Conkey agar media by spread plate technique, with the help of sterile glass rods. The plates were incubated at 37oC for 24 hours.

Culture and Isolation of pure colonies of C.jejuni

Individual colonies were identified from the Spread plates, based on their morphology [7,9]. These colonies were then grown on Nutrient agar plates by quadrant streaking technique to obtain pure individual colonies to carry out further works. Streak plates allow bacteria and fungi to grow on a semi-solid surface to produce discrete colonies. These colonies can be used to help identify the organism, purify the strain free of contaminants, and produce a pure genetic clone. In order to obtain well-isolated discrete colonies, the quadrant streak technique should be used. This allows sequential dilution of the original microbial material [broth culture or colonies on a plate or slant] over the entire surface of a fresh plate. As the original sample is diluted by streaking it over successive quadrants, the number of organisms decreases. Usually by the third or fourth quadrant only a few organisms are transferred on the inoculating loop and these produce a few isolated colonies.

The inoculating loop was flamed until it is red hot and then allowed to cool. A small amount of bacterial growth [either a loopful from a broth culture or a single colony from a plate or slant] was removed with the sterile inoculating loop. The inoculating loop was immediately streaked very gently over a quarter of the plate using a back and forth motion. The loop was flamed again and allowed to cool. Going back to the edge of area 1 that was just streaked, the streaks were extended into the second quarter of the plate. The procedure was repeated for the entire quadrant. The plates were then inverted and incubated at 37^{o}C for 24 hours. The pure colonies were then identified by Gas Chromatography-Mass Spectrophotometry method.

Bioinformatics tools used to identify genes in C.jejuni

CD-HIT stands for Cluster Database at High Identity with Tolerance [10,11,12]. The program [CD-HIT] takes a fasta format sequence database as input and produces a set of 'non-redundant' [nr] representative sequences as output. In addition cd-hit outputs a cluster file, documenting the sequence 'groupies' for each nr sequence representative. The idea is to reduce the overall size of the database without removing any sequence information by only removing 'redundant' [or highly similar] sequences. CD-HIT uses a 'longest sequence first' list removal algorithm to remove sequences above a certain identity threshold. Additionally the al gorithm implements a very fast heuristic to find high identity segments between sequences, and so can avoid many costly full alignments. KEGG [Kyoto Encyclopedia of Genes and Genomes] is a collection of online databases dealing with genomes, enzymatic pathways, and biological chemicals [13-15]. The metabolic pathway database records networks of molecular interactions in the cells and we used it to identify enzymes in carbohydrate metabolic pathway.

GOLD is a program for calculating the docking modes of small molecules in protein binding sites and is provided as part of the GOLD Suite, a package of programs for structure visualisation and manipulation [Hermes], for protein-ligand docking [GOLD] and for post-processing and visualisation of docking results [16,17]. We have used GOLD in this study because, it is very highly regarded within the molecular modeling community for its accuracy and reliability.

Pfam is a database of protein families that includes their annotations and multiple sequence alignments generated using hidden Markov models. 74% of protein sequences have at least one match to Pfam. This number is called the sequence coverage. The Pfam database contains information about protein domain and families. Pfam-A is the manually curated portion of the database that contains over 10,000 entries. For each entry a protein sequence alignment and a hidden Markov model is stored. These hidden Markov models can be used to search sequence databases with the HMMER package written by Sean Eddy. Because the entries in Pfam-A do not cover all known proteins, an automatically generated supplement is provided called Pfam-B. Pfam-B contains a large number of small families derived from clusters produce by an algorithm called ADDA. Although of lower quality, Pfam-B families can be useful when no Pfam-A families are found.

RESULTS AND DISCUSSION

In our initial studies we identified and confirmed the identity of C jejuni from spoiled food source. Based on the literatures, carbohydrate metabolic pathway was selected for the drug target study. CD-HIT was performed to generate the non-paralogous sequences. Nucleic acid and amino acid sequences were extracted from KEGG database. The solved genes from the KEGG pathways were retrieved and the CD-HIT was performed. The output of CD-HIT was the base results for drug target identification.

The non-paralogous sequence obtained from CD-HIT was given as input to EMBOSS-CUSP server which generate the codon table and saved with extension ".cut" which was given as input along with CD-HIT results to EMBOSS-CAI server generating highly expressed gene. Highly expressed genes were identified with the value >0.7. From the above results the genes which holds value >0.7 in EMBOSS-CAI and genes present in the upstream position of the metabolic pathways were short listed for further analysis of drug target identification.

The template selection was done by performing BLAST against protein data bank [PDB] to find the structural homologs. The percentage of similarity between the target and template was selected in the range of 40% to 90%, for which the target and the template should have the same function. The domain analysis was done for the target and the template sequence to find whether they share the same function. The pfam database was used to find the domains present in the sequence. The pfam result clearly indicates that the target and the template sequence have the same Citrate synthase family [Table 1].

The molecular docking was performed with the Streptococcus pyogenes inhibitor and the modeled proteins acting as drug targets. Potential ligands were screened for the inhibitor study using GOLD. The analog binds with the protein with a positive fitness [Figure 1], which proves that the series of analogs have a potential activity in inhibiting Streptococcus pyogenes by down regulating the protein responsible for the survival of the bacteria. The inhibitor used was Nitazoxanide, the molecular docking results show a fitness score of 44.9 between Nitazoxanide and Citrate Synthase [Table 2]. Our results demonstrated identification of target site on citrate synthase using inhibitor Nitazoxanide, which could potentially be used for drug development in fighting against C.jejuni.

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Table 1
Target Sequence

Significant Pfam-A Matches

Show or hide all alignments.

Family	Description	Entry type	Clan	Envelope		Alignment		HMM		Bit score	E-value	Predicted active sites	Show/hide alignment
				Start	End	Start	End	From	To				
Citrate_synt	Citrate synthase	Domain	n/a	47	411	47	411	1	356	439.7	5.4e-132	266,364,307	Show

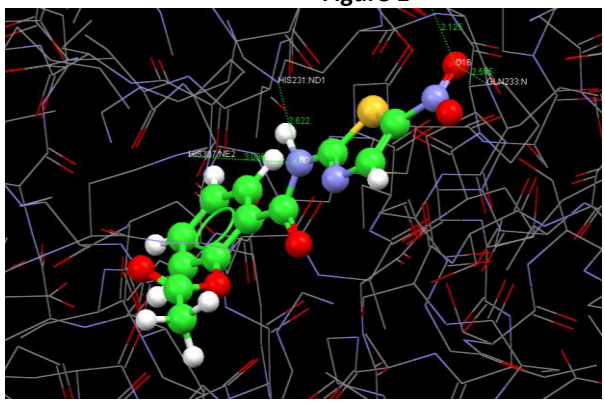
Template Sequence

Significant Pfam-A Matches

Show or hide all alignments.

Family	Description	Entry type	Clan	Envelope		Alignment		HMM		Bit score	E-value	Predicted active sites	Show/hide alignment
				Start	End	Start	End	From	To				
Citrate_synt	Citrate synthase	Domain	n/a	53	418	53	418	1	356	448.4	1.2e-134	272,371,313	Show

Figure 1



3 –Dimensional interaction of Inhibitor (Nitazoxanide) complex with Citrate Synthase

Table 2

Atom in protein	Atom in ligand	Hydrogen bond distance	Score
HIS 231:NDI	N8	2.622	44.9
OLU 232:OEI	O16	2.125	44.9
GLNN 233:N	O16	2.585	44.9
HIS 307:NE2	N8	3.096	44.9

GOLD score representing the interaction of Nitazoxanide inhibitor with Citrate synthase.

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