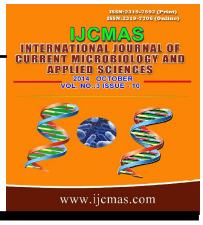
International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 3 Number 10 (2014) pp. 290-300**
http://www.ijcmas.com



Original Research Article

Identification of HT-29 plasma membrane proteins as binding glycoproteins of enterotoxigenic *Escherichia coli* (ETEC) colonization factor antigen I (CFA/I)

B.Srinivasa Reddy[1,2] and P.Rama Bhat[3*]

[1]BioGenics, Veena Plaza, P.B.Road, Unkal, Hubli, Karnataka, India
[2]Research & Development Centre, Bharathiar University, Coimbatore, Tamil Nadu, India
[3]PG Department of Biotechnology, Alva's College, Moodbidri-574 227, Karnataka, India
**Corresponding author*

Keywords

HT-29 plasma membrane proteins, Entero-toxigenic *Escherichia coli* (ETEC)

A B S T R A C T

Colonization factors are important protein components of Enterotoxigenic *Escherichia coli* which are involved in adhesion to the intestinal epithelium and initiate pathogenesis. Among the several colonization factors identified so far, colonization factor antigen I (CFA/I) is the prominent fimbrial antigen present on almost one-third of the ETEC isolates. CFA/I fimbriae isolated from Enterotoxigenic *Escherichia coli* strain SB5 was assessed for the binding proteins in adhesion assays and by western blot analysis. Adhesion assays revealed the adherence of CFA/I fimbriae to the plasma membrane proteins which was affected by the unbiotinylated CFA/I as well as by periodate oxidation of membrane proteins. Two plasma membrane proteins of approximately 66 and 58 kilodaltons molecular weight were detected in western blot analysis that bind to the biotinylated CFA/I fimbrial protein. On pretreatment of HT-29 plasma membranes with sodium metaperiodate, the binding of biotinylated CFA/I protein to the plasma membrane proteins was significantly reduced which led to conclude that these proteins are glycoproteins. The structural studies of these CFA/I binding proteins will be instrumental in understanding the fimbrial interaction with the binding proteins and also in developing chemical analogues with an inhibitory role in the bacterial adhesion process.

Introduction

Enterotoxigenic *Escherichia coli* (ETEC) are the causal agents of diarrhea in infants and also in the cases of traveler's diarrhea (Black, 1990). The clinical symptoms of pathogenesis are initiated by heat-labile (LT) and/or heat-stable enterotoxins expressed by bacteria (Sack, 1975; Gyles, 1992; Spangler, 1992).

Bacterial adhesion to intestinal epithelial cells is a prerequisite phenomenon for colonization and subsequently for exhibiting pathogenesis (Cheney and Boedecker, 1983; Levine, 1987). In ETEC and also in most of the infectious organisms, bacterial adhesion is accomplished by specific adhesion proteins that are expressed by bacteria in the

form of fimbrial structures which act as virulence factors. These fimbrial structures are referred as colonization factors (CFs) (De Graaf, 1990). As many as twenty five CFs were identified in human ETEC strains (Wolf, 1997). These were designated as coli surface antigens (CSs) based on their chronological identification. These colonization factors are multi subunit structures of coli surface antigens as alone or as combinations exception being CFA/I which is a single fimbrial structure.

CFA/I was the first colonization factor to be characterized by studying the prevalence of the ETEC strains bearing CFA/I (Ahrén, 1986), plasmid bearing the CFA/I gene and its expression (Ahrén, 1986; Grewal, 1997), hemagglutination pattern of ETEC strains bearing CFA/I colonization factor (Evans, 1975, 1978; Gonzales and Blanco, 1985). Attempts were also made to understand the target receptor proteins for CFA/I in different cell models. Erythrocytes were studied to understand the human ETEC colonization factors interaction with cell surface proteins involved in hemagglutination by utilizing naturally occurring glycopeptides and oligosaccharides. These glycopeptides and oligosaccharides were able to inhibit the hemagglutinations of CFA/I- and CFA/II-expressing strains (Neeser, 1988). Studies revealed that sialic acid, sialic acid-containing glycopeptides (Bartus, 1985; Gonzales and Blanco, 1985), the GM2-like glycoconjugate (Faris, 1980; Buhler, 1991; Lena Jansson, 2006) or the asialo-GM1 (Oro, 1990) are glycopeptides on erythrocytes facilitate binding of CFA/I colonization factor in the process of hemagglutination.

Enterocytes represent the intestinal mucosa and these brush border enterocytes are the target of ETEC and other pathogenic bacteria (Forstner, 1978; Walker, 1985; Falkow, 1992). ETEC adhesion to isolated human duodenal enterocytes in vitro established an improved adhesion assay for studying the colonization factor target receptor (Knutton, 1984a,b; Knutton, 1985). Human colon adenocarcinoma cell line as an invitro model of the mature enterocyte of the small intestine was explored for understanding the ETEC adhesion as well as target receptors for colonization factors (Lindahl, 1982; Wenneras, 1990; Kerneis, 1992; Kerneis, 1994).

In this report, the binding of colonization factor antigen I (CFA/I) to human colon adenocarcinoma cell line, HT-29, was studied in adhesion assays as well as western blotting method. The adhesion of biotinylated CFA/I to the HT-29 plasma membranes was prevented by unbiotinylated CFA/I in a dose dependent manner. Two prominent membrane protein bands were identified when probed with biotinylated CFA/I of approximately 66 and 58kDa which were not observed on treatment of the membrane proteins with sodium metaperiodate indicating that the colonization factor binding to these glycoproteins involve the carbohydrate moiety of the glycoprotein.

Materials and Methods

Reagents: Biotinamidocaproate N-Hydroxysuccinimide ester (Cat. B2643), Streptavidin–Peroxidase from *Streptomyces avidinii* (Cat.No – S5512) and 3,3',5,5'-Tetramethylbenzidine for adhesion assay were purchased from Sigma Aldrich. 10X TMB Substrate and protein markers were purchased from Bangalore Genei, Bangalore, Karnataka, India.

Bacterial Strains and growth conditions: An Enterotoxigenic *Escherichia coli* strain

SB5 – GenBank Accession number KM114609 (078, ST) was isolated from diarrheal stool samples from a diagnostic laboratory. The isolated bacterium was tested for mannose-resistant hemagglutination by human, bovine and chicken erythrocytes. The erythrocytes were suspended in phosphate buffered saline (pH 7.2) in the presence and absence of 1% mannose. A glass slide with bacterial cells mixed with erythrocyte suspensions was assessed for hemagglutination.

Colonization factor Antigen I isolation

ETEC fimbrial purification was performed by method described previously (Evans, 1979; Anantha, 2004). ETEC SB5 strain was grown in CFA agar at 37^0C overnight. The cells were harvested in 100mM phosphate buffer, pH 7.2. The cells were homogenized in a blender and centrifuged at 12,000 x g for 30min. The obtained supernatant was left at 4^0C for 3 days and subjected to ammonium sulphate precipitation at 20% saturation. The 20% saturated protein solution was centrifuged to collect the precipirate and ammonium sulphate was added to achieve a final 40% saturation. The resultant precipitate was collected by centrifugation and resuspended in phosphate buffer. After 24h dialysis, the protein solution was further purified by chromatography on a DEAE-Sephadex column. The protein purity was determined by SDS-PAGE followed by coomassie staining and protein quantification by the Bradford assay.

Cells, culture conditions and membrane preparations: Intestinal epithelial cell carcinoma, HT-29 cells were used for the study. These cells were cultured in DMEM supplemented with 10% inactivated fetal calf serum in a glucose-free medium and 1% penicillin/streptomycin at 37^oC in a 10% CO_2/90% air atmosphere. Glucose- free medium was utilized for the process of cell differentiation. The cultures were maintained in T-25 flasks with routine passages using 0.25% trypsin EDTA. Five confluent T-25 flasks were taken for isolation of plasma membrane preparation.

Tris-buffered saline solutions (TBSS) containing 0.16 M NaCl, 3 mM $MgCl_2$, 5 mM KC1 in 10 mM Tris-HCl (pH 7.4 at 4°C) was used throughout the membrane isolation steps (Millette et al., 1980). Cells were first swollen in hypotonic medium by the addition of 0.8 ml of homogenizing buffer (TBSS diluted 1/10 with 10 mM Tris-HCl, pH 7.4 at 4°C) for 5 min. After homogenization, 0.08 ml 10 x TBSS was added to approximate isotonicity and to stabilize the nuclei. Unbroken cells, nuclei and large aggregates of debris were removed by centrifugation for 30sec at 1000g. Supernatant was collected in a fresh tube for further isolation procedure. Plasma membranes were isolated from the supernatant material by centrifugation on discontinuous sucrose density gradients in TBSS. The supernatant was mixed with equal volume of 80% sucrose w/v to yield a 3ml of 40% sucrose solution containing membranes. The 40% sucrose material was layered on top of 3ml of 45 % sucrose w/v in TBSS. Five millilitres of 30% sucrose (w/v) in TBSS were then layered above the 40% sucrose, followed by 1 x TBSS to fill the tube. Gradients were centrifuged at 25,000 x g at 4°C for 90 min. Then the plasma membrane pool was collected, washed and resuspended in TBSS buffer.

Labeling of colonization factor antigen I

Biotinylated CFA/I was prepared by incubating the biotin ester (Sigma, B2643) with colonization factor antigen I in sodium phosphate buffer, 100mM, pH 7.4. Biotin

ester was incubated with CFA/I at a concentration of 15 to 20 moles biotin ester / mole of protein and incubated for 1hour at room temperature. The reaction was stopped by adding primary amine solution to quench the free biotin and the solution was then subjected to dialysis at 4^{o}C overnight to remove unbound biotin ester.

Biotinylated Adhesion assays and western blotting analysis: Biotinylated adhesion assays were performed using isolated plasma membrane protein from HT-29 cells. The plasma membrane protein suspended in 50mM carbonate-bicarbonate buffer (pH 9.6) was added to microtiter plates at a concentration of 2.5µg protein per well and then incubated overnight at 4^{o}C. The contents of the wells were then aspirated and washed with PBS containing 0.05% tween 20 (PBST). The wells were blocked using 1% BSA in carbonate: bicarbonate buffer and incubated for 37^{o}C for 45min. The BSA solution was then discarded and each well was washed four times with PBST. A 100µl biotinylated CFA/I was added at a concentration of 0.3µg per well and plate was incubated at 37°C for 1hr. The wells were washed with PBST for three times. Horseradish peroxidase conjugated to streptavidin (100µl of 1:1000 dilution in PBS) was added to each well and incubated for 45min at RT. The wells were rinsed three times with PBST and the chromogenic peroxidase substrate 3,3',5,5'-Tetramethylbenzidine was added to detect the bound horseradish peroxidase conjugated to streptavidin. BSA was incubated in the microtiter wells instead of plasma membranes and unbiotinylated CFA/I instead of biotinylated CFA/I were controls included in the adhesion assay.

The reagents and the plasma membrane protein used for biotinylated adhesion assay were also utilized for western blotting experiment. A 25µg plasma membrane protein was loaded on 12% SDS-PAGE and then transferred to PVDF membrane. The filter was blocked with 5% skimmed milk powder (Tris buffered saline with 0.05% tween 20 - TBST) for 90min at RT with shaking. The blocking buffer was discarded and the membrane was washed five times with TBST. The biotinylated CFA/I was added at 0.3µg per ml TBS to the membrane and incubated for 90min at RT with shaking. The membrane was washed with TBST and horseradish peroxidase conjugated to streptavidin added at a dilution of 1:4000, to the membrane and incubated for 45min at RT. The ST-HRP solution was discarded and membrane was washed and incubated with the chromogenic substrate TMB leading to detection of biotinylated CFA/I bound plasma membrane proteins.

Sodium metaperiodate treatment

Periodate oxidation of plasma membrane proteins was performed by treating with 10mM sodium metaperiodate in 0.2M sodium acetate (pH 4.5) for 1h at RT. A control protein pool was also treated with 10mM sodium iodate in 0.2M sodium acetate (pH 4.5) (Erickson, 1992). After incubation of membrane proteins with sodium metaperiodate and relevant control sample, was centrifuged at 25,000 x g, 20min at 4°C. The membrane proteins were washed with PBS and collected as described above. The treated membrane protein pool was used for adhesion assays as well as for western blot analysis.

Results and Discussion

Adhesion Assay using HT-29 plasma membranes

Colon Adenocarcinoma cell line, HT-29 plasma membranes were used to understand

the binding receptors for biotinylated CFA/I in adhesion assay. Biotinylated CFA/I exhibited adhesion to HT-29 plasma membranes (Fig. 1) and treatment of these membranes with sodium metaperiodate, an oxidizing agent of carbohydrate moiety of glycoproteins, led to significant reduction of CFA/I adhesion to the plasma membranes (Fig. 2). The adhesion of biotinylated CFA/I was also studied in the presence of increasing amounts of unbiotinylated CFA/I (Fig. 1). The reduction in adhesion of biotinylated CFA/I to the plasma membranes in the presence of increasing amounts of unbiotinylated CFA/I can be attributed to the competition to the specific binding sites on the plasma membranes. The robustness of the adhesion assay using biotinylated CFA/I was confirmed by relevant controls such as wells coated with BSA instead of HT-29 plasma membranes (Fig. 2). These results indicate that there are membrane binding sites available for CFA/I and these membrane binding sites are specific for CFA/I. These binding sites are impacted by periodate oxidation of the carbohydrate moieties on the membrane proteins suggesting that these binding proteins could be glycoproteins.

Identification of colonization factor antigen binding proteins

The identification of binding proteins for CFA/I was explored by western blot analysis. The biotinylated CFA/I was found to bind two HT-29 membrane proteins which were of molecular weight approximately 66kDa, 58kDa (Fig. 3). The specificity of these binding proteins was examined by incubating the transferred proteins on pvdf membrane with unbiotinylated CFA/I. A 100 fold excess of unbiotinylated CFA/I was used in the analysis. The binding proteins were detected specifically in the sample lane where only biotinylated CFA/I was used and these binding proteins were undetected and significantly reduced in the lanes where excess amounts of unbiotinylated CFA/I was employed along with biotinylated CFA/I highlights the specificity of these binding proteins for CFA/I (Fig. 4).

Identification of CFA/I binding proteins as glycoproteins

The biotinylated CFA/I adhesion assay using sodium metaperiodate treated membranes suggested the possibility of glycoproteins as binding proteins of CFA/I. The binding proteins that were identified in the figure 3 were further studied by treating the HT-29 plasma membranes with sodium metaperiodate. As evident from the western blot analysis that the proteins that were detected by the biotinylated CFA/I were not detected in the sodium metaperiodate treated HT-29 plasma membrane proteins (Fig. 5). These studies indicate that the identified proteins by the biotinylated CFA/I were glycoproteins and demonstrate that carbohydrate component of the glycoproteins are involved in the CFA/I interaction with the binding proteins. This result indicates that the plasma membrane proteins approximately 66kDa and 58kDa molecular weight are binding proteins of CFA/I and these proteins are possibly of glycoproteinaceous in nature.

In the present study, an effort was made to identify the binding proteins for one of most prominent colonization factor, CFA/I, by utilizing an *in vitro* epithelial cell model - HT-29, a human colon adenocarcinoma cell line. Initial studies by researchers with ^{14}C-labeled ETEC strain expressing CFA/I reported that CFA/I expressing ETEC strains were unable to bind to HT-29 cell monolayers whereas ^{14}C-labeled ETEC strain expressing CFA/II showed adhesion

to the HT-29 cell monolayers (Neeser, 1989). However, it was demonstrated that ^{14}C labeled ETEC strains expressing CFA/I adhered to the differentiated HT-29 cells compared to undifferentiated HT-29 cells (Kerneis, 1992). Based on these reports, differentiated HT-29 cells were cultured and used for our studies in identifying CFA/I binding proteins. It was observed in our adhesion assays using HT-29 plasma membranes that biotinylated CFA/I displayed a significant adhesion to these plasma membranes indicating that there are receptors or binding sites available on the HT-29 plasma membrane. These results are in correlation with the reports that differentiated HT-29 cells express binding proteins for CFA/I colonization factor.

Binding properties of purified CFA/I and CFA/I bearing strains in invitro experiments using intestinal brush borders and intestinal cell lines were examined (Knutton et al., 1985; Neeser et al., 1989; Kerneis et al., 1994). Studies using glycosphingolipids demonstrated that CFA/I binding involve glycosphingolipids (Lena Jansson, 2006). CFA/I protein structural studies with the mutant subunits of CFA/I specifically indicated the binding component of CFA/I crucial for interaction with the human intestinal binding sites (Baker, 2009).

Though the genetics and protein structure of CFA/I and other fimbriae was studied extensively, the target cell receptors of these fimbriae is less known. Studies using CFA/I and three components of CFA/II binding to electrophoretically separated rabbit intestinal brush border and HT29 cell lines was performed (Wenneras, 1990). It was revealed that CFA/I and CFA/II fimbriae bound to proteins of molecular weights between 30 and 35kDa. In our study using differentiated HT29 cells and CFA/I, binding proteins of molecular weight of approximately 58 and 66kDa were observed. These differences reported in the CFA/I binding proteins using HT-29 cell membranes could be because of the differentiation process associated with the cells. It was reported earlier that attachment of ETEC to the enterocytic brush border was due to expression of CFA specific receptors during the cell differentiation process of human intestinal cells. The expression and accumulation of ETEC CFA receptors is a growth-related phenomenon (Kerneis, 1992) and also reported to be dependent on state of differentiation of human colon cancer cells in culture.

In conclusion, our studies revealed two CFA/I binding proteins of approximately 58 and 66kDa molecular weight proteins and these proteins are glycoproteins which are in agreement with previous findings that the CFA/I fimbriae bind to the carbohydrate sequences that are present in human small intestinal glycosphingolipids and glycoproteins.

Further understanding of these CFA/I binding proteins will provide insights into the (fimbriae and its relevant) structural details of the binding proteins which may be instrumental in understanding the fimbrial interaction with binding proteins and also designing new chemical entities or analogues which interfere with the adhesion of ETEC strains bearing CFA/I fimbriae.

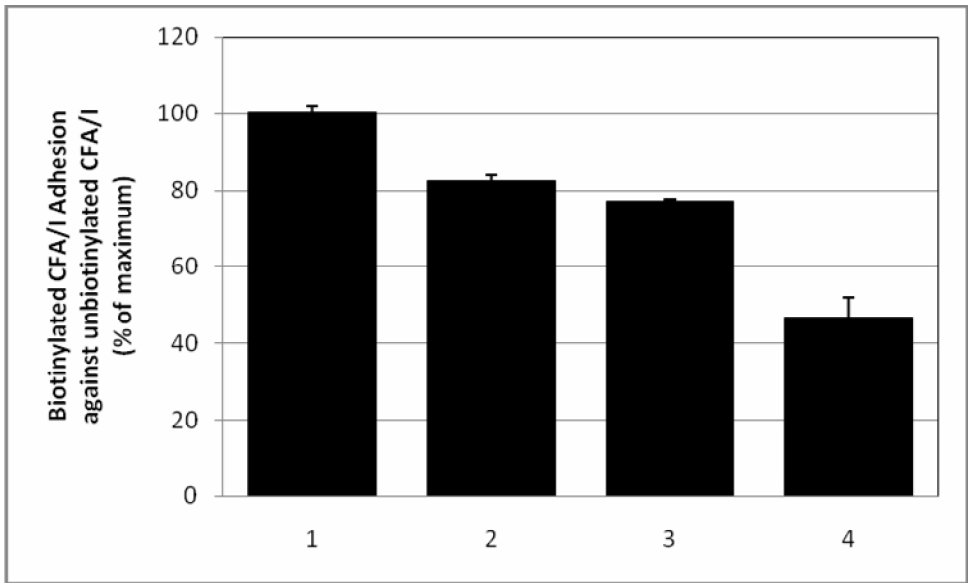


Fig.1 Binding of biotinylated CFA/I to plasma membrane proteins from HT29 cells – Adhesion assay was studied by coating 2.5µg of membrane proteins onto wells of the microtiter plates and incubated with biotinylated CFA/I (Column 1) and unbiotinylated CFA/I was also included at an amount of 1µg, 3µg and 30µg along with biotinylated CFA/I (Columns 2,3,4). Vertical bars indicate standard deviation.

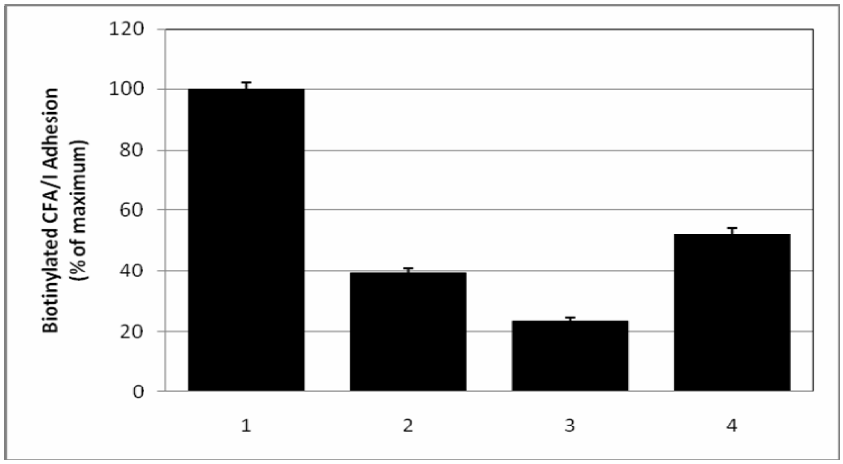


Fig.2 Binding of biotinylated CFA/I to sodium metaperiodate treated plasma membrane proteins from HT29 cells – Column 1 represent the adhesion observed in the untreated membranes, column 4 represent the adhesion pattern in the sodium metaperiodate treated membranes. Non specific adhesion pattern with the reagent system was observed in column 2 and column 3 which represent HT29 membrane incubation in BSA coated wells and BSA coated wells alone respectively. Vertical bars indicate standard deviation.

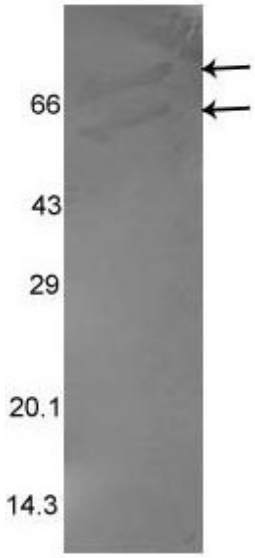


Fig.3 Identification of CFA/I binding proteins – HT29 plasma membrane proteins (25µg per lane) was subjected to SDS-PAGE (12% polyacrylamide) and transferred to PVDF membrane. Binding proteins were detected by the biotinylated CFA/I and developed by the Streptavidin conjugated horseradish peroxidase detection system as mentioned in the materials and methods. The detected binding proteins were pointed by the arrows indicating the location of the binding proteins. Protein molecular weight markers are also labeled.

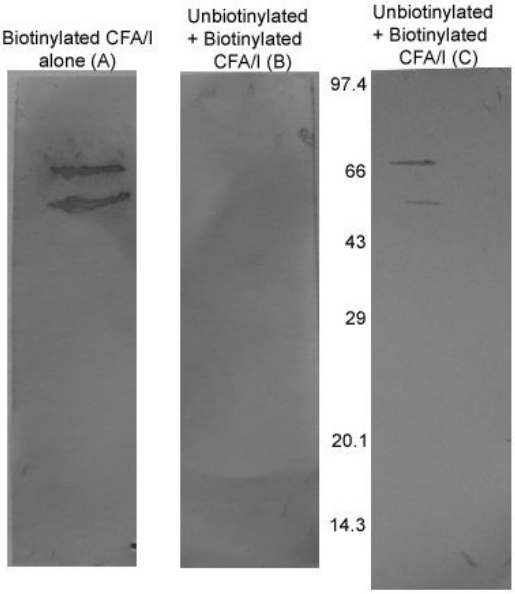


Fig.4 Specificity of binding proteins for CFA/I – The specificity of binding proteins for CFA/I was assessed by incubating the PVDF transferred proteins with biotinylated CFA/I (lane A) and protein transferred onto PVDF, B and C labeled lanes were incubated with 100 fold excess of unbiotinylated CFA/I and developed as mentioned in the materials and methods. Protein molecular weight markers were labeled on the blots.

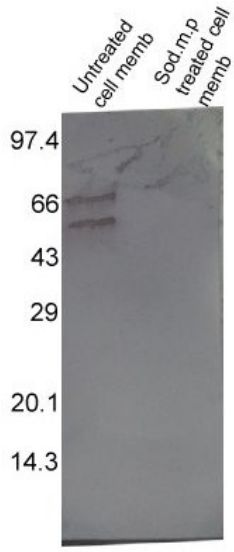


Fig.5 CFA/I binding proteins are glycoproteins - The membrane proteins were treated with 10mM sodium metaperiodate for inactivation of carbohydrate moieties by oxidation. The untreated and treated samples were subjected to SDS-PAGE followed by western transfer onto PVDF which was incubated with biotinylated CFA/I and developed as mention in the materials and methods. Sodium metaperiodate treatment of plasma membranes led to the loss of binding of CFA/I to the plasma membrane proteins.

Acknowledgements

The author acknowledges the support extended by Dr. Girish Babu K for his suggestions and comments while preparing the manuscript.

References

Ahrén, C. M., Gothefors, L, Stoll, L. B, Salek M. A and Svennerholm, A M. 1986. Comparison of methods for detection of colonization factor antigens on Enterotoxigenic *Escherichia coli*. J. Clin. Microbiol. 23(3): 586.

Anantha R.P, McVeigh, A.L, Lee, L.H, Agnew, M.K, Cassels, F.J, Scott D.A, Whittam, T.S and Savarino, S.J. 2004. Evolutionary and functional relationships of colonization factor antigen I and other class 5 adhesive fimbriae of Enterotoxigenic *Escherichia coli.* Infect. Immun. 72:7190-201.

Baker, K.K., Levine, M.M, Morison, J, Phillips, A. and Barry, E. M. 2009. CfaE tip mutations in enterotoxigenic *Escherichia coli* CFA/I fimbriae define critical human intestinal binding sites. Cell Microbiol. 11: 742-754.

Bartus, H., P. Actor, E. Snipes, Sedlock, D. and Zajac, I. 1985. Indication that the eythrocyte receptor involved in enterotoxigenic *Escherichia coli* attachment is a sialoglycoconjugate. J. Clin. Microbiol. 21:951–954.

Black, R. E. 1990. Epidemiology of travelers' diarrhea and relative importance of various pathogens. Rev. Infect. Dis. 12(Suppl. 1):S73–S79.

Buhler, T., H. Hoschutzky and Jann, K. 1991. Analysis of colonization factor antigen I, an adhesin of enterotoxigenic *Escherichia coli* O78:H11 fimbrial morphology and location of the receptor-binding site. Infect. Immun. 59: 3876–3882

Cheney, C. P. and Boedecker, E. C. 1983.

Adherence of an enterotoxigenic *Escherichia coli* strain, serotype O78:H11, to purified human intestinal brush borders. Infect. Immun. 44: 514–518.

De Graaf, F. K. 1990. Genetics of adhesive fimbriae of intestinal *Escherichia coli*. Curr. Top. Microbiol. Physiol. 151: 29–53.

Erickson A.K., Willgohs, J.A, McFarland,S. Y, Benfield, D.A and Francis, D.H. 1992. Identification of two porcine brush border glycoproteins that bind the K88ac adhesion of *Escherichia coli* and correlation of these glycoproteins with the adhesive phenotype. Infect. Immun. 60: 983-988.

Evans, D. G., and D. J. Evans, Jr. 1978. New surface-associated heat-labile colonization factor antigen (CFA/II) produced by enterotoxigenic *Escherichia coli* of serogroups 06 and 08. Infect. Immun. 21: 638-647.

Evans, D. G., Evans, D. J. Jr., Clegg, S. and Pauley, J. A. 1979. Purification and characterization of the CFA/I antigen of enterotoxigenic *Eschenchia coli*. Infect. Immun. 25: 738-748.

Evans, D. G., Silver, R. P., Evans, D. J. Jr., Chase, D. G. and Gorbach, S. L. 1975. Plasmid-controlled colonization factor associated with virulence in *Escherichia coli* enterotoxigenic for humans. Infect. Immun. 12: 656-667.

Falkow, S., Isberg R.R and Portnoy, D.A. 1992. The interaction of bacteria with mammalian cells. Ann. Rev. Cell Biol. 8: 333-63.

Faris, A., M. Lindahl, and Wadstrom, T. 1980. GM2-like glycoconjugate as possible erythrocyte receptor for the CFA/I and K99 haemagglutinins of enterotoxigenic *Escherichia coli*. FEMS Microbiol. Lett. 7:265–269

Finlay, B.B. and Falkow, S. 1990. Common themes in microbial pathogenicity. Microbiol. Rev. 53: 210-30.

Forstner, J.F. 1978. Intestinal mucins in health and disease. Digestion 17: 234-63.

Gonzales, E. A., and Blanco J. 1985. Comparative study of inhibition of mannose-resistant hemagglutination caused by CFA/I, CFA/II, K88, and K99-positive *Escherichia coli* strains. FEMS Microbiol. Lett. 29:115–121.

Grewal, H. M. S., Valvatne, H, Bhan, Dijk, M. K, Gaastra, L. V. and Sommerfelt, H. 1997. A new putative fimbrial colonization factor, CS19, of Human Enterotoxigenic *Escherichia coli* Infect. Immun. 65: 507–513

Gyles, C. L. 1992. *Escherichia coli* cytotoxins and enterotoxins. Can. J. Microbiol. 38:734–746.

Hall, R. H., Maneval, D. R. Jr., Collins, J. H., Theibert, J. L. and Levine, M. M. 1989. Purification and analysis of colonization factor antigen I, coli surface antigen 1, and coli surface antigen 3 fimbriae from enterotoxigenic *Escherichia coli*. J. Bacteriol. 171: 6372–6374.

Kerneis, S., Chauvire, G., Darfeuille-Michaud A., Fourel V. C. M.H., Joly, B.. 1992. Expression of receptors of enterotoxigenic *Escherichia coli* during enterocytic differentiation of human intestinal epithelial cells, Caco-2 and HT-29, in culture. Infect. Immun. 60: 2572-80.

Kerneis, S., Bernet, M. F., Coconnier, M. H. and Servin, A. L. 1994. Adhesion of human enterotoxigenic *Escherichia coli* to human colonic mucus-secreting HT-29 cell subpopulation in culture. Gut. 35**:**1449–1454.

Knutton, S., Lloyd, D. R., Candy, D. C. A. and McNeish, A. S. 1984(a). *In vitro* adhesion of enterotoxigenic *Escherichia coli* to human intestinal epithelial cells from mucosal biopsies. Infect. Immun. 44: 514-518.

Knutton, S., Lloyd, D. R., Candy, D. C. A. and McNeish, A. S. 1984(b). Ultra structural study of adhesion of enterotoxigenic *Escherichia coli* to

erythrocytes and human intestinal epithelial cells. Infect. Immun. 44: 519-527.

Knutton, S., Lloyd, D. R., Candy, C. A. and McNeish, A. S. 1985. Adhesion of enterotoxigenic *Escherichia coli* to human small intestinal enterocytes. Infect. Immun. 48:824-831.

Lena Jansson, Joshua Tobias, Michael Lebens, Ann-Mari Svennerholm and Susann Teneberg. 2006. The Major Subunit, CfaB, of Colonization Factor Antigen I from Enterotoxigenic *Escherichia coli* is a Glyco-sphingolipid Binding Protein. Infect. Immun. 74: 3488-3497.

Levine, M. M. 1987. *Escherichia coli* that cause diarrhea: enterotoxigenic, enteropathogenic, enteroinvasive and enteroadherent. J. Infect. Dis. 155: 377–389.

Lindahl, M., Faris, A. and Walstrom, T. 1982. Colonization factor antigen on enterotoxigenic *Escherichia coli* is a sialic-specific lectin. Lancet 11: 280.

Millette, C. F., O'Brien, A.D, and Moulding, T.C. 1980. Isolation of plasma membranes from purified mouse spermatogenic cells. J. Cell Sci. 43: 279-299.

Neeser J., R., Chambaz A., Golliard M., Link-Amster H., Fryder V. 1989. Adhesion of colonization antigen factor antigen II-positive enterotoxigenic *Escherichia coli* strains to human enterocyte-like differentiated HT-29 cells: a basis for host-pathogen interactions in the gut. Infect. Immun. 57: 3727-3734.

Neeser, J.R., A. Chambaz, K. Y. Hoang, and H. Link-Amster. 1988. Screening for complex carbohydrates inhibiting hemagglutinations by CFA/I- and CFA/II-expressing enterotoxigenic Escherichia coli strains. FEMS Microbiol. Lett. 49:301-307.

Oro, H. S., Kolsto, A. B., Wenneras, C. and Svennerholm. A. M. 1990. Identification of asialo-GM1 as a binding structure for *Escherichia coli* colonization factors antigens. FEMS Microbiol. Lett. 72:289–292.

Sack, R. B. 1975. Human diarrheal disease caused by enterotoxigenic *Escherichia coli*. Ann. Rev. Microbiol. 29: 333–353.

Spangler, B. D. 1992. Structure and function of cholera toxin and the related *Escherichia coli* heat-labile enterotoxin. Microbiol. Rev. 56: 622–647.

Walker, W.A. 1985. Role of the mucosal barrier in toxin/microbial attachment to the gastrointestinal tract. In: Microbial toxins and diarrhoeal disease. London: Pitman, Ciba Foundation Symposium, p. 112.

Wenneras, C., Holmgren, J.and Svennerholm, A. M. 1990. The binding of colonization factor antigens of enterotoxigenic *Escherichia coli* to intestinal cell membrane proteins. FEMS Microbiol. Lett. 66:107-112.

Wolf, M.K. 1997. Occurrence, distribution, and associations of O and H serogroups, colonization factor antigens, and toxins of enterotoxigenic *Escherichia coli.* Clin. Microbiol. Rev. 10: 569 – 584.

Zweibaum, A., Pinto, M., Chevalier, G., Dussaulx, E., Triadou, N., Lacroix, B., Haffen, K., Brun, J. L. and Rousset, M. 1985. Enterocytic differentiation of a subpopulation of the human colon tumor cell line HT-29 selected for growth in sugar-free medium and its inhibition by glucose. J. Cell. Physiol. 122: 21- 29.