

7. Studies on *Escherichia coli*

7.1 Studies on the binding of *Escherichia coli* heat-stable enterotoxin to the intestinal epithelial cells and brush border membranes of different animals.

Investigator: M.K. Chakrabarti

The principal objective of this study was to determine the presence and density of the STa receptor in intestinal epithelial cell and brush border membranes of different animals and to purify and characterize the receptor for STa from a high-density receptor system. Moreover the exact mechanism of action of STa was also to be explored. We reported earlier that binding of ^{125}I -STa to the brush border membranes of rat, rabbit (*J. Diarr. Dis. Res.* 17, 28-33, 1999), hamster and guineapig (*Indian J. Med. Res.*, 113, 5-10, 2001) was specific, time and temperature dependent. A single class of receptors was present in all the tested animals and the number of receptors remained lower in hamster in comparison to rat, rabbit and guineapig. Autoradiographic demonstration of SDS-PAGE of intestinal brush border membranes showed STa binding proteins of apparent MW of 160 KDa in rat 118 KDa in guineapig, 140 and 38 KDa in rabbit and 65 KDa in hamster. We also reported that STa binds to a single class of receptors in COLO-205 human colonic carcinoma cell. Binding was specific, time and temperature dependent. STa binding protein with MW of 95 KDa was detected in this cell line (*FEMS Microbiol.Lett* 156, 79-83,1997). STa was found to stimulate G-cyclase in COLO-205. It has been found that besides stimulating cGMP STa also involves two potential intracellular signal. It increases rapidly inositol triphosphate and cytosolic free calcium in COLO-205 cells prelabelled with myo[2- ^3H] inositol resulted in a rapid rise of [^3H] inositol triphosphate . Using fluorescent indicator, Fura 2AM, intracellular free Ca^{2+} has been found to increase 5.12 fold compared to control. Suspension of cells in calcium was chelated with EGTA. This effect was not observed with cells that were pretreated with dantrolene which suggest that the intracellular calcium rise might be due to mobilisation of intracellular stores. This study demonstrated for the first time a change in cytosolic calcium in cultured human colonic cell by STa, which was accompanied by inositol tri-phosphate activation (*Biochim.Biophys.Acta*, 1403, 1-4, 1998). The involvement of protein kinase C (PKC) in the mechanism of action of STa in COLO 205 had been shown. STa treatment causes translocation of PKC from cytosol to membrane fraction of COLO 205 in a Ca^{2+} dependent manner and PKC might have some role in the regulation of guanylate cyclase (*Toxicol Appl Pharmacol*, 206(1), 9-16, 2005). These findings have been further supported by the fluorescence ratio imaging studies by using fura-2AM. Moreover, involvement of nitric oxide in the mechanism of action of Ecoli-STa in COLO-205 cells was determined by using the nitric oxide probe 4,5 diaminofluorescein-2 diacetate (DAF 2DA) as a probe. It was found that Ecoli STa increases the intracellular nitric oxide level in COLO-205 cell line. It was found that Ecoli STa increases the intracellular nitric oxide level in COLO-205 cell line by activation of intracellular nitric oxide synthase. For purification and characterization of Ecoli STa receptor the gene was amplified and cloned in TOPO-TA cloning vector and was partially sequenced.

For better understanding of structural heterogeneity among *E. coli* STa receptor, during the reported period an attempt has been made to compare the *E. coli* STa receptor (STaR) gene from COLO-205 cell line with STaR gene from two other colonic cell line such as HT-29 and CACO-2. Therefore, to amplify and clone *E. coli* STaR gene from HT-29 and CACO-2 cell line, at first total RNA was isolated from the cell line. Isolated RNA was then used to make cDNA by reverse transcription. The template RNA was subjected to reverse transcription in

the presence of MMLV Reverse Transcriptase, Random decamer, dNTP mix, Rnase inhibitor and RT buffer. The mixture was incubated at 44°C for 1hr and then again incubated at 92°C for 10 min. E coli StaR gene from HT-29 and CACO-2 cell line was then amplified separately from these RT product by polymerase chain reaction with the primer designed from reported E coli StaR gene sequence of human. Amplified product then subjected to agarose gel electrophoresis and two bands were found with the molecular size of 2.6 kb and 3.5 kb respectively. Further studies are going on for sequencing and cloning of these amplified products.

7.2 Molecular characterization of multidrug resistance strains of enterotoxigenic *Escherichia coli* (ETEC) isolated from an outbreak of diarrhoea in Ahmedabad.

Investigator: Dr. T. Ramamurthy

In this study, we have analyzed 17 ETEC strains isolated from a diarrhoeal outbreak in Ahmedabad during January 2000 for mutation in the QRDR of *gyrA* and *parC* genes, resistance gene cassettes in the integrons, fluoroquinolone efflux and other genes mediating resistance to different antimicrobials. The antimicrobial susceptibility assay results showed that the ETEC strains are resistant to ampicillin (100%), cephalothin, tetracycline, cotrimoxazole, nalidixic acid, norfloxacin, ciprofloxacin (94% each), chloramphenicol (59%), streptomycin, kanamycin (52% each). In addition, most of the ETEC strains were also resistant to other antibiotics (Table 7.2.1). All the ETEC strains harboured *elt* and expressed LT in the CHO cell assay. The MICs of nalidixic acid resistance was high (2000 µg/ml) and for fluoroquinolones, the MIC ranged between 250 and 1000 µg/ml (Table 7.2.2).

DNA sequencing of the 190-bp PCR product covering the QRDR of *gyrA* demonstrated presence of mutations at codons 83 in quinolone and fluoroquinolone resistant strains compared to the sensitive strain AV195 (Table 7.2.2). Mutation at codon 83 was a C→T transversion in the codon TCG that resulted substitution of leucine for serine. Except for one strain (E19), the second mutation was noted in all the strains at position 87 (G→A transversion of codon GAC), which resulted in an asparagine substitution for an aspartate. A 265-bp PCR amplicon of *parC* was also analyzed in this study. Majority of the quinolone resistant strains had a mutation at codon 80 (G→T translation of codon AGC), resulting substitution of isoleucine for serine. A second mutation at position 84 (E→G) in the *parC* was detected in E13 and AV185, in which glutamic acid was replaced by glycine. In 6 strains, mutation was detected at position 108 (A→V), where alanine was substituted by valine. In 3 strains (E2, E14, and AV185) the mutation at position 108 was absent. Strain AV185 had a third mutation at position 90 (A→V), where alanine was replaced by valine.

The *gyrA* mutations that frequently affect residue serine 83 and aspartate 87 are common among fluoroquinolone resistant *E. coli*. For the expression of high-level resistance, acquisition of a second *gyrA* mutation and a *parC* mutation seems important. With the sequenced regions of QRDR in this study, correlation could not be made with the increased MIC of quinolone/fluoroquinolones and amino acid substitutions in topoisomerases.

Efflux pumps play an important role in intrinsic resistance of *E. coli* to fluoroquinolones. The fluoroquinolone accumulation kinetics before and after the addition of CCCP was almost similar in most of the resistance strains (Table 7.2.2) indicating the involvement of other resistance mechanism(s). Interestingly, most of the ETEC strains (64.7%) harboured the

newly described *aac(6')-Ib-cr* gene, which encodes the fluoroquinolone-modifying enzyme aminoglycoside acetyltransferase (Table 7.2.2).

ETEC strains carrying the integrons were screened for the presence of contiguous resistance gene cassettes. Of the 10 *IntI1* probe positive strains, 6 carried two cassettes and the resistance genes detected in these strains were *dfrA17* and *aadA5*, which confers resistance to trimethoprim and spectinomycin/streptomycin using dihydrofolate reductase and adenylyltransferase, respectively. In addition to these resistance gene cassettes, 2 strains (E9 and E17) gave an additional amplicon with a size of 1009 bp, which was identified as *aadA1*, conferring resistance for aminoglycosides. Isolates E3 and E6 strains carried 1009 and about 800 bp with complete and incomplete *aadA1* gene cassette, respectively. In 7 ETEC strains, *intI2* was detected, of which 4 were also positive for *IntI1*. All the *intI2* positive strains carried three cassettes (2449 bp) as those found in Tn7, namely *dfrA1*, *sat1*, and *aadA1* conferring resistance to trimethoprim, streptothricin, and streptomycin/spectinomycin, respectively. Existence of *sat1* has not been reported in ETEC (Table 7.2.1).

The plasmid patterns and the location of the *intI1* gene in the plasmid were not uniform (Table 7.2.1). *EcoRI* and *EcoRV* were used for digestion of chromosomal DNA, since the *intI1* did not have the corresponding restriction sites for these enzymes. In the Southern hybridization assay, class 1 integron was detected both in the chromosome and large plasmid in most of the strains (Table 7.2.1), however, class 2 integron was detected only in the chromosome in all the *intI2* positive ETEC strains. In E15, *intI1* was detected in the large plasmid, however, this strain did not amplify any resistance gene cassette.

Our finding has shown that the *dfr* and *aad* gene alleles encoding dihydrofolate reductases and adenylyltransferase, respectively were present in both the classes of integrons. The *dfr* alleles *dfrA1* and *dfrA17* were detected in 71% of the ETEC strains. In 50% of the *intI1* positive ETEC, *dfrA17* was detected along with *aadA5* cassette. The *dfr* is located behind the 5'-conserved segment, which is closest to the promoter, thereby providing high-level and conditional resistance. It is likely that selection of cassettes carrying *dfr* genes has occurred among ETEC as trimethoprim in combination with sulfamethoxazole (cotrimoxazole) is used frequently in the treatment of diarrhoea and other infections.

In addition to the integrons, the other resistance genes such as alleles of *tet*, *catA1*, *strA*, *bla_{TEM}*, and *aphA1-Ia* encoding for resistance for tetracycline, chloramphenicol, streptomycin, ampicillin, and kanamycin, were detected in 94%, 71%, 59%, 35% and 29% of the ETEC strains, respectively (Table 7.2.1). Among *tet* gene alleles, *tetB* was detected in 53% of the ETEC strains. The *tetB* gene that encodes an efflux protein and confers resistance to tetracycline was predominant in majority of the ETEC strains. *tetA* and *tetB* alleles were detected in 4 strains whereas *tetBE*, *tetABE*, *tetA* were detected in one strain each (Table 7.2.1). The other tested *tet* alleles such as *tetC*, *tetD* and *tetY* were not detected in any of the ETEC strains (data not shown). When tested for the MIC of tetracycline by agar dilution technique with strains harbouring different *tet* alleles, *tetA* or *tetB* alone had the MIC of 100 µg/ml, whereas *tetBE* and *tetABE* had 150 µg/ml (data not shown).

An analysis on the prevalence of different resistance gene combinations showed that ETEC strains harbouring *bla_{TEM}* gene was detected with *catA1* gene in 5 out of 6 strains and strains harbouring *aphA1-Ia* were also positive for *tetAB* or *tetABE*. In one streptomycin resistant strain (E2) *strA* gene was negative but carried *aadA5*, which was associated with the class 1 integron structure. The 4 strains, E2, E8, E10 and E16 were sensitive to streptomycin and did

not harbour *aadA1* or *strA*. *aphA1-Ia* was detected along with different *tet* alleles other than *tetB*. We have detected the aminoglycosides (neomycin and kanamycin) resistance gene (*aphA1-Ia*) in 5 strains (29.4%), even though 59% of the ETEC were resistant to kanamycin. It is likely due to the cross-resistance caused by most of the aminoglycoside resistance genes.

Even though the antimicrobial therapy is generally supportive for the treatment of ETEC infection, its rational use is difficult to implement in diarrhoea endemic regions especially during outbreaks. As evidenced from this study, innate gene mutations and acquisition of multidrug resistance genes through mobile genetic elements might have contributed to the emergence of multidrug resistance in ETEC. This study reinforces the necessity of utilizing molecular techniques in the epidemiological studies to understand the nature of resistance responsible for antimicrobial resistance in different species of pathogenic bacteria.

Table 7.2.1. Resistance profile, integrons and drug resistance gene cassettes in ETEC strains

Strain (Serogroup)	Resistance profile	Resistance gene cassette		Other resistance gene	Location of <i>intI1</i>
		<i>IntI1</i>	<i>IntI2</i>		
E2 (O146)	ACeCxTCoNxNaNe	<i>dfrA17, aadA5,</i>	-	<i>tetB</i>	Chromosome, large plasmid
E3 (O1)	ACeCfCtCxChTCoSxNaNe	<i>aadA1</i>	<i>dfrA1, sat1,</i> <i>aadA1</i>	<i>catA1, tetB, strA</i>	Chromosome Plasmid (8.6 kb)
E4 (O1)	ACeAmKCxChTCoSxNaNe	<i>dfrA17, aadA5</i>	-	<i>catA1, tetAB, strA,</i> <i>aphA1-Ia, aac(6')-Ib-cr</i>	Large plasmid
E6 (O8)	ACeCxChTCoSxNa	<i>aadA1</i>	-	<i>bla_{TEM}, catA1, tetB,</i> <i>strA</i>	Chromosome, large plasmid
E7 (O146)	ACeKCtCxTCoSxNa	<i>dfrA17, aadA5</i>	<i>dfrA1, sat1,</i> <i>aadA1</i>	<i>bla_{TEM}, tetB, strA,</i> <i>aac(6')-Ib-cr</i>	Chromosome, large plasmid
E8 (ONT)	ACeGCfKCtCxChTCoSxNa	<i>dfrA17, aadA5</i>	-	<i>bla_{TEM}, tetB, catA1,</i> <i>aac(6')-Ib-cr</i>	Chromosome, large plasmid
E9 (O146)	ACeGCxChTCoSxNa	<i>dfrA17, aadA5,</i> <i>aadA1</i>	<i>dfrA1, sat1,</i> <i>aadA1</i>	<i>bla_{TEM}, catA1, tetB,</i> <i>strA, aac(6')-Ib-cr</i>	Chromosome, large plasmid
E10 (O1)	ACeCfKCtCxChTCoSxNa	<i>dfrA17, aadA5</i>	-	<i>catA1, tetB, aac(6')-Ib-cr</i>	Chromosome
E13 (O1)	ACeGCfKCtCxChTCoSxNaNe	-	<i>dfrA1, sat1,</i> <i>aadA1</i>	<i>caA1, tetAB, strA</i> <i>aphA1-Ia, aac(6')-Ib-cr</i>	
E14 (O1)	ACeGCfKCtCxChTCoSxNaNe	-	<i>dfrA1, sat1,</i> <i>aadA1</i>	<i>catA1, tetAB, strA,</i> <i>aphA1-Ia, aac(6')-Ib-cr</i>	
E15 (O1)	ACeCxTNxNa	-	-	<i>tetBE, aac(6')-Ib-cr</i>	large plasmid
E16 (O1)	ACeCfChTCoSxNaCx	<i>dfrA17, aadA5</i>	-	<i>catA1, tetB</i>	Chromosome
E17 (O146)	ACeGKCxChTCoSxNa	<i>dfrA17, aadA5,</i> <i>aadA1</i>	<i>dfrA1, sat1,</i> <i>aadA1</i>	<i>bla_{TEM}, catA1, tetB,</i> <i>strA</i>	Chromosome, large plasmid
AV185 (O1)	ACoCfCxKTNxSNa	-	<i>dfrA1, sat1,</i> <i>aadA1</i>	<i>catA1, tetAB, strA,</i> <i>aphA1-Ia, aac(6')-Ib-cr</i>	
AV189 (O25)	ACeGCfKCtCxTNxNa	-	-	<i>tetA, aac(6')-Ib-cr</i>	
AV193 (ONT)	ACeCoCxNxNaGKT	-	-	<i>bla_{TEM}, catA1, strA,</i> <i>tetABE, aphA1-Ia,</i> <i>aac(6')-Ib-cr</i>	
AV195 (O1)	ACeCo	-	-	-	

Abbreviation; ONT untypable, ampicillin (A), chloramphenicol (Ch), co-trimoxazole (Co), gentamycin (G), neomycin (Ne), tetracycline (T), streptomycin (S), nalidixic acid (Na), cephalothin (Ce), amikacin (Am), ceftazidime (Cf), kanamycin (K), ceftriaxone (Ct), ciprofloxacin (Cx) and norfloxacin (Nx).

Table 7.2.2. MICs, amino acid substitutions in the QRDRs and fluroquinolone efflux of ETEC strains

Strain	Serotype	MIC ($\mu\text{g/ml}$)			Amino acid substitution		Accumulation of fluroquinolones ($\mu\text{g/mg}$ [dry weight] of cells)			
		Na	Cx	Nx	GyrA	ParC	Cx		Nx	
							Before addition of CCCP	After addition of CCCP	Before addition of CCCP	After addition of CCCP
E2	O146	2000	250	250	S83→L, D87→N	S80→I, A108	0.064 ± 0.0011	0.089 ± 0.0028	0.161±0.007	0.502±0.0032
E3	O1	2000	250	250	S83→L, D87→N	S80→I, A108→V	0.067 ± 0.003	0.089 ± 0.0025	0.166±0.003	0.461±0.0021
E4	O1	2000	500	500	S83→L, D87→N	S80→I, A108→V	ND	ND	ND	ND
E7	O146	2000	1000	1000	S83→L, D87→N	S80→I, A108→V	0.014 ± 0.0013	0.020 ± 0.0019	0.043±0.0010	0.047±0.0022
E9	O146	2000	1000	500	S83→L, D87→N	S80→I, A108→V	ND	ND	ND	ND
E13	O1	2000	500	500	S83→L, D87→N	S80→I, E84→G	0.009 ± 0.0012	0.020 ± 0.0020	0.024±0.0011	0.044±0.0014
E14	O1	2000	1000	500	S83→L, D87→N	S80→I, A108	0.012 ± 0.0015	0.014 ± 0.0035	0.036±0.0017	0.040±0.0033
E15	O1	2000	250	250	S83→L, D87→N	S80→I, A108→V	ND	ND	ND	ND
E16	O1	2000	250	200	S83→L, D87→N	S80→I, A108→V	ND	ND	ND	ND
E17	O146	2000	250	250	S83→L, D87	S80, A108	0.013 ± 0.0011	0.022 ± 0.0021	0.046±0.0014	0.047±0.0024
AV185	O1	2000	1000	500	S83→L, D87→N	S80→I, E84→G, A90→V, A108	0.031 ± 0.005	0.090 ± 0.0011	0.133±0.002	0.531±0.0010
AV195	O1	4	2	2	S83, D87	S80, A108	0.088 ± 0.007	0.096 ± 0.009	0.564±0.004	0.590±0.006

ND, not done

7.3 Detection and characterization of different colonization factors for molecular typing of Enterotoxigenic *Escherichia coli*

Investigator: N.S. Chatterjee

Enterotoxigenic *Escherichia coli* (ETEC) is an important cause of diarrheal disease in humans, affecting children and adults. In particular, ETEC is a cause of morbidity and mortality in children up to 5 years of age in developing countries. ETEC strains have two major virulence determinants: the enterotoxins (the heat-labile toxin [LT] and the heat-stable toxin [ST]) and the colonization factor antigens (CFAs).

Over 20 distinct, human-specific ETEC adhesins or colonization factor antigens (CFAs) have been described. In many geographic areas, the most common CFs individually expressed by ETEC strains are CF antigen I (CFA/I), CFA/II, which is composed of coli surface antigen CS3 alone or in combination with CS1 or CS2 and CFA/IV, which is composed of CS6 alone or in combination with CS4 or CS5. To cause disease, ETEC must first adhere to the epithelium of the small intestine by means of the CFAs and then produce secretory diarrhea due to the effects of the enterotoxin(s). The overall goal is to develop a simple and specific method for detection of different colonization factor antigens (CFAs) for typing enterotoxigenic *Escherichia coli* (ETEC), identify the most prevalent CFAs and characterize them. This will also help us in tracking the movement of ETECs round the globe.

We have developed a PCR-based method to detect common CFAs ETEC, which illustrates usefulness of this method in the epidemiology of diarrhea. We used 44 ETEC strains and 10 non-ETEC strains to establish this method. This method could efficiently and correctly identify presence and expression of CFA genes in ETECs. Our results also suggested that serologically untypable strains could be classified by this method. CS6 was found to be more prevalent in the Indian strains classified by PCR-based method, as well as by antibody method.

Since limited information is available regarding the characteristics of CS6, we attempted to purify and characterize this protein. We have purified CS6 to homogeneity from a CS6-expressing ETEC. Heat-saline extract was prepared and was subjected to ammonium sulphate precipitation. 45-60% fraction was subjected to chromatographic separation. The identity of these subunits was confirmed by N-terminal sequencing. Purified CS6 had two very closely associated subunits- C_{ss}A (18.5 kDa) and C_{ss}B (15.9 kDa) as determined by mass spectrometry. Subunit C_{ss}A had stearic acid and palmitic acid modifications determined by gas-liquid chromatography. CS6 was found to oligomerize in solution during purification. Calorimetric studies showed that these two subunits dissociated at 60°C without denaturaton. C_{ss}B-subunit was immunogenic, but C_{ss}A did not produce any antibody response. Further functional characterization is in progress.