5 Studies on *Vibrio cholerae* Phages

Section highlights

- In this Institute, studies on cholera phages are continued. NICED is the only institute engaged in study on cholera phages. All isolates of *V.cholerae* from different parts of the country are being sent to NICED for confirmation, serotyping, biotyping and phage typing. This year, a total of 570 strains of *V.cholerae* strains were received from different parts of the country. Of these, 398 (69.8%) confirmed strains as *V.cholerae* O1 biotype ElTor were included in phage typing study. All except 4 strains belonged to Ogawa. A total of 387 strains of *V.cholerae* O1 were grouped under type 4 with the conventional scheme of Basu and Mukerjee. Remaining 11 strains were found to be untypeable. All these strains were found to be typeable with the new scheme and were clustered into a number of distinct types of which majority were grouped under type 27 (83.2%) followed by type 26 (2.5%), type 13 (2.5%), type 23 (2.2%), respectively.

- Last year, a total of 35 strains of *V.cholerae* O139 received from different parts of the country were included in phage typing study. All the strains of *V.cholerae* were found to be typeable with the *V.cholerae* O139 phage typing scheme. Majority of the strains was clustered under type 1.

- From the typing scheme, N-4 ElTor O1 phage was selected for molecular characterization. Most of the restriction enzymes could not digest the phage DNA. Of these, Hind III was used to digest the DNA partially. An attempt was made to map the genome partially.

5. Studies on *Vibrio cholerae* Phages

5.1. Further application of phage typing scheme for discriminating strains of *Vibrio cholerae* O139

Investigator :

B.L. Sarkar

The phage typing scheme specific for *V. cholerae* O139 is developed in this institution. This scheme is routinely in use for discriminating strains of *V. cholerae* O139 received from different parts of the country. A total of 35 strains of *V. cholerae* O139 were included

in phage typing study table 5.1.1. Of these, 8 strains were received from Maharashtra and 26 strains from Tamil Nadu state. All of these strains were found to be typeable. Majority of the strains could be clustered under type 1.

A representative number of strains were compared with other molecular typing systems like Random amplified polymorphic DNA (RAPD) and Pulse field gel electrophoresis (PFGE). RAPD could not differentiate the strains belonging to different phage types. Also, the strains could not be differentiated from a single phage type. However, the strains belonging to single phage type could further discriminate the strains into different subtypes. It was observed that PFGE was much more sensitive compared to conventional phage typing systems.

5.2. Nationwide screening of phage types of *V. cholerae* O1 biotype ElTor

Investigator :

B.L. Sarkar

This year, a total of 570 strains of *V. cholerae* were received from different parts of the

Mothers meeting in the rural field area

country for serotyping, biotyping and phage typing. Of these, 398 (69.8%) representative strains of *V. cholerae* O1 biotype ElTor were included in phage typing study table 5.2.1. This year, highest number of strains of *V.cholerae* O1 were received from Punjab state. Majority of the strains belonged to Ogawa. For the last couple of years, Ogawa was the dominant serogroup. Only 4 strains were grouped under Inaba. A total of 11 strains were found to be untypeable with the conventional scheme of Basu and Mukerjee. Remaining 387 strains *V.cholerae* O1 were clustered under type 4. Using the new scheme, all of these strains were found to be typeable and could be clustered into a number of distinct types of

which majority were grouped under type 27 (83.2%) followed by type 26 (2.5%), type 13 (2.5%), type 23 (2.2%), 25 (1.7%) respectively. It has been observed that phage type 27 of *V.cholerae* O1 strains was the predominant through out the country.

5.3. Molecular analysis of *Vibrio cholerae* bacteriophages: cloning and sequencing of phage DNA

Investigator :

B.L. Sarkar

One of the phage, N-4 is a ElTor O1 phage of the new phage typing scheme is selected for molecular characterization. The high titre phage stock (1011 pfu/ml) was prepared. Phage DNA was isolated and digested with different enzymes. A total of twenty eight restriction enzymes were used. Unfortunately, DNA could be digested with only four enzymes, Hind III, Acc I, Hae III and EcoRV. Additionally, EcoT14I, EcoT22I, TthHB 81 and MspI have newly found which could digest the phage DNA. It has been calculated that the length of the DNA seems to be approximately 30kb. Since, the phage DNA is resistant to a large number of enzymes, it may be assumed that the DNA is highly methylated. This posed a serious problem in constructing a physical map of the phage genome. So far, Hind III was used to digest the DNA partially. Further information could not be obtained since majority of the bands are of small size. Presently, Hind III and other enzymes were employed to clone the phage DNA and an attempt was made to map the genome physically.

Table 5.1.1. Phage typing results of *V.cholerae* O139 strains during 2002-2003

State	Name of the Institute	Number of strains	Phage type
Gujrat	State Public Health Laboratory, Baroda	1	1
Maharashtra	Indira Gandhi Medical College, Nagpur	2	2
	Nagpur Reference Pathology Laboratory, Nagpur	1	1
	State Public Health Laboratory, Pune	3	3
	Mahatma Gandhi Institute of Medical Sciences, Sewagram	2	2
Tamil Nadu	CD Hospital, Chennai	23	23
	Madurai Medical College, Madurai	3	3
	Total	35	35

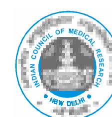
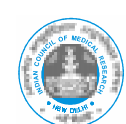


Table - 5.2.1
Phage typing results of *V.cholerae* O1 biotype ElTor strains during 2002-2003

State	Name of the Institute	No. of Strain Recived	Biotype		Serotype			Basu & Mukerjee	
			El	Cl	In	Og	T2	T4	Ut
Delhi	1. National Institute of Communicable Diseases, Delhi	18	18	–	4	14	–	18	–
Gujarat	1. Govt. Medical College, Surat	10	10	–	–	10	–	10	–
	2. State Public Health Laboratary, Borada	48	48	–	–	48	–	48	–
Karnataka	1. Karnataka Institute of Medical Sciences, Hubli	7	7	–	–	7	–	7	–
	2. Jawaharlal Nehru Medical College, Belgaum	7	7	–	–	7	–	6	1
Madhya pradesh	1. N.S.C.B. M. Medical College, Jabalpur	1	1	–	–	1	–	1	–
Maharastra	1. Govt. Medical College, Aurangabad	23	23	–	–	23	–	21	2
	2. Regional Public Health Laboratory Aurangabad	41	41	–	–	41	–	41	–
	3. Indira Gandhi Medical College, Nagpur	18	18	–	–	18	–	16	2
	4. Regional Public Health Laboratory,Nagpur	8	8	–	–	8	–	8	–
	5. Grant Medical College hospital,Mumbai	6	6	–	–	6	–	6	–
	6. Govt. Medical College, Nagpur	8	8	–	–	8	–	8	–
	7. Govt. Medical College, Nanded	1	1	–	–	1	–	1	–
	8. State Public Health Laboratory, Pune	3	3	–	–	3	–	3	–
	9. Mahatma Gandhi Institute of Medical Sciences, Sewagram	4	4	–	–	4	–	4	–
	10. B.J. Medical College, Pune	1	1	–	–	1	–	1	–
Punjab	1. C.M.C. Hospital, Ludhina	57	57	–	–	57	–	54	3
	2. Dayanand Medical College, Ludhina	10	10	–	–	10	–	10	–
	3. Govt. Medical College, Patiala	1	1	–	–	1	–	1	–
	4. Medical College, Amritsar	2	2	–	–	2	–	2	–
	5. Govt. Medical College and Hospital, Chandigarh	57	57	–	–	57	–	55	2
Rajasthan	R.N.T. Medical College, Udaipur	8	8	–	–	8	–	8	–
Tamil nandu	1 C.D. Hospital, Chennai	20	20	–	–	20	–	19	1
	2. King Institute of Preventive Medicine, Chennai	37	37	–	–	37	–	37	–
	3. Madurai Medical College	2	2	–	–	2	–	2	–
	Total	398	398	–	4	394	–	387	11

Strains received during 2001-2002

New Phage Tysting Scheme																	
2	3	4	6	7	12	13	14	16	19	20	21	22	23	24	25	26	27
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	18
–	–	–	–	–	–	–	1	–	–	–	–	–	–	–	–	–	9
–	–	3	–	–	1	4	–	–	–	–	–	–	1	–	–	2	37
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	7
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	2	5
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1
–	–	–	–	–	–	1	–	–	–	–	–	–	–	–	3	1	18
–	–	1	–	1	–	–	–	–	1	1	–	–	–	–	1	3	33
–	–	–	–	–	–	1	–	–	1	–	–	–	–	–	2	1	13
–	–	–	–	–	–	–	–	1	–	–	–	1	–	–	–	–	6
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	6
–	–	–	–	–	–	1	–	–	–	–	–	–	–	–	–	–	7
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	3
–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	3
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1
–	1	3	–	2	–	–	–	1	1	–	1	–	6	1	1	–	40
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	10
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	2
–	–	–	–	1	–	3	–	–	–	–	–	–	1	–	–	–	52
–	2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	6
–	1	–	1	–	–	–	–	–	1	–	–	–	–	–	–	–	17
–	–	–	–	–	–	–	1	2	–	–	–	–	–	–	–	–	34
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	1
–	5	7	1	4	1	10	2	4	4	1	1	1	8	1	7	10	331



NICED
National Institute of Cholera and Enteric Diseases

6 Studies on *Shigella* Species

Section highlights

- A bivalent hybrid non-invasive stable vaccine strain was constructed against *Shigella dysenteriae* 1 and *Salmonella enterica* serover Typhimurium. In rabbit intestinal mucosa infected by the hybrid vaccine strain did not show lesions and the entire villi were conserved.
- Mu like temperate phages isolated from a strain of *Pseudomonas* spp (DR-11) transferred drug resistances (five) to the plasmidless strain of *Shigella flexneri* (Pl- 35).
- Murine macrophages essentially require Toll-Like receptor 2 to recognize porin, a potential vaccine candidate, of *S.dysenteriae* type 1. The protein up-regulates CD80 on Mφ surface and induces the cell to release IL-12 suggesting that porin would promote Th1-type response directly and profoundly with IFN-γ.

6. Studies on *Shigella* Species

6.1. Genetic studies on virulence mechanisms of *Shigella dysenteriae* 1 in relation to vaccine development

Investigator :

R. Kumar

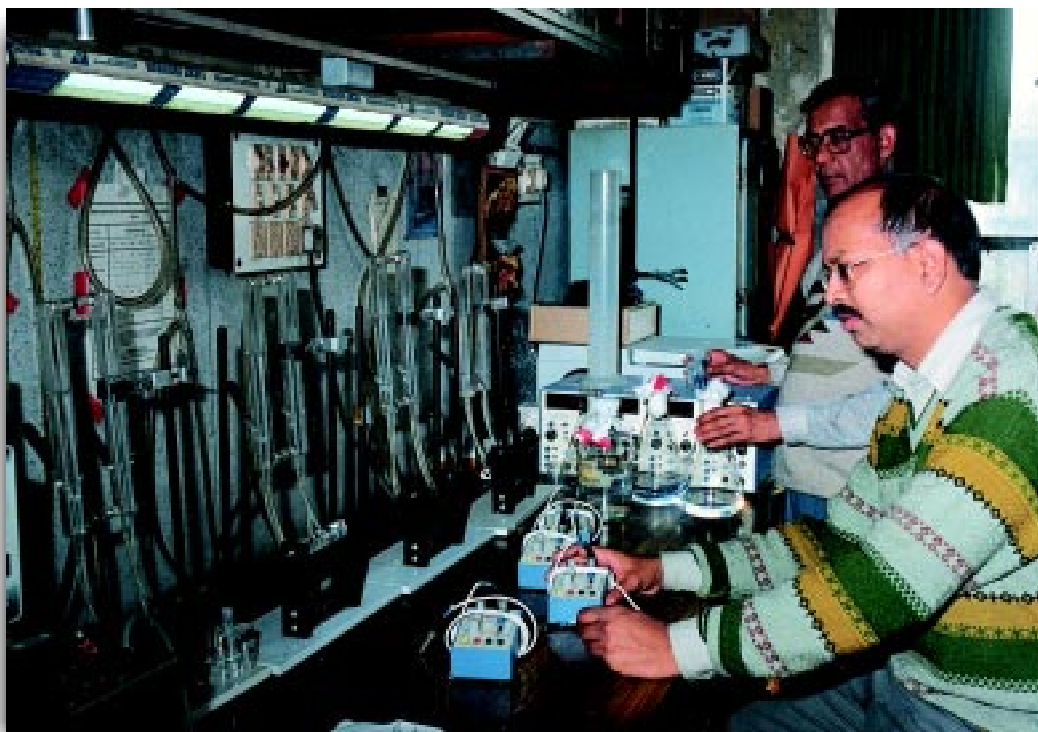
Bacillary dysentery and its association with poor hygienic standards have been known for centuries. *Shigella* spp. produce a major enteric disease (bacillary dysentery) responsible over 200 million infections annually with 650000 deaths due to its high infectivity, improvement of hygienic standard alone can reduce the spread of dysentery. The possibility of other preventive means such vaccines against shigellosis have been explored over the past 50 years. Recently, increased understanding of the molecular biology of *Shigella* spp. and the possibility of designing well characterized vaccine strains have increased interest in the field. Antimicrobial resistance has complicated the treatment of shigellosis since the 1940's, when sulfa resistance among *Shigella* organisms was first recognized in Japan. Since then, resistance to a variety of antimicrobial agents used to treat shigellosis has forced repeated re-evaluation of treatment recommendation.

Genetic characterization of the major virulence factors in *S.dysenteriae* 1 helped in designing vaccine strains. The primary step in pathogenesis of bacillary dysentery caused by *S.dysenteriae* 1 is adhesion and later on invasion of the human colonic mucosa. At the end of 1996, a meeting was organized at WHO, Geneva by the Steering Committee on Diarrhoeal Disease Vaccines, which was placed *Shigella* vaccine development first in line of priorities.

A special emphasis given for development of a vaccine against *S.dysentery* type 1. The prophylactic measure would be prevent the *Shigellae* from invading the mucosal lining of the intestine. The least approach for construction of *Shigellae* vaccine is to expressing the surface components such as LPS and a few selected immunogenic Ipa antigens (BC). However, multicopy IpaH (60kDa) probably play an important role in relation to mucosal invasion of the intestine which might be considered for vaccine development.

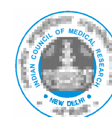
This study was initiated with the objectives: i) to construct a stable (100%) hybrid strain without interfering the plasmid gene(s) of *S.dysenteriae* 1; ii) to find out the expression of Ipa BC and H gene products in the hybrid strain; iii) to check the expression of *Shigella* LPS biosynthesis in hybrid strain; iv) to examine the hybrid strain for invasive property; and also v) to find out whether any other genes/proteins involved in relation to virulence mechanism of *S.dysenteriae* 1.

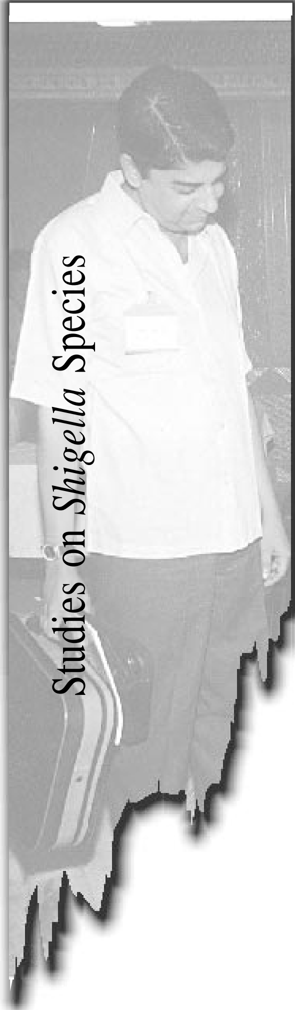
Hybrid strain of *Shigella dysenteriae* 1 was constructed which was 100% stable and carrying LPS biosynthesis genes (rfb and rfc genes) of *Salmonella enterica* serover typhimurium, Ligated ileal loops were prepared in rabbit, which were inoculated with 10^8 CFU/ml each of the hybrid strain and invasive *S.dysenteriae* 1 strain used as positive control. *Escherichia coli* K12 was also used as a negative control. After 18 hrs of inoculation, the fluid accumulation ratios were 0.2 and 1.6 for hybrid and invasive strains of *S.dysenteriae* 1 respectively. Rabbit intestinal mucosa infected with hybrid *S.dysenteriae* 1 strain showed the presence of intact villus tips and unruptured intestinal mucosa whereas total necrosis of intestinal mucosa and villi in the infecrted region were observed in inoculation of *S.dysenteriae* 1.



Scientist working with Ussing chamber

A strain of *Shigella flexneri* was isolated from I.D. Hospital, Kolkata which contained 5 plasmids ranging in sizes from 180 kb to 3 kb. These strain showed resistance to 18 drugs out of 22 drugs tested. All 5 plasmids were eliminated from *S.flexneri* by us using acridine orange (AO) as curing agent. The all plasmid cured strain (Pl- 35) was sensitive against 22 antibiotics examined earlier. A temperate phage from *Pseudomonas* spp. was isolated by us which infected the plasmidless (Pl- 35) strain of *S.flexneri* and forms plaque on soft agar containing Luria broth. The phages carried 5 antibiotic resistance property of





Pseudomonas spp. and transmit into Pl- 35 strain of *S.flexneri*. The head size of the phage was 65 nm, tail portion was 200 nm and one long variable (400 nm - 700 nm) thick tail appendage.

6.2. Molecular characterization of multi-drug resistant *Shigella flexneri* in kolkata

Investigator :

S.K. Niyogi

Shigellosis is a major public health problem in developing countries. Increased incidence of antibiotic resistance in *Shigella* spp. constitute a major concern. High frequency of resistance of *Shigella flexneri* to many of the first line antimicrobial agents (multi drug resistant) have been reported in recent years from Kolkata. Most of the conventional typing methods are based on the phenotypic properties of the micro-organisms and offer little strain discriminatory information. The objective of this study is to analyse clonal relationships among isolates of multi drug resistant *Shigella flexneri* using different molecular typing methods to determine changes at the genetic level and to understand their implications in the epidemiology of the disease process.

During the period under study a total of 759 stool samples collected from Dr. B.C. Roy Memorial Hospital for Children, Kolkata were screened for detection of *Shigella* spp. To isolate *Shigella* spp. stool samples were inoculated onto MacConkey, XLD, HEA and SS agar plates (Difco, USA) and the resulting colonies which exhibited characteristics of *Shigella* spp. were identified by conventional biochemical methods. Subsequently serogroups and serotyping was identified by slide agglutination using commercially available poly and monovalent antisera (Denka Seiken Co, Japan).

Antimicrobial susceptibility tests were performed by an agar diffusion disk methods as advocated by the National Committee for Clinical Laboratory Standards. Mueller Hinton agar was obtained from Difco, Detroit, USA and antimicrobial disks were obtained from Hi Media Ltd. India. MIC of the strains against different antimicrobial agents were determined by E-test (AB BIODISK, Solna, Sweden).

Out of 759 stool specimens, 63 (8.3%) were positive for *Shigella* spp. Among *Shigella* strains, 33 (4.3%) were *Shigella flexneri*, 2 (0.26%) were *Shigella boydii*, 18 (2.3%) were *Shigella sonnei* and 10 (1.3%) was *Shigella dysenteriae*.

Ninety one percent of the *Shigella flexneri* were found to be multi-drug resistant. One strain, NK 2788, *Shigella flexneri* type 3b has MIC >32 mcg/ml and >256 mcg/ml against ciprofloxacin and norfloxacin respectively. Digestion of chromosomal DNA with the restriction endonuclease XbaI produced clearly resolvable restriction endonuclease analysis (REA) pattern after PFGE. Different REA pattern was identified amongst the isolates strains *Shigella flexneri* strains originated from different clones.

6.3. Antigenic recognition of *Shigella dysenteriae* outer membrane proteins using human convalescent sera and to evaluate their role in cell-mediated immune response in shigellosis

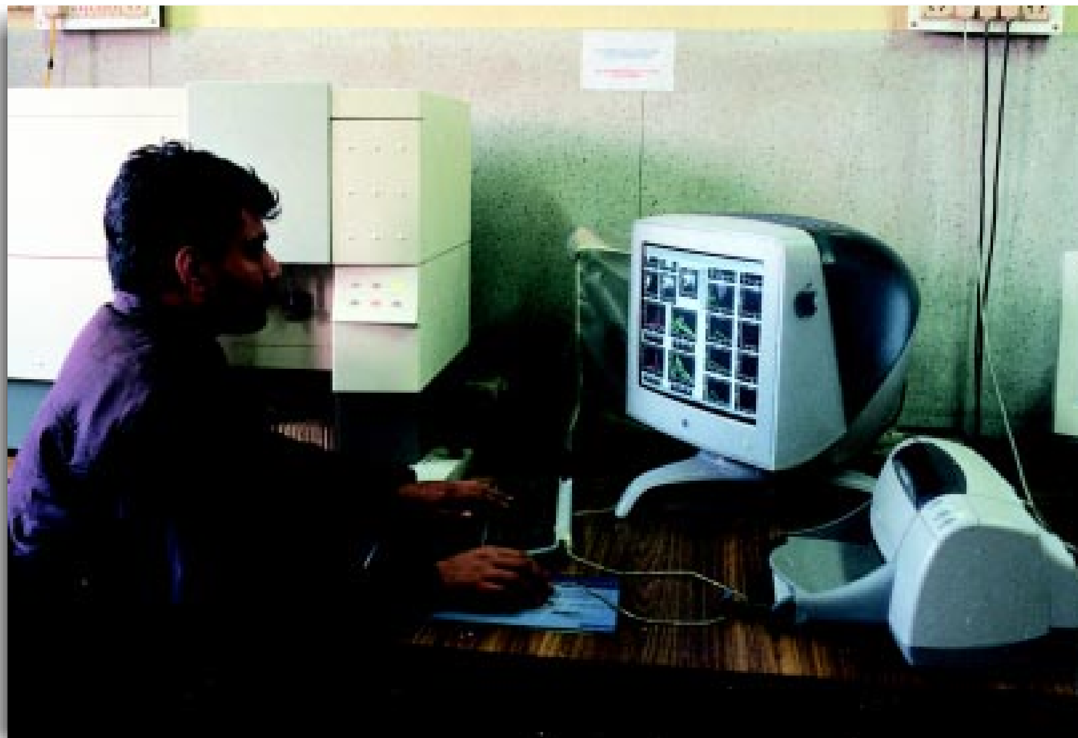
Investigator :

A.K. Sinha

The study was undertaken with two primary objectives, namely to find out the major immunodominant components after antigenic recognition of the Outer Membrane Proteins (OMPs) from *S.dysenteriae* 1 and to evaluate their role in cell-mediated immunity in shigellosis.

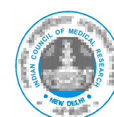
Previously, the use of anti-IL-2 in generation of O-2, sinergestically enhanced the pro-inflammatory cytokine like TNF-α and Th1 subset was reported. Later on, experiment was carried out to understand the T-cell components in response to generate the antigen specific signals, when T-cells were continuously exposed to major antigenic fraction (57 kDa), a rising spurt in lymphocyte proliferation, specific T-cell dependent humoral immune responses and proportionately the expression of CD4 (26.8%) and CD25+ T-cells (22.5%). Out of total CD3+ T-cells were found significantly dominant over CD8 (4.7%) on day 28. This increase in *Shigella* specific CD4+ T-cells and CD25+ T-cells in target group of immunized mice were almost comparable to that corresponding cell numbers as observed in control mice (17.3% and 18.9%) and in other stimulated groups. Later, cytokine kinetics had evaluated in events, 57 kDa antigenic fraction produced a change in kinetics of antigen specific response.

Upregulation of IL-2 and its recovery after day 14 (1293.8 pg/ml) is due to poor internalization of IL-2/IL2R and re-expression of cytokines, as a result expressing CD25 (IL-2R) by the targeted cells for generating signals after immunization and re-stimulated with 57 kDa antigenic fraction. Reciprocal relation of IL-2 and Migration Inhibition Factor (MIF) indicated that 57 kDa antigen might not inhibit the migration of the macrophages towards the activated response in turn it provoked IL-2 secretion with low degree of internationalization to its receptor which is required for the activation and maturation of CD4+ T-cells and concomitantly provides in initiation of phosphotyrosine mediated signal, as activation of CD22 (20%) is associated.



Student working with flow cytometer (FACS Calibur)

The above results indicate that 57 kDa antigenic fraction significantly triggers antigen specific memory T-cells towards the activated responses of Th1. Further studies are in progress to evaluate the role of major antigenic fraction in monitoring the antigenic-specific signals to establish its immuno-prophylactic nature against such infection.



Studies on *Shigella* Species

6.4. Immunoregulatory functions of porin of *Shigella dysenteriae* type 1

Investigator :

T. Biswas

Western blot analysis showed that 21-day old sera obtained from three patients convalescing from shigellosis after *S. dysenteriae* type 1 infection, recognized specifically the purified porin monomer of 38,000 of *S. dysenteriae* type 1 and interacted strongly with it. Since human, the only natural host of *S.dysenteriae* type 1, recognized the protein through humoral immune response, it is of great significance to study the surface-exposed outer membrane antigen keeping it in view as a potential vaccine candidate. $CD11b^+$ peritoneal cavity (PerC) macrophages (MΦ) were treated with 1µg/0.2×10^6 cells of porin alone, 20 U/0.2×10^6 cells of mouse IFN-γ, or porin plus IFN-γ. Analysis of relative fluorescence intensity after 24 h incubation with porin or IFN-γ, demonstrated that CD80 expression increased significantly compared to control. The up-regulation of the costimulatory molecule on $CD11b^+$ MΦ was augmented substantially by porin plus IFN-γ. The increment of CD80 expression by the cells was 22% in presence of porin compared to control, which got enhanced by 1.6-fold to 36% in the presence of porin and IFN-γ. However, the analysis of relative fluorescence intensity showed that 1µg of porin could not express CD86 on $CD11b^+$ PerC MΦ, supporting porin mediated selective up-regulation of the B7-1 (CD80) member of the B7 family. One µg of porin released 7.25 pg, and 20U of IFN-γ released 8 pg of IL-12 from 0.2×10^6 MΦ. Addition of porin in combination with IFN-γ released 19 pg of IL-12 by the cells, an increase by 2.6-fold of the IL-12 secretion than found by either porin or IFN-γ alone ($p<0.005$). ELISA for quantitation of IL-10 showed that porin could not induce the release of the cytokine from PerC MΦ. Therefore, the specific induction of IL-12 would promote Th1-type response by porin directly and profoundly in combination with IFN-γ.

RT-PCR was conducted using Toll-like receptor (TLR)2, TLR4, MyD88 and GAPDH specific primers with total RNA isolated from PerC MΦ of BALB/c mice after incubation for 24h with or without porin. Cells grown in presence of porin showed a 2-fold increase in TLR2 and a 2.3-fold increase in MyD88 mRNA expression compared to the untreated cells. TLR4 mRNA expression remained unaffected with porin treatment. The recognition of porin by MΦ through the expression of TLR2, known for recognition of several patterns of bacterial structures other than LPS, emphasizes that requirement of TLR2 is essential for recognition of *S. dysenteriae* type 1 porin.

7 Studies on *Escherichia coli*

Section highlights

- Information regarding Shiga toxin producing *E.coli* (STEC) in our country are scanty. Studies on STEC using molecular techniques showed the high prevalence among cattle population in Kolkata. Characteristication of virulence genes of STEC showed that most of the isolates habour different virulence genes responsible for diarrhoea and HUS. These STEC are clonally diverse.
- *E.coli* STh binds to its receptor and causes IP3 mediated calcium mobilization resulted in translocation of protein kinase C from cytosol to membrane associated site. Translocated PKC then phosphorylate some membrane proteins which may include guanylate cyclase.

7. Studies on *Escherichia coli*

7.1. Search for Shiga toxin producing *Escherichia coli* including O157: H7 strains in animals, animal products and hospitalised acute diarrhea cases in Kolkata

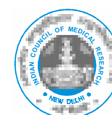
Investigator :

S. Dutta

In recent years Shiga like toxin producing *Escherichia coli* (STEC) has been implicated as an emerging cause of acute diarrhoea. Of which, O157:H7 strain is widely recognised as an aetiological agent of several diarrhoeal outbreaks. Cattle have long been regarded as the principal reservoir of O157:H7 strains, though the animal usually remain asymptomatic. STEC strains were also found prevalent in the gastro intestinal tracts of other domestic animals, including sheep, pig, goat, dog and cat. STEC including O157:H7 can enter the human food chain from a number of animal sources, most commonly by contamination of meat with feces or intestinal contents after slaughter. In developing countries like India, STEC has not been identified as a major aetiological agent of diarrhoea. But recently some strains of STEC including O157:H7 serotype were isolated from dairy cattle, beef samples marketed in Kolkata and from hospitalised diarrhoea cases.

The objectives of the study were: i) to determine if dairy cattle are potential sources of Shiga toxin producing *Escherichia coli* (STEC) including O157: H7 serotype; ii) to ascertain the presence of STEC in foods (beef, pork, milk) marketed in Kolkata, and in animal handlers residing in and around Kolkata.

Investigations were also performed to determine the role of STEC in causing acute diarrhoea among hospitalized children. Attempts were made to study the relatedness of the isolated STEC strains by their antibiotic resistance profiles, plasmid profiles and subtyping by various molecular methods like RAPD and PFGE.



Twenty strains of STEC were isolated from dairy cattle and beef samples and clinical samples screened in this study from Kolkata. Clinical samples yielded only three STEC isolates. Of 20 strains 12 belonged to O157:H7 serotype. All O157: H7 strains were multidrug resistant and possessed important virulent factors, which included Shiga toxin production and the presence of *eaeA* and *hlyA* genes. Out of 20 strains 13 strains possessed *stx1*gene whereas only 1 strain showed presence of *stx2* genes, and 3 strains showed presence of both *stx1* and *stx2* genes. Three strains did not have any of the toxin genes. When tested for the plasmid profiles, it was observed that all strains had a 60 MDa heavy plasmid, but overall there was no uniformity among plasmid profiles of the strains. Pulsed field gel electrophoresis (PFGE) typing was carried out after digesting the genomic DNA of the strains with *Xba1* restriction enzyme and running the digested DNA in multidirectional electric fields using CHEF DR III apparatus (BIO-Rad). The PFGE profiles of the strains revealed that 9 of 20 (45%) strains were closely related and constitute PFGE type 1 profile. In rest of the strains less than 70% similarity was observed indicating their multiclonal origin. All strains were multidrug resistant. As a further extension of the previous work RAPD molecular typing was performed for all strains using published four universal primers and the profiles also showed varied pleomorphism among those strains.

Therefore viewing all results it may be concluded that the circulating strains of STEC in Kolkata are heterogeneous and originated from more than one clone, although one clone might be predominating. No homology also could be established between strains isolated from clinical and environmental source.

Scientist working in bacteriology lab

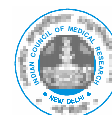
7.2. Studies on the binding of *Escherichia coli* heat-stable enterotoxin to intestinal epithelial cells and brush border membranes of different animals

Investigator :

M. K. Chakrabarti

In developing countries enterotoxigenic *Escherichia coli* that produce heat stable toxins (STa) may be responsible for 50% to 80% of the reported cases of diarrhoea. STa are also a major cause of diarrhoea in laboratory and domestic animals. The heat stable toxin bind to cell surface receptors in the intestine, which subsequently leads to an activation of guanylate cyclase. There are controversies whether guanylate cyclase and STa receptor are same protein or two different proteins. STa receptor is a distinctly different protein from guanylate cyclase. Although guanylate cyclase coupled STa receptors have been reported to exist only in intestinal epithelial cells of mammals, however, recently it was shown that guanylate cyclase coupled STa receptors are expressed in epithelial cells throughout the body of the north American opossum. It was also reported that receptors for the *E.coli* STa are present throughout the digestive tract of chicken, with binding activity present not only in the intestinal epithelium but also in the intestinal smooth muscle. It is, therefore, remains possible that guanylate cyclase coupled STa receptor are also expressed in the epithelial cells of other animals. STa bind to a single class of high affinity receptors present on rat intestinal epithelial cells and brush border membranes. Most of the studies on STa-receptor were done with rat brush border membranes and epithelial cell. However, the STa receptors are present in low numbers in rat enterocytes.

The objectives of this study were: i) to determine the presence and density of the STa receptor in intestinal epithelial cell and brush border membranes of different animals and ii) 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. Binding of ^{125}ISTa to the brush border membranes of rat, rabbit was specific, time and temperature dependent was reported by us earlier. A single class of receptors were 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. STa binds to a single class of receptors in COLO-205 human colonic carcinoma cell also reported by us. Binding was specific, time and temperature dependent. STa binding protein with MW of 95 kDa was detected in this cell line. 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[$^{2-3}$H] inositol resulted in a rapid rise of [^{3}H] 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. 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. To explore further the calcium signaling induced by STa treatment in COLO-205, during the reported period fluorescence ratio imaging, was done



by using fura-2AM where excitation and emission spectra changes as function of calcium concentration. Changes in calcium concentration are often occur within ms, therefore, the speeds at which intracellular calcium signaling processes occur is generally monitored by fluorescence ratiometric analysis. Ratiometric analysis reveals calcium kinetics of different cells in a single microscopic field. Imaging also permits the separate analysis of calcium wave of each cells. For the mesurement of [Ca^{2}+]i using dual wavelength ratiometric analysis, COLO-205 cells were grown on glass coverslips precoated with polylysine. The cells adhered to coverslips then loaded with fura-2AM at room temperature under dark condition for 30 min. Fura-2 loaded cells, suspended in normal free (NF) saline were then placed under microscope. Individual cell was selected to measure the individual calcium signal upon addition of toxin. This set up was employed when such experiments were required in which different drugs or buffers were applied to the same cells. In COLO-205 cells, STa in the presence of 1.8 mM $CaCl_2$ showed an immediate increase in [Ca^{2}+]i to 522 nM which was corroborated with the result observed in fluorimeter. The same population of cells when treated with only Thapsigargin (TG, a calcium ATPase inhibitor), a transient rise in [Ca^{2}+]i was observed that had a tendency towards base line. TG showed a sustained rise of [Ca^{2}+]i in the same cells suspended in NF saline containing 1mM $CaCl_2$. This was again confirmed using 2 µM thapsigargin and thapsigargin plus STa. Here, Thapsigargin showed a transient increase in [Ca^{2}+]i that was down regulated towards base line in about 15 minutes. When TG plus STa was given no further increment of [Ca^{2}+]i was observed suggesting that like TG, STa deplete the intracellular calcium store resulting in rise in [Ca^{2}+]i. The result obtained by using TG and TG plus 1.8 mM $CaCl_2$ treated cells suggested that STa raise [Ca^{2}+]i by depleting [Ca^{2}+]i store that may potentiate further entry of calcium across the plasma membrane. When cells were treated with metacholine a most selective ligand for muscarinic type3 (M3) receptors, showed a transient rise in [Ca^{2}+]i followed by a sustained rise in [Ca^{2}+]i which is very much similar to the effect of STa on COLO205. These observations suggested that receptor of STa might be a muscarinic receptor. So it may be concluded that both calcium efflux and influx are involved in the rise of [Ca^{2}+]i in COLO-205cells following STa treatment.

7.3. Antibiotic Resistance, Virulence Genes and Molecular Profiles of Shiga Toxin Producing *Escherichia coli* Isolated From Diverse Sources in Calcutta, India

Investigator :

A. Khan

During the past decade, Shiga toxin-producing *Escherichia coli* (STEC) has evolved from a clinical novelty to a global public health concern. STEC infections have been reported from many countries and can cause a spectrum of human illness ranging from symptom-free carriage to severe bloody diarrhoea and even to life threatening sequel such as hemolytic uremic syndrome. STEC is a serologically diverse group of food borne, zoonotic, pathogen, of which the serotype O157:H7 has been epidemiologically significant worldwide because of its notoriety of being associated with life threatening disease.

In this study, two powerful molecular typing techniques were used to examine a collection of STEC strains isolated from diverse sources in Kolkata. One of the objectives was to determine the relatedness of STEC strains isolated from different sources and thereby obtain clues on whether transmission occurs between human and bovine sources in this part of the country. Of the 63 stains isolated during the study period, 40 (63%) were from cow stool samples, 19 (30%) from human stool samples and the remaining 4 (6%) strains were isolated from beef samples obtained from a city abattoir. The STEC strains were tested for resistance

against 15 antimicrobial agents. Resistance was observed most commonly to ampicillin (25%), tetracycline (24%) and streptomycin (14%) and less frequently to cephalothin (11%), co-trimoxazole (9%), nalidixic acid (6%) and neomycin (3%). Multidrug resistance was seen in 14 strains and there was no common resistance pattern among the strains.

In STEC strains, the virulence genes *stx1*, *stx2* and *eae* are chromosomal encoded while E-*hlyA, etpD* and *katP* are plasmid encoded virulence marker genes. Of the 63 strains tested by PCR, 36% carried *stx1* and 19% carried *stx2*, while 44% strains gave positive amplicons for both *stx1* and *stx2*. Nineteen percent of the STEC strains possessed *stx1*, *stx2* and E-*hlyA* genes, 5% strains carried *stx2* and E-*hlyA* genes while one strain (P-179-10) isolated from a cow stool sample carried the *stx1* and E-*hlyA* genes (Table 7.3.1). Intimin is the only intestinal adherence factor identified to date for STEC and the *eae* gene, encoding intimin, is widely distributed in STEC strains. *eae* gene was found in 14% of strains and was exclusively associated with *stx1* positive strains in this study. The *etpD* and *stx1* genes were detected in 3 strains isolated from cows. Two strains, which carried *stx1* and *eae* were also positive for *etpD* and *katP*. Two strains, AK-38 and P-33-2-26, which were isolated from cow stool samples, carried all the potential virulence genes (*stx1*, *eae*, E-*hlyA, etpD* and *katP*). Only one STEC strain (AK-47), which was isolated from a human stool sample, harboured 4 virulence genes (*stx1*, *eae*, E-*hlyA,* and *katP*). A strain (AK-17) isolated from beef sample gave positive amplicons for *stx1*, *stx2, eae*, E-*hlyA,* and *katP*. Overall, STEC strains possessing both *stx1* and *stx2* (24%) was the most prevalent toxin genotype followed by strains carrying *stx1*, *stx2* and E-*hlyA* (19%) (Table 7.3.1). Thus, it appears that the STEC in this part of world lack some factors that are essential for it to become a frequent cause of diarrhea.

The STEC strains were characterized by RAPD-PCR to analyze the genetic diversity among the strains. The RAPD profile of strains SD8, SD10 and SD12, isolated from different cow stool samples during different time periods were identical (Fig 7.3.1; panel I, lanes 4, 5, 6). Three sets of bovine isolates (SD2 and SD5 [Fig 7.3.1; panel I, lanes 2, 3], SD4 and SD7 [Fig 7.3.1; panel II, lanes 2, 3], AK48 and AK54 [Fig 7.3.1; panel IV, lanes 2, 3]), which had the same virulence genotype had the same RAPD profile while SD1 and SD3 gave the same profile in RAPD-PCR (Fig 7.3.1; panel II, lanes 4, 5), despite having a different virulence genotype (Fig 7.3.1). Two human strains (SDH2 and SDH3) were closely identical in RAPD profile with only one band difference. Interestingly, two strains (AK33 and AK36) that were isolated from human and cow stool samples, showed an identical RAPD profile (Fig 7.3.1; panel III, lanes 2, 3).

The *Xba*I pattern by PFGE was analyzed to determine the clonal relatedness among 25 STEC strains that gave identical or nearly similar DNA fingerprints in RAPD-PCR and had similar virulence genotype (Fig 7.3.2). SD2 and SD5, SD4 and SD7, AK 48 and AK 54, which gave the same RAPD profile also showed similar PFGE (Fig 7.3.2 [panel II, lanes 2, 5] [panel II, lanes 5, 7] and [panel IV, lanes 2, 3]). SD8, SD10 and SD12 had identical RAPD profile but SD10 showed a band difference as compared to SD8 and SD12 in PFGE (Fig 7.3.2; panel I, lanes 2, 3, 4). However, stains AK33 and AK36, which were isolated from human stool and cow stool sample respectively, possessing the same RAPD profile, showed different PFGE profiles (Fig 7.3.2; panel III, lanes 2, 3). Likewise SD1 and SD3, which also showed identical RAPD profile but showed different PFGE profile (Fig 7.3.2, panel II, lanes 2, 4). This study indicates that clones present in bovine sources are not transmitted to humans in Kolkata setting. One of the possible reasons could relate to food habits in this part of the country where beef consumption is relatively low.

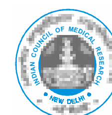


Fig 7.3.1. RAPD-PCR results of STEC strains that exhibited identical DNA banding patterns, Panel 1, lane 2 and 3 are strains SD2 and SD5; lanes 4-6 are strains SD8, SD10 and SD12 respectively. Panel II, lane 2 and 3 are SD4 and SD7, lane 4 and 5 are SD1 and SD3 respectively. Panel III, lane 2 and 3 are AK33 and AK36 respectively. Panel IV, lane 2 and 3 are AK48 and AK54 respectively. Lane 1 in each panel in 1 Kb DNA ladder marker.

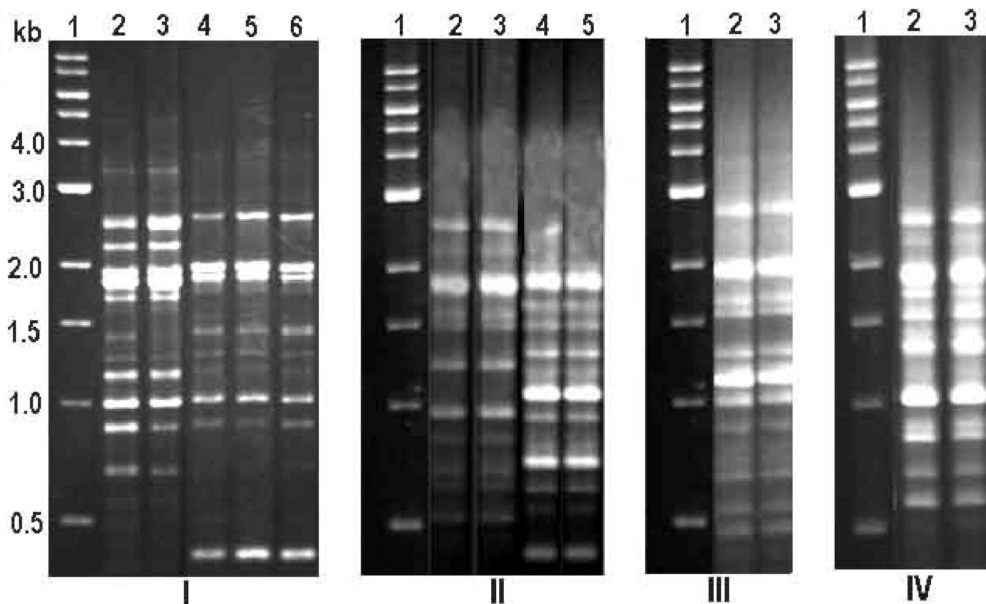


Fig. 7.3.2. PFGE profiles of *XbaI* digest of STEC strains. PFGE of STEC strains that exhibited identical profile in the RAPD-PCR and different virulence gene analysis are shown from panels I to IV and V and VI respectively. Lane 1 in all the panels and 7 in panel V are the l ladder molecular size markers.

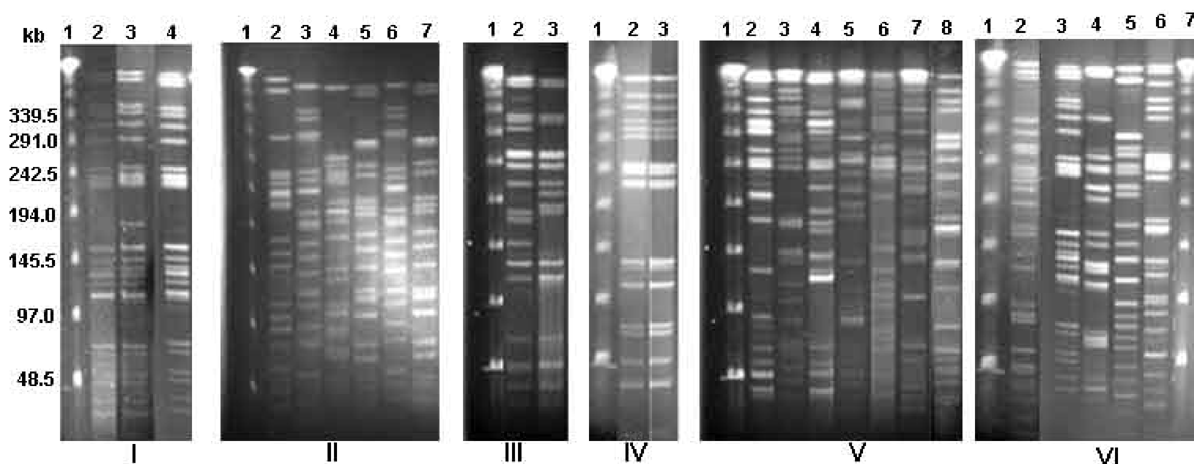
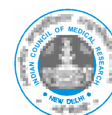


Table 7.3.1 Virulence genes profile of STEC strains.

Virulence genes	Number of strains with virulence genes			Total percentage of strains (%)
	Human	Cow	Beef	
stx1	6	5	0	11 (17.5)
stx2	3	4	0	7 (11.0)
stx1 and *stx2*	4	9	2	15 (23.8)
stx1 and E-*hlyA*	0	1	0	1 (1.6)
stx2 and E-*hlyA*	0	3	0	3 (4.8)
stx1, *stx2* and E-*hlyA*	3	9	0	12 (19.0)
stx1 and *eae*	1	2	1	4 (6.4)
stx1 and *katP*	1	1	0	2 (3.2)
stx1, *katP* and *eae*	0	1	0	1 (1.6)
stx2, katP and E-*hlyA*	0	2	0	2 (3.2)
stx1, stx2, katP and E-*hlyA*	0	0	1	1 (1.6)
stx1, eae, katP and E-*hlyA*	1	0	0	1 (1.6)
stx1, eae and *etpD*	0	1	0	1 (1.6)
stx1, eae, katP, etpD and E-*hlyA*	0	2	0	2 (3.2)

Mothers meeting at a rural field area



7.4. Studies to determine the role of enteroaggregative *Escherichia coli* (EaggEC) for causing diarrhoea with reference to its virulence properties

Investigator :

S. Dutta

The objective of the study was to study the difference in virulent attributes of EaggEC strains isolated from both diarrhoea cases and control children in a hospital based case control study conducted in the year 1996-97. Twelve sole isolates of EaggEC from cases of acute diarrhoea and eight isolates from controls were subjected to test for HeLa cell adhesion assay, surface pellicle formation, invasion assay, haemagglutinin, secretary protein profiles respectively. Plasmid profiles of those isolates were also tested. Genetic virulence determinants of both the groups were determined by PCR and compared. It was seen that all identified EaggEC strains showed negative result, when tested for LT, ST, VT genes by PCR method. But some strains from cases were shown positive for *set* gene (encoding for ShET1), the only difference obtained from the strains isolated from control children. Salt aggregation tests (SAT) performed with all strains using various molar concentration of ammonium sulphate. Almost all strains showed agglutination (+++) with 3.0 (M) and lower concentration of ammonium sulphate, indicating their adhesive properties. Almost 60% strains were positive for EAST. All strains were negative for *hly* and *eae* genes. All strains were found non-invasive. Almost 80% strains showed mannose resistant haemagglutinin activity with human Gr "O" RBCs, sheep RBC and chicken RBC at various dilutions. Total secreted protein content and outer membrane protein content of the strains were examined by running SDS-PAGE. The results were compared and analysed. Surprisingly, it was observed that strains both from cases and controls had identical OMP profile with major band at 38 kDa region (Fig. 7.4.1). All strains were found to possess a 60 Mda heavy plasmid, which was reportedly responsible for their adherence properties. Therefore, it was concluded that there was not much difference in the known virulence characteristics of case and control strains. But search should be continued to identify any putative virulence factor for this newly emerged bacterial enteric pathogen.

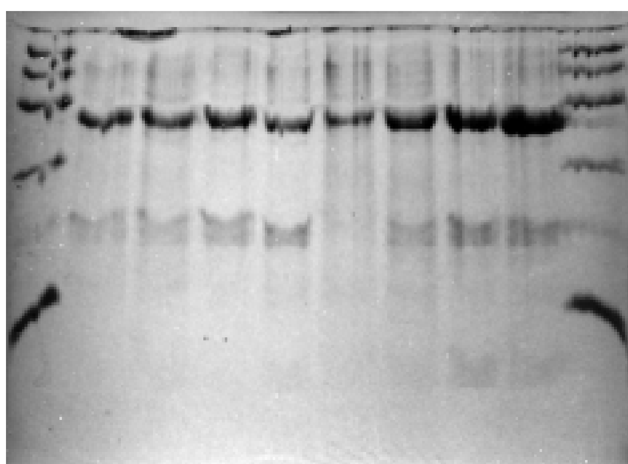


Fig. 7.4.1 : SDS-PAGE of outer membrane protein profile of EAggEC strains showing major band at 38 KD region

8 Studies on Other Bacterial Enteropathogens

Section highlights

- Purified *Yersinia enterocolitica* heat stable enterotoxin (YSTa) was found to stimulate intracellular calcium level in isolated rat enterocytes.
- In a study for better visualization of *Helicobacter pylori* in gastric mucosa modified Gemsa, Giemenez stains and a special stain Alpas were evaluated in addition to Haematoxylene and Eosine stain.
- One hundred and fifty strains of *Salmonella enterica* serotype Typhi isolated from clinical cases by examine for antimicrobial susceptibility testing. Most (84%) isolates were still resistant to chlorophenicol, ampicillin, tetracycline and co-trimoxazole.

8. Studies on Other Bacterial Enteropathogens

8.1 Mode of action of *Yersinia enterocolitica* heat stable enterotoxin (YSTa) in rat intestinal epithelial cell

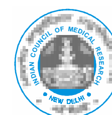
Investigator :

M.K. Chakrabarti

Yersinia enterocolitica, one of the aetiological agents of human gastroenteritis particularly in children in developed countries, is known to secrete a heat-stable enterotoxin (ST) called Y-ST that is similar to other methanol-soluble STs and the biological expression of which can be detected by the suckling mice assay.

Although lots of studies have been made regarding the genetic and chemical characteristics of Y-STs, and the amino acid sequence homology have been elucidated among different subtypes of Y-STs but no emphais have been made regarding the functional aspects of Y-STs. Among the different types of Y-STs, the Y-STa have been postulated to be involved in the cyclic GMP-mediated signal transduction resulting in the stimulation of chloride secretion and/or inhibit absorption and thus promote intestinal fluid secretion, since studies indicate that Y-STa increases cyclic GMP- levels in mouse intestine and cultured cell lines. However, no work has yet been made to evaluate the involvement of other signal transduction molecules, besides cyclic GMP, as the second messanger in the mechanism of action of Y-STa. Therefore, it would be interesting to find out the involvement of other signal transduction molecules such as IP_3, DG and Ca^{2+} in the mechanism of action of Y-STa.

The principal objective of this study was to evaluate the mechanism of action of heat stable enterotoxin secreted by *Yersinia enterocolitica.* At the initial stage of this study



Studies on Other Bacterial Enterpopathogens

Yersinia enterocolitica heat stable enterotoxin (YSTa) was purified from the culture filtrate using ammonium sulfate precipitation, DEAE Sephacel and Sephacryl S-100 HR column chromatography. Fractions were tested for enterotoxicity by suckling mice assay. It was found that purified YSTa raised [Ca^{2}+]i in a dose dependent manner and the optimal level of [Ca^{2}+]i was achieved by incubating cells with 10 ng YSTa . During the reported period the endogenous source of intracellular calcium rise in rat enterocytes after incubation with YSTa was evaluated. For the measurement of [Ca^{2}+]i fluorescent dye fura-2 was used as a probe of intracellular free calcium. Enterocytes (suspended in calcium free BSS) were incubated with 10 µM FURA-2AM at 37°C with constant shaking in the dark. After 40 min the cells were washed and resuspended in calcium free BSS and transferred to a quartz cuvette of Hitachi spectrofluorometer (model-3010). Fluorescence (F) was measured at 37°C with excitation at 340 nm and emission fixed at 495 nm. Maximum (Fmax) and minimum (Fmin) fluorescence were measured in presence of 0.1% Triton X-100 and $MnCl_2$ respectively. The [Ca^{2}+]i was then obtained using the formula [Ca^{2}+] = 224(F-Fmin)/(Fmax-F) where 224 is the association constant of calcium with FURA-2 at physiological pH. When Fura-2 loaded cells were reacted with 10 ng YSTa, rise of intracellular calcium concentration [110 $\pm$ 5 nM] was found to occur compared to control cells [48 $\pm$ 2 nM]. Again when the extra cellular calcium was chelated with EGTA, YSTa at similar dose raised the intracellular calcium concentration [102 $\pm$ 3 nM]. In another experiment cells were pretreated with 50 µM dantrolene, an IP3 induced calcium release inhibitor and it was found that YSTa induced rise in [Ca^{2}+]i was abolished. These observations provided evidence that YSTa could release calcium from intracellular stores.

Phosphoinositide specific phospholipase C (PLC) activity in rat jejunal enterocyte was then measured by treating the cells with YSTa. Rat intestinal mucosal scrapings were homogenized with 10 mM Tris-HCL buffer pH 7.5 containing 0.1% bovine serum albumin and centrifuged at 600 g for 10 min. Supernatant was then collected and used for enzyme assay. Intestinal mucosal homogenate (approx 160-200 µg protein) pretreated with YSTa was incubated at 37°c for 20 min in a 0.5 ml medium containing 10 mM phosphatidylinositol, 100 mM sodium borate and 0.8% sodium deoxycholate. The reaction was stopped by adding 2.5 ml chloroform: methanol: HCL (66:33:1). After centrifugation an aliquot (0.4 ml) was transferred from the methanol water layer in to a test tube. Inorganic phosphate was measured spectrophotometrically at 660 nm after hydrolyzing the myoinositol 1,2 cyclic phosphate. It was observed that YSTa increased the phospholipase C activity in a dose dependent manner and optimal level of phospholipase C activity was achieved (1.75 $\pm$ 0.08 PLC unit/ mg protein) by 10 ng YSTa compared to control (0.14 $\pm$ 0.03 PLC unit/ mg protein).

8.2. Correlation of histology with genotypes of *Helicobacter pylori* isolated from cases of peptic ulcer, non ulcer dyspepsia,gastric carcinoma and lymphoma Histologic special strain for Hpy1; modified giemsa, gimenez stain

Investigator :

D.R. Saha

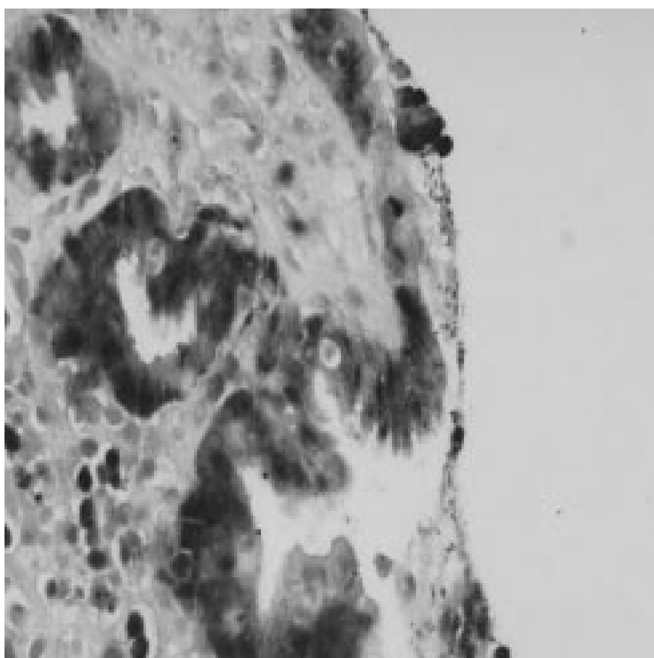
The discovery of *H.pylori* totally altered the concepts of the etiology of gastritis, as it has become apparent that infection with this organism is the major cause of non-autoimmune chronic gastritis. The present level of interest in *H.pylori* infection is high because of its etiologic importance in peptic ulcer disease and in gastric malignancy. Infection due to *H.pylori* appears to be a major risk factor for gastric Non-Hodgkin's lymphoma and MALT lymphoma. MALT lymphoma regresses in about half of the cases when *H.pylori* infection

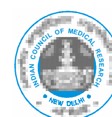
is eradicated with antimicrobial agents. Two important bacterial factors CagA and VacA are associated with *H.pylori* infection.

This project was undertaken to determine the association and tissue response to *Helicobacter pylori* with different diseased conditions along with the virulence genetic pattern of the organism. Endoscopic biopsy samples were collected from fundus and antrum of the stomach from the patients of S.S.K.M hospital, Kolkata. Five bits of tissue were taken, of which one bit was used for Rapid urease test, two bits were kept in Brucella broth with 15% glycerol for culture and two bits were kept in buffered formalin for histopathological examination. Formalin treated tissues were processed for paraffin embedding and serial thin sections were prepared, stained by Haematoxylene and Eosine (H&E) to see the histological changes by light microscopy. For better visualization of *H pylori* in gastric biopsies,few special stains were done .

In addition to routine H&E stain, other two stains were used e.g. modified Giemsa stain and Gimenez stain for microscopic examination of gastric biopsies. The bacteria was deep blue against a light bluish background with the use of modified Gimsa stain and the bacteria was dark red magenta against a pale bluish background with Giemsa (Fig. 8.2.1). The metaplastic stain (ALpas) was used for sections where metaplasisa was detected by H&E stain.Acid mucin turned blue and neutral mucin red /magenta ,nuclei pale blue with this method.Another special stain i.e.,immuno stain has been standardized and is being used specially in cases where bacterial load is scanty. Further study is in progress.

Fig 8.2.1 showing magenta coloured bacteria against a faint bluish background.





8.3. Environmental isolates of *Aeromonas* spp. harboring the *cagA* gene of *Helicobacter pylori*

Investigator :

S. Datta

The *cag* pathogenicity island (*cag* PAI) is a well-known virulence marker of *Helicobacter pylori* that is believed to be acquired initially by horizontal gene transfer from an unrelated species and over its evolution have adopted various intermediate types by chromosomal re-arrangement mediated by direct repeats and IS605. The *cag* PAI is a 40 kb DNA segment with 31 predicted open reading frames, of which the gene *cagA*, localized to the 3' end of the island, encodes an immunodominat antigen CagA, and often used as a marker of *cag* PAI. At present, however, nothing is known about the evolution of *cag* PAI or how it was acquired by *H. pylori*. It is often found that the virulence genes are dispersed among the environmental strains of the same or distant species and can be ferried about, given the fact that most of them are located on mobile elements. Thus it is possible that there may be an environmental reservoir of the genes of *cag* PAI that needs to be investigated. This prompted us to conduct a study for determining the prevalence of *cagA* in the aquatic microflora.

Mothers meeting in the field area of NICED

During September 2000 to March 2001, about 100 ml of water samples were collected from four fresh water ponds and a fresh water lake of Kolkata. A total of 41 environmental strains from these samples were found to possess *cagA*-like gene by colony hybridization test (Fig. 8.3.1). The PCR amplicon of the *cagA* gene of *H. pylori* reference strain 26695 amplified with primers Cf2 (5'GAATCAGTATTTTTCAGAC) and Cr2 (5' GGGTTGTATGATATTTTCC) was used as specific probe for *cagA*. These *cagA*-positive colonies had been formed on LA plates in normal atmospheric conditions, which preclude *H. pylori* growth that require blood or serum supplemented plates and has demanding

atmospheric requirements (5% O_2; 10% CO_2 and 85% N_2). Of these 41 strains, 14 yielded the desired 208 bp amplicon with Cf2 and Cr2 indicating the presence of *cagA*-like gene. For further analysis, only those *cagA* carrying strains were selected that were positive by both PCR and colony hybridization test. Twelve of these 14 strains were also positive by PCR using another set of *cagA*-specific primers cagA5 (5'-GGCAATGGTGGTCCTGGAGCTAGGC) and cagA2 (5'- GGAAATCTTTAATCTCAG TTCGG) that gave the appropriate amplicon of 324 bp while the remaining two yielded negative results. The primer pairs Cf2 and Cr2 and cagA5 and cagA2 were designed from different regions of *cagA* and hence PCR positivity using both primers strengthened our finding of the presence of *cagA*-like gene in these environmental isolates.

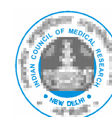
Western blot analysis with CagA specific antibody was used to detect the presence of surface-exposed CagA in the whole-cell protein of PCR positive strains. However, none of the test environmental strains yielded positive results although a 128 Kda protein band was observed in positive control strain *H. pylori* 26695 indicating the presence of CagA.

The negative Western blot result prompted to repeat the PCR analysis of the strains with the same pair of primers, as used before, to determine whether CagA was actually not expressed or whether there was loss of *cagA* in these strains. Culture material (10 µl) from the glycerol stock of each of the 14 strains was deployed to inoculate 5 ml of LB and after overnight incubation at 37°C; about 105 cells from these strains were subjected to PCR analysis with Cf2 and Cr2 primers. Interestingly, no amplification product was observed in 6 strains that were initially positive by PCR. But for each of the remaining 8 strains the expected 208 bp fragment was detected. However, chromosomal DNA prepared directly from the glycerol stocks of the strains using DNA extraction kit (Qiagen) was found to retain *cagA* as determined by PCR using primers Cf2 and Cr2. This highlights the possibility of gene loss upon subculture in the six strains that were negative by PCR for the *cagA* gene.

With the whole cell protein of these 6 stable strains, Western blot experiment was repeated again but as before, none of these strains expressed CagA in spite of the presence of the *cagA*-like gene. The *cagA* specific PCR products of 5 out of the above 6 strains were sequenced directly and compared with the published sequence of 26695 (accession number AE000511) in the Genbank database and four *cagA* sequences of clinical *H. pylori* strains of Calcutta. All the sequences obtained were 99-100% similar to each other and 97-98% identical to the corresponding portion of *cagA* of 26695 and Calcutta *H. pylori* strains (Fig. 8.3.2).

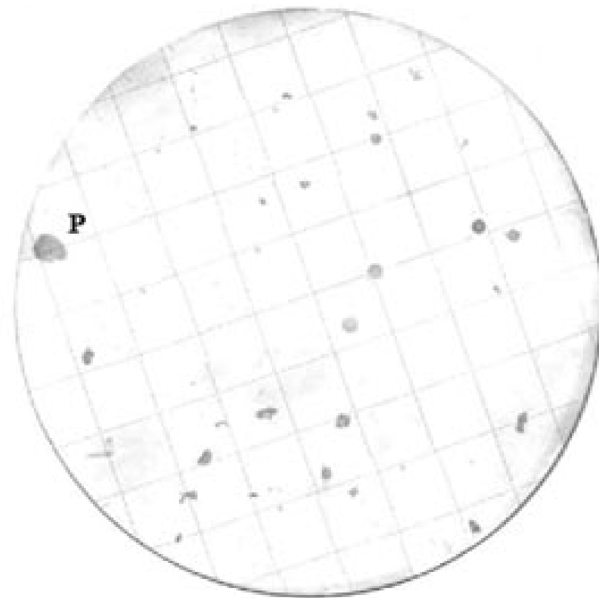
For presumptive identification of the environmental strains, oxidase tests were first performed and all the six strains yielded positive results. The results of the API 20E test identified the strains as *Aeromonas hydrophila;* analytical profile indices being 3047124 (for three strains) and 3047134, 3047125 and 1047124 for each of the remaining three strains. All the strains were also resistant to 150 µg/ml concentration of vibriostatic agent, 2,4-diamino-6, 7-diisopropylpteridine (0/129). Five strains were chosen for precise identification using the 16 S rDNA sequence and data analysis system. Of the five strains, one (C-15) was identified as *A.veronii* while another (C-8) was closely related to it (Fig. 8.3.3). However the other three strains (C-45, CE3 and C-92) grouped within the cluster of *A. enteropelogenes* and *A. caviae* in phylogenetic analysis (Fig. 8.3.3).

Thus in this study, *Aeromonas* spp. from aquatic sources in Kolkata were found to harbor silent *cagA*-like genes as demonstrated by colony hybridization, PCR analysis and nucleotide sequencing. The presence of *cagA*-like gene in *Aeromonas* spp. poses the question as to



whether gene transfer between *H. pylori* and *Aeromonas* is directly responsible for their presence. Since both *H. pylori* and *Aeromonas* spp. are known to survive in raw water and since both are human enteric pathogens, such a possibility could not be ruled out.

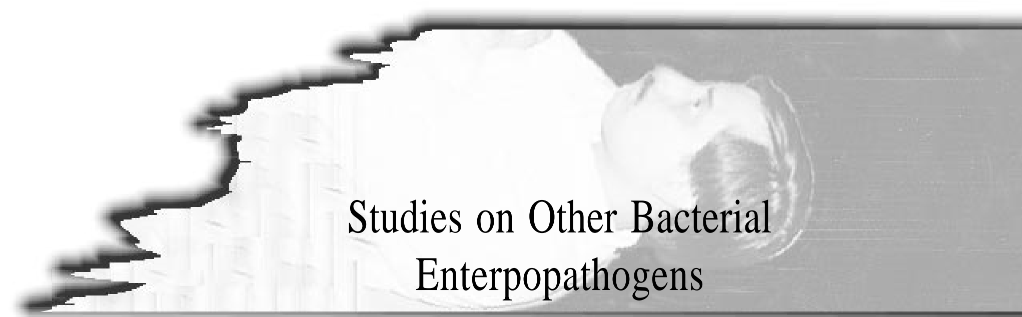
Fig.8.3.1 Colony hybridization test for the detection of *cagA* positive environmental isolates. P: positive control strain *H. pylori* 26695.



```
                  *        20         *        40         *        60         *
CE3    : .........................................................................
26695  : .................A.......................................................
C-15   : .........................................................................
C-8    : .........................................................................
C-45   : .........................................................................
I-18   : ................G........................................................
I-30   : ................G........................................................
I-47   : ................G........................................................
I-77   : ................G........................................................
C-92   : .........................................................................
         TTTAATCAACAAAGAC cTCTCATTGATGTAGAATCTTCCACAAAGAGCTTTCAGAAATTTGGGGATCAGCGT

          *       100         *       120         *       140         *       160
CE3    : .............................................CG..........................
26695  : .........................................................................
C-15   : .........................................................................
C-8    : .........................................................................
C-45   : .........................................................................
I-18   : .........................................................................
I-30   : .........................................................................
I-47   : .........................................................................
I-77   : .........................................................................
C-92   : ...................G.....................................................
         AGTTGGGTGTCCCATCAAAa GATCCGTCTAAAATCAACACCCGAtcGATCCGAAATTTTATGGAAAATATCA
```

Fig. 8.3.2 : Alignment of 169 bp sequence within *cagA* gene of five environmental isolates *of A. hydrophila* (C-8, C-15, C-45, C-92 and CE3) and four clinical *H. pylori* strains (I-18, I-30, I-47 and I-77) determined in this study and *H. pylori* reference strain 26695 (accession number AE000511). The alignments of all the sequences were carried out using the CLUSTAL-X software program.



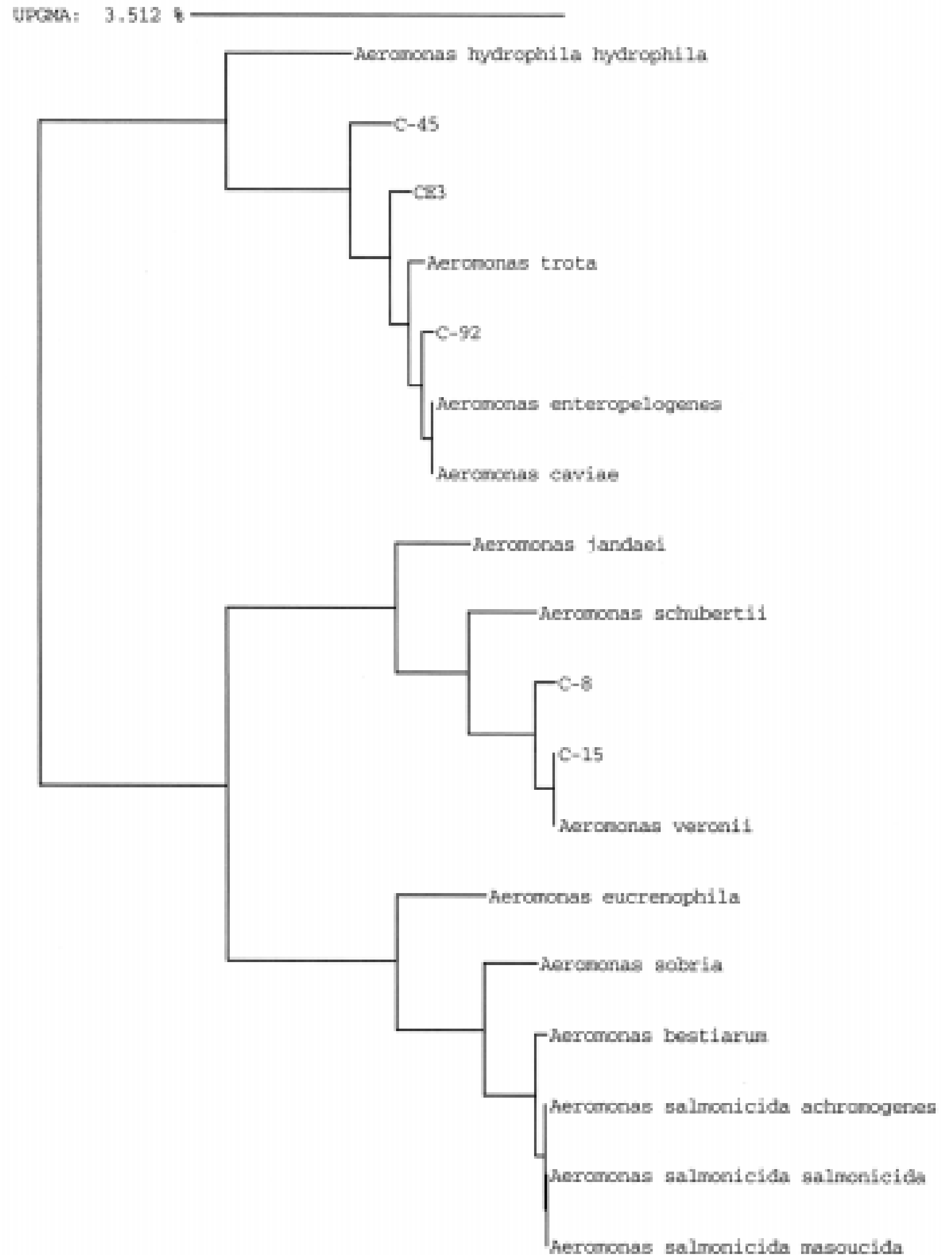


Fig. 8.3.3. : Species identification of five environmental strains by sequencing of 500 bp region of 16S rDNA. Three (C-45, CE3 and C-92) of the five strains, which were analyzed, clustered with *A. caviae* and *A. enteropelogenes* when compared with 16S rDNA sequences in the Micro Seq database while one (C-15) was identified as *A. veronii* and another (C-8) was closely related to it.

8.4. Differentiation of *Salmonella enterica* Serotype Typhi by molecular methods

Investigator :

M.R. Saha

In many developing countries typhoid fever remains an important cause of morbidity and mortality with an estimated annual global incidence of 21 million cases and more than 70,00,000 deaths. The epidemiological investigations are used to determine the source and spread of *S.enterica* Serotype Typhi but due to absence of reliable and sufficiently discriminated native methods of differentiating individual strain beyond the species level are not possible. The methods which have been used include antibiotic resistance patterns, biochemical reactions, phage typing, biotyping and plasmid analysis. The present study was undertaken to determine spatial and temporal variation of clonal type in Kolkata with the retrospective strains of *S.enterica* Serotype Typhi isolated since 1990 and strains of recent origin by pulsed field gel electrophoresis. Isolates of *S.enterica* Serotype Typhi by blood cultures clinically diagnosed typhoid fever cases admitted to Dr. B.C. Roy Memorial Children Hospital before administrating any antimicrobial were included. The organisms were isolated by blood culture using standard methods. The total of 30 isolates of the same organisms were analysed by phage types and pulsed field gel electrophoresis. The total of ten different phage types were discernable. All isolates were resistant to chloramphenicol, ampicillin, tetracycline and cotrimoxazole. The restriction endonucleases xba1 and spe1 were then used to perform PFGE analysis on *S.enterica* Serotype Typhi isolated from massive outbreak of typhoid fever in Kolkata during 1990-92 and from sporadic cases occurring in the year 1993-2002. Among the epidemic isolates and sporadic isolates patterns of pulsotypes were different from one another. Different pulsotypes of the isolates were observed of which 5 strains showed similar pulsotype 1. Four strains showed similar pulsotype II. Five strains showed pulsotype III and 16 different patterns of pulsotypes were observed. Considering 10 different phage types and 30 different pulsotypes indicate that the epidemic as well as sporadic cases of *S.enterica* Serotype Typhi were different in its origin. The PFGE were more discriminatory than that of phage types in the present study.