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Original Article

Production, Purification and Characterization of Chicken Egg Yolk Monoclonal Antibody Against Colonization factor antigen -1 of Enterotoxigenic *Escherichia coli* Causing Diarrhea

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Enterotoxigenic *Escherichia coli* (ETEC) causes diarrhea in both humans and animals. The contaminated food and water are the most common vehicles for ETEC infection. The colonization factor antigen (CFA-1) is a fimbriae protein that promotes adherence of the ETEC strain to the epithelium of the small intestine of the host. In this study IgY proteins were produced against the CFA-1 of ETEC in immunized white leg horn chickens. The collection of antibody was found higher in 25% and 50% ammonium precipitated samples. The isolated CFA-1 protein (0.5 mg/ml) was purified by dialysis (0.42 mg/ml), gel-filtration (0.08 mg/ml), and ion-exchange chromatography (0.065 mg/ml). The CFA-1 protein obtained was submitted to pBLAST which matched with CFA-b with accession no.-AAC41415.1. The CFA-1 gene was cloned in pUC18 vector, and transformed into *Escherichia coli* DH5α strain. In conclusion, we have successfully purified and characterized monoclonal antibodies (IgY proteins) against CFA-1 of enterotoxigenic *E. coli* that may be useful for diagnosis of widely occurring *Escherichia coli* infections.

Keywords: Enterotoxigenic *Escherichia coli*, diarrhea, colonization factor antigen

Enterotoxigenic *Escherichia coli* (ETEC) are predominant facultative anaerobe of the human colonic flora and recognized cause of diarrhea in the developing world. Acute infectious diarrhea is the second most common cause of death in children living in developing countries, surpassed only by acute respiratory diseases accounting for approximately 20% of all childhood deaths (1). ETEC was estimated to cause 210 million infectious cases, leading to the death of 380000 children under the age of 5 years mostly in developing countries (2). Successful adhesion of ETEC to the small intestine mucosa is a precondition for the development of diarrhea, and the pathogenecity is mediated through specific surface structures called pili or fimbriae (3). Traveler's diarrhea is usually contracted from contaminated food and water (4). The practical use of chiken antibodies isolated from egg yolks (IgY) in research and diagnostics is limited due to complex and time consuming purification steps (5). Plasmid-encoded colonization factors (CFs) and one or more plasmid encoded enterotoxins that induce a secretory diarrhea are the major determinants of ETEC virulence (6). CFs are clearly essential to disease initiation, CF-a and CF-b have been suggested to be involved in interactions

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with and /or invasion of the host cells, and in bacterial cell-cell interactions that could promote aggregation and biofilm formation (7). As fimbriae mediate the first step in the pathogenesis of traveler's diarrhea, and considering their antigenic potential, they may be of value in new of vaccine development (8). ETEC strains cause diarrhea through the action of heat-labile enterotoxin (LT) and heat-stable enterotoxin (ST). These strains may express either one type of enterotoxin, or both of them. LTs of *E. coli* are oligomeric toxins that are closely related in structure and function to the cholera enterotoxin (CT) expressed by vibrio cholerae (9). The fimbrial colonization factor antigen (CFA) of enterotoxigenic *E. coli* was purified and characterized (10). DNA vaccines were used to induce immune responses against an enterotoxigenic bacterial pathogen (11). Targeting virulence factors such as toxins and CFs are the most effective approaches for ETEC vaccine development strategy (11, 12). ETEC isolates were typed for CFA expression with a dot blot assay using a panel of 12 monoclonal antibodies against CFA/1, CS1 to CS8,CS12,CS14, and CS17 (13-15). It was reported that LT expressing ETEC isolates may cause severe diarrhea, given that they were isolated from patients with severe dehydrating diarrhea (16). A number of vaccine approaches are currently being pursued with a combined toxin –CFA approach (17). Both LT and ST generate net secretion of ions and water, resulting in watery diarrhea by the activation of the cystic fibrosis transmembrane regulator (CFTR) chloride channel due to the increase of cyclic AMP (cAMP) or cyclic guanosine monophosphate (cGMP) levels (18). Acute diarrheal disease is an important health problem among children less than 5 years of age in developing countries in Africa, Asia and Latin America (19). In this study, CFA-1 gene was cloned and transformed into pUC18 vector and DH5α, respectively. Also, monoclonal antibody (IgY proteins) against CFA -1 of enterotoxigenic *E. coli* that may be useful for diagnosis of widely occurring *E. coli* infections was successfully purified and characterized.

Materials and methods

Sample collection and CFA-1 protein isolation from ETEC

The ETEC culture was procured from culture collection of Bio Genics, Hubli, Karnataka, and was routinely grown on tryptone soy agar plates in broth at 37 °C for 24 h. ETEC was washed 3 times with phosphate buffer saline (PBS) and heated at 60 °C for 30 min in water bath, and immediately sonicated at 180-200 frequency for 10 min. CFA-1 protein was collected after centrifugation at 10000 rpm.

Purification and identification of CFA-1 protein

CFA-1 protein was purified by ammonium sulphates (25%, 50%, 75% saturation level), dialysis (5 mM sodium phosphate buffer (pH 7.3) for 16 h), gel-filtration (50 mM sinking pre-beta-lipoprotein (SPB), gel filtration matrix activated at 50 °C for 30 min) and ion- exchange chromatography (500 mM SPB-elution buffer and 50 mM SPB buffer reservoir). CFA-1 samples were read at 595 nm absorbance using Bradford reagent. Purified CFA-1 protein was analysed on 10% SDS-PAGE. The gel was fixed in fixing solution (methanol, acetic acid, distilled water 4:1:5) for 2 h with continuous shaking, and washed 3 times (30 min each) with 50% ethanol. After that, the gel was treated with pre-treatment solution (0.05 g sodium thiosulphate in 25 ml distilled water) for 1 min followed by 3 washes in distilled water (20 s each). Soaked gel was put in silver nitrate solution (0.05g AgNo3 and 19 µl formaldehyde in 25 ml distilled water) for 2 min followed by 2 washes with distilled water (20 s each). The gel was then immersed in developer (3 g Na2Co3, 25 µl formaldehyde, and 0.002 g sodium thiosulphate in 50 ml distilled water) till the development of brown bands. ELISA was performed to find out the maximum and minimum antibody titre in different samples.

Immunization of chickens

23 weeks old white leg horn chicken were taken for immunization with immunogen (0.5 ml antigen dissolved in PBS and 0.5 ml complete freunds adjuvants) given intramuscularly. After 4 weeks a booster dose (0.5 ml antigen with PBS and 0.5 ml incomplete freund's adjuvants) was given intramuscularly. After immunization, eggs were collected with an interval of 2 weeks.

Isolation and purification of IgY

Egg's (9 weeks old) yolk was diluted with distilled water (1:9 vol/vol), pH was adjusted to 5.0 with 0.1 N HCL for overnight at 4 °C. The yolk was precipitated with ammonium sulphate, and then undergone SDS-PAGE and ELISA to find out the desired IgY proteins. IgY proteins were purified by affinity chromatography and washed with 20% ethanol and 20 mM PBS. Eluted sample was preserved in 100 µl Tris-HCl pH 8.8.

PCR amplification and analysis of CFA-1 gene

The 18 h old culture of *E. coli* (50 ml) was used for the isolation of plasmid DNA by different solutions (solution A: 50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA; solution B: 0.2 N NaOH, 1% SDS; solution C: 3 M potassium acetate; solution – D: isopropanol). PCR reaction mixture (2 µl template DNA, 1 µl 10x buffer, 0.4 µl dNTPs, forward 5'-CTTACCACCAAGCTTATGAAA TTTAAA-3' and reverse 5'-CTGCCCGGGTCA GGGTCCCAAAGT-3' primers (1 µl each), 0.3 µl Taq DNA polymerase, and 4.3 µl sterile water) was used to amplify DNA through PCR. The confirmed amplify product of CFA-1 gene was sequenced by Bioserve Biotechnologies, Hyderabad.

Cloning and transformation of CFA-1 gene

E. coli DH5α culture with 100 ml LB medium was centrifuged, and the pellet was suspended in 0.1 M CaCl2 solution aseptically for competent cell preparation. pUC18 vector was used for cloning with reaction mixture (1 µl 10x restriction enzyme buffer, 1 µl pUC18, 0.3 µl Hind III restriction enzyme, and 7.7 µl HPLC water) in water bath at 37 °C for 1 h. The amplified CFA-1 gene was enzymatically digested using the following reaction mixture (1 µl 10x restriction enzyme buffer, 8.7 µl PCR product, and 0.3 µl Hind III restriction enzyme). The digested vector was used to insert the amplified product in multiple cloning site (10 µl digested vector, 2.5 µl ligation assay buffer, 0.2 µl T4 DNA ligase, 10 µl PCR product, and 2.3 µl HPLC water at 16 °C over night). The 200 µl competent cells and 15 µl recombinant DNA plasmid were mixed and placed on ice for 20 min, heat shock was then applied on water bath at 42 °C in the presence of LB- broth medium. The plates were then observed for blue (non recombinant) and white (recombinant) colonies between 24-48 h in the presence of LB-ampicillin (60 µg/ml) with X-gal (50 µl, 2% in DMF) and isopropyl β-D-1-thiogalactopyranoside (IPTG) (10 µl, 100 mM).

Results

Purification of CFA protein

The concentration of CFA-1 protein was 0.42 mg/ml after dialysis. Due to gel filtration column, the CFA proteins were collected in 25 fractions of 1.5 ml each. The highest optical density (OD) of 0.760 was noticed in the 13th fraction. The active fraction of the highest OD was found in 11-16th fraction. Further, ion-exchange column analysis revealed higher protein concentration of CFA in the fraction tube 10-16th as shown in figure 1 and table 1.

The CFA-1 proteins had a molecular weight around 29 kDa by SDS-PAGE analysis (figure 2).

Purification of IgY

ELISA test of different samples (control, ETEC water soluble fraction, 25%, 50%, and 75% ammonium sulphate precipitates, and supernatant from ammonium sulphates) showed that 25% and 50% ammonium precipitated samples had more antibody titre in comparison with other samples as shown in figure 3. The IgY proteins were of molecular weight around 34 kDa in the SDS-PAGE.

Isolation of CFA-1-gene.

The presence of CFA-1 gene was confirmed

on agarose gel as compared to a molecular marker (figure 4A). The. The expected size of CFA gene after PCR amplification was 483 bp as shown in figure 4B. The sequence was observed to possess fimbrial CS1 superfamily domain as confirmed by matching with database.

Cloning and transformation of CFA-1 gene

The white colonies indicated the successful transformation of the CFA-1 gene as shown in figure 5.

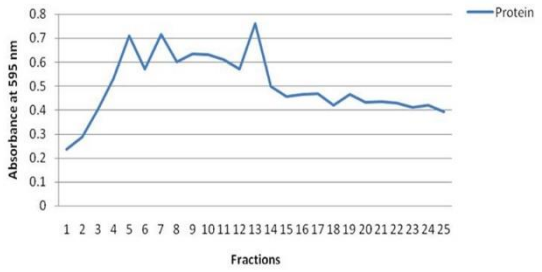


Figure 1. Protein content of gel filtration column eluted fractions.

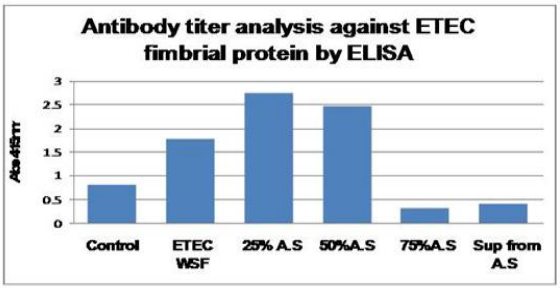


Figure 3. Antibody titre analysis against ETEC fimbrial protein by ELISA.

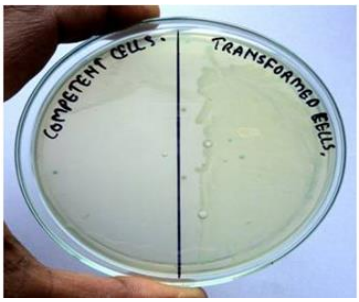


Figure 5. Transformed cells on TS agar medium. Blue and white colonies correspond to non-recombinant and recombinant vectors, respectively.

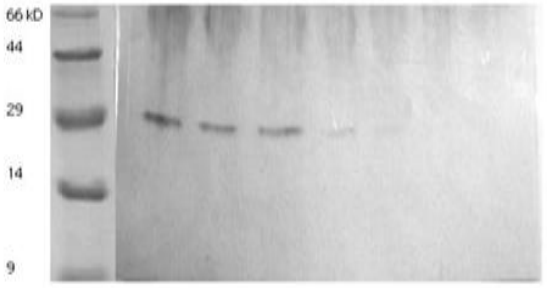
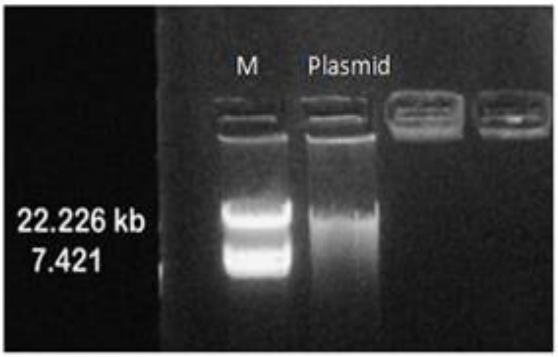


Figure 2. CFA bands on SDS-PAGE.



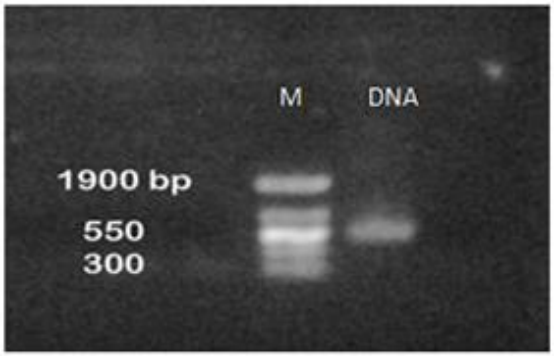


Figure 4. DNA analysis on agarose gel. A: left panel showing pUC18 linear band; B: right panel showing the CFA-1 amplified PCR product. M: molecular weight size marker.

Table 1. Protein concentration at different purification steps

Purification step	Protein Concentration (mg/ml)	Yield (%)
Crude	0.5	100
Dialysis	.42	84
Gelfiltration	.08	16
Ion-exchange	.065	13

Discussion

ETEC has a significant prevalence in various sample types such as fecal, in and around hospital, waste materials, sewage water and polluted drinking water. CFA-1 is a polymer of identical subunits with an N-terminal valine, 37% hydrophobic amino acids residues and 11 residues of proline per molecules. CFA-1 protein was purified by using ammonium sulphates precipitates dialysis, gel-filtration, and ion-exchange chromatography. The isolated antigen protein was then prepared for the chicken immunization. Immunized chickens are known to produce antibody (IgY) effectively in their egg yolk (20). IgY antibody is also relatively stable under various conditions including heat, pressure, acid, alkali, and proteolytic enzymes (21). The water soluble fraction containing IgY was purified from egg yolk using the water dilution method (22). Finally CFA-1 gene was isolated from ETEC by phenol method and then analysed by gel-electrophoresis with standard marker. The gene sequence was virtually translated with the help of ORF finder (http://www.ncbi.nlm.nih.gov/projects /gorf/) and the amino acid sequence was also deduced. A sequence of 170 amino acids was obtained with a CD from base 1 to 152. The protein obtained was submitted to pBLAST which matched with CF-b antigen with accession no.-AAC41415.1. The sequence was observed to possess fimbrial CS1 superfamily domain confirming the function of isolated gene. The gene was cloned into pUC18 and transformed successfully in *E. coli* DH5α. The synthetic ligand for IgY affinity purification consisted of a tetrameric tripeptide (Arg-Thr-(Tyr) 4-k2-k-G), named- TG19318, synthesized by solid phase peptide synthesis using a peptide synthesizer.

In conclusion, the CFs obtained in the present study may be used as biomarker for the study of ETEC adhesion, and for the generation of antibodies. The antibody generation can be conveniently used for the diagnosis and therapy of ETEC widely occurring in man as well as in livestock.

Conflict of interest

The authors declared no conflict of interest.

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