Special Issue on S. N. De and
Cholera Enterotoxin

Cholera Research in India: An Overview

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Cholera, a severe diarrhoeal disease leading to acidosis and death, is still a public health problem in India. *Vibrio cholerae*, a non-invasive gram negative bacterium is the etiological agent of cholera. The bacterium colonizes the small intestine and its pathogenicity is due mainly to the production of an enterotoxin which binds to an epithelial ganglioside receptor and promotes the secretion of water into the small intestine by stimulating adenylate cyclase activity[1]. India has a long tradition of cholera research and several pioneering studies led to early breakthroughs in this area.

Assay for Cholera Toxin: Rabbit Ileal Loop Method

Realizing the importance of adherence of the *V. cholerae* cells to the intestinal surface, its motility and toxin production as the major factors for pathogenesis of this organism, De and Chatterjee[2] carried out extensive studies to understand the mechanism of action of this bacterium on the intestinal mucosal membrane. They had demonstrated that severe inflammatory reaction with distension of the gut with fluid and intense congestion with haemorrhagic necrotic reaction takes place in the wall of the intestine following infection of *V. cholerae* into the tied loop of the intestine of a rabbit. One of the major outcomes of this study was the development of an assay for cholera toxin using the ligated rabbit ileal loop method which is still being used today[2]. The ileal loop method has several advantages. The metabolism of the small bowel is of aerobic glycolytic type rather than the anaerobic glycolytic type characteristic of the highly immature animal. This method can be used for active immunization studies and it is more sensitive to the toxin. Besides, the reaction is subject to relatively precise quantitation.

Haemolytic Activity of *V. cholerae*

In the course of these studies, De *et al*[3] had established the haemolytic activities of *V. cholerae* and other related vibrios and had developed a technique for estimating the O and H agglutinins in the serum of cholera patients[4]. The importance of the demonstration of haemolytic activity has recently been realized while examining the *tox* mediated regulation of the toxinogenicity of *V. cholerae*. Haemolysin production is now considered one of the criteria to distinguish between classical vibrios and their biotype *eltor*, the former being nonhemolytic (*hly*$^-$). Evidences have recently been presented that the *hly* gene is tightly linked to the *tox* locus which transregulates expression of hemolysin[5].

Excretion of Cholera Toxin

Another pioneering discovery made in the late fifties was the demonstration for the first time that the cholera toxin is exported out of the cell[6]. Using the rabbit ileal loop assay, it was shown that similar responses were obtained using the cell free culture supernatant and live vibrios. Dutta and his collaborators[7,8] showed that it is possible to reproduce the clinical appearance of cholera as well as certain histological and biochemical features of the disease in infant rabbits by oral administration of the toxin or parenteral administration of live vibrios thereby demonstrating the role of cholera toxin in experimental cholera. It was shown that if a virulent culture of *V. cholerae* or the culture free supernatant is administered intra-intestinally to weanling rabbits, the rabbits developed profuse diarrhoea and extreme dehydration simulating the clinical symptoms of cholera in human[9]. This method of reproducing the disease in the infant rabbits has been widely used in the study of cholera toxin.

Phage Typing Scheme of *V. cholerae*

In the early sixties attempts were made to use phages infecting *V. cholerae* cells in the treatment of cholera. Dutta and Panse[10] tried to evaluate the efficacy of cholera bacteriophage on the infant rabbit model of cholera under controlled laboratory conditions.

N. K. DUTTA

Cholera research in India reached a high point in the 1950's and Nirmal Kumar Dutta (1913-1982) was a major figure in this area. In the course of his studies carried out at the Haffkine Institute, Bombay, his group made important contributions towards developing the infant rabbit model for cholera and in demonstrating the role of toxins. His key paper entitled 'Role of cholera toxin in experimental cholera', with M. V. Panse and D. R. Kulkarni appeared in the Journal of Bacteriology (79, 594 - 595) a few months after De's classic paper in Nature. The paper by R. A. Finkelstein in this issue provides a brief account of this work and its importance in Finkelstein's eventual purification of cholera toxin.

N. K. Dutta had a distinguished career and served at the Haffkine Institute, Bombay from 1949-1971. He rose to be the Director of the Institute between 1967 and 1971. He subsequently served as Deputy Director General of ICMR for one year. He received many awards and honours including the Fellowships of the Indian National Science Academy and the National Academy of Medical Sciences. He was the General President of the Zoological Society in 1963. He was elected Fellow of the National Academy of Sciences, India in 1964 and the Royal Society, London in 1971.

The phage samples were tested for lytic activity on the hypertoxinogenic strain 569B and a number of locally isolated *V. cholerae* strains. All the phages had lytic activity for the strains of *V. cholerae* tested. However, such efforts were not successful in the long run. Later on, studies from various laboratories demonstrated the possibility of phage typing scheme for classical vibrios. Mukherjee[11] serologically distinguished cholera phages into four different groups and for the first time developed the phage typing scheme of *V. cholerae*. Phage typing was defined by the pattern of susceptibility of classical vibrios to these four groups of phages[11,12]. Phages belonging to group I, III and IV can lyse both smooth and rough strains of *V. cholerae*. Phages belonging to group II are specific for smooth strains. For infection of rough strains secondary growth overlay the lytic area. Phages belonging to group IV can infect and lyse all strains of classical vibrios but none of the *eltor* biotype[11]. Phages belonging to four serological groups are morphologically distinct[13,14].

The phage typing scheme of *V. cholerae* proposed by Mukherjee has been immensely helpful from epidemiological considerations. It has been useful in differentiating whether the epidemic is caused by *V. cholerae* or its biotype *eltor*. With the isolation of *eltor* vibrios from pandemics of cholera, phage typing of *eltor* strains were also carried out by Mukherjee and his colleagues. The *eltor* scheme, however, has never been thoroughly tested in the field.

This work gained international recognition and the WHO established an international center for vibrio phage typing at the then Indian Institute of Experimental Medicine (presently, Indian Institute of Chemical Biology), Calcutta. The center rendered valuable help to public health laboratories in India as well as other countries all over the world in identifying the causal strains of cholera epidemics and tracing their routes of spread.

Discovery of *P* Factor

Several variants of *V. cholerae* were reported during the early phase of cholera research. To characterize these variants, Bhaskaran[15-19] undertook genetic studies on this organism. Information on genetics of *V. cholerae* was almost non-existent primarily because of the lack of demonstrable exchange systems. Bhaskaran[15,16] demonstrated conjugation in *V. cholerae* and identified a fertility factor designated as *P* factor in *V. cholerae* strain 129. Later on this factor was found to be present in several strains of *V. cholerae*. Cells of strains possessing this factor (P^+ strains) produced plaque-like clearing when tested on strains devoid of *P* factor (P^- strains). It was possible to produce recombinants by mating genetically marked P^+ and P^- strains of *V. cholerae*[17-19]. The

strain carrying the *P* factor functions as the donor cell while the P^- strain serves as a recipient. The *P* factor discovered by Bhaskaran in *V. cholerae* is similar to the sex factor *F* of *Escherichia coli* in several respects. Both exist as autonomous replicons capable of depressed self-transfer and can also mediate low frequency transfer of bacterial genes[17]. However, unlike *F* factor, *P* factor cannot induce Hfr donors and hence high frequency mobilization of chromosomal genes from donor to recipient cells is not possible in *V. cholerae*. Treatments that readily eliminate *F* factor, do not have any detectable effect on *P* factor. Parenthetically, Bhaskaran's studies on the genetics of cholera marks the beginning of a new era in cholera research which attracted molecular biologists and geneticists who started probing the molecular basis of pathogenicity.

The importance of genetic analysis of *V. cholerae* realized by Bhaskaran in the early sixties for purposefully addressing the problem of toxinogenicity has been appreciated in recent years and several laboratories in India and abroad have given emphasis to understand the mechanism[6] of regulation of gene expression in general and toxin production in particular in this organism. Transfection of *V. cholerae* cells by phage DNA was demonstrated[20], cholera-phages have been used as a tool to study the genetics of the host and the DNA repair mechanisms operative in this organism have been delineated.

DNA Repair Mechanisms

In the last decade the DNA repair mechanisms operative in *V. cholerae* cells have been examined in great detail. These cells are 7–10 fold more sensitive to UV light then *E. coli* B cells. Although *V. cholerae* cells are proficient in reversing the UV light induced DNA damage by photoreactivation they do not possess efficient dark repair mechanisms[21]. The hypertoxinogenic strain 569B is most efficient in repairing DNA damage. The mild and nontoxinogenic strains completely lack the excision repair mechanism due to a defect in the UV endonuclease activity[21]. Atempts to demonstrate Weigle reactivation of UV irradiated choleraphages were not successful although UV induced filamentation of host cells was observed[22].

Using interspecific complementation of an *E. coli* recA mutant with plasmids containing the gene bank of *V. cholerae*, the recA gene of *V. cholerae* was identified, which codes for a protein of MW 39,000[23]. These cells lack the umuDC gene functions[24] which explains why UV irradiated choleraphages cannot be Weigle reactivated and why these cells are UV-nonmutable even when functions of recA and uvrA genes are present in these cells.

In spite of its inefficiency in repairing DNA damage induced by physicochemical agents, the spontaneous mutation frequency of *V. cholerae* for any given marker is comparable to that of other organisms. The spontaneous mutation frequency is directly related to the ability of a cell to repair mismatch in its DNA. The methyl-directed DNA mismatch repair mechanism and the gene functions involved in this process have recently been examined. The product of the DNA adenine methylase (dam) gene, responsible for strand discrimination to ensure that the mismatch is repaired on the newly synthesized strand, has been characterized and a mutation in this gene has been reported[25]. The mutS and mutL genes of *V. cholerae*, the products of which are required for recognition of the mismatch in the DNA, have been cloned and the complete nucleotide sequence of the mutL gene has been determined[26].

Genetics of Choleraphages

Another facet of research on cholera during the last five years has been an examination of the intracellular replication of choleraphage ϕ149, a representative strain of group IV phage, for two major reasons: firstly, to investigate whether choleraphages can be used as tools to study the genetics of the host and secondly, to find the reason for differential sensitivity of this group of phages towards *V. cholerae* and *V. eltor* cells. The intracellular replication of the circularly permuted DNA of phage ϕ149[27](102 kbp) involves a concatemeric DNA structure which serves as the substrate for the synthesis of mature phage DNA which is eventually packaged by a head full mechanism starting from a unique pac site in the concatemeric DNA. Packaging of DNA into phage involved binding of the concatemeric DNA to the cell membrane[28-30]. The mature phage DNA has single strand interruptions along its length repairable by T4 DNA ligase[27]. The phage DNA codes for fifty phage specific proteins of which nineteen are structural proteins. These proteins appear during the infection cycle in two distinct phases – early and late. Of the 26 early proteins, 11 are DNA-binding proteins[31]. The phage replication is sensitive to phosphate ion concentration in the growth medium. When infection is carried out in high phosphate medium none of the late proteins is synthesized[31]. *V. eltor* phage e4 codes for five different amino acids[32]. The tRNA gene cluster has been cloned and expressed to produce phage coded tRNAs[33]. Choleraphage ϕ149 also codes for 12 different amino acids[34].

Phage ϕ149 makes an abortive attempt to replicate in *V. cholerae* biotype *eltor*. Although no infectious centers are produced at any time during infection, the host macromolecular syntheses were shut off and the host DNA was degraded. Synthesis of monomeric phage DNA continued similar to that observed in the permissive host. However, concatemeric DNA intermediates produced were unstable and could not be chased to mature phage DNA[35]. A 1.8 kb *V. eltor* DNA fragment has been identified and cloned which codes for two proteins of MW 22,000 and 14,000. When the recombinant plasmid carrying the 1.8 kb DNA fragment was conjugally transferred to *V. cholerae* cells and the transformed cells were infected with phage ϕ149, no mature phage particles were produced and the concatemeric DNA intermediate was unstable as observed for infection in *V. eltor* cells (unpublished observation).

Adherence Factor

It was realized that for the development of an effective vaccine, cell surface components responsible for adherence of the cells to the intestinal surface need to be identified. Srivastava *et al*[36,37] have identified a 22 kd protein coded by a 1.3 kb DNA fragment of *V. cholerae* cells which reacts with antisera prepared against an adhesive antigen. Antibodies directed against this protein blocked adhesion of *V. eltor* strain KB207 to the rabbit intestine.

Cholera Vaccine

Mukherjee and his collaborators investigated for the first time the possibility of a live oral vaccine for protection against cholera. An oral vaccine based on naturally non-virulent strain of *eltor* biotype was tried as a vaccine on a limited number of human volunteers. Although it was initially found to be safe for human use and apparently stimulated the protective antibodies, eventually it turned out to be not so useful as a vaccine.

Sinha and Srivastava[38,39] showed that the pathogenicity of *V. cholerae* was significantly reduced in strains carrying the *P* plasmid in which a *V. cholerae* plasmid designated as *V* plasmid, is introduced. When the *P* and *V* plasmids were conjugally transferred in a virulent strain of *V. cholerae* KB9, a large number of isolates were obtained which are non-pathogenic. It was suggested that the loss of pathogenicity was due to the inability of these cells to synthesize cholera toxin. In view of the fact that both *P* and *V* plasmids are stably maintained in the nonpathogenic KB9 cells, and that these cells are immuno-

S. Mukherjee (far right) and colleagues drinking oral vaccine during a test at the Indian Institute of Experimental Medicine, Calcutta. (From 'A Decade in Retrospect.' Indian Institute of Experimental Medicine 1966–1976).

genic, this strain can serve as a potential live oral vaccine for cholera.

Recent attempts to develop an efficacious oral vaccine are directed primarily towards constructing attenuated nontoxinogenic *V. cholerae* strains using recombinant plasmids carrying the cholera toxin gene from which specific sequences encoding the A subunit are deleted *in vitro,* retaining intact the gene for the B subunit. Clinical trials of these attenuated strains showed that although the vaccine was effective, it caused mild-to-moderate diarrhoea upon initial immunization. Use of *E. coli*, a normal inhabitant of the small intestine, carrying the cloned cholera toxin genes as an oral vaccine strain for cholera may eliminate such side-effects. Keeping this in mind, Dasgupta *et al*[40] had taken the strategy of using *E. coli* cells as the host for cloning attenuated cholera toxin genes. However, the problem of using *E. coli* as an oral vaccine strain has been the inability of these cells to excrete the products of the cloned cholera toxin genes which probably accumulate within the periplasmic space. An *E. coli* mutant with a leaky cell surface phenotype has been isolated by transposon mutagenesis which can excrete the toxin protein synthesized in the cells. This strain may have some potential as a live oral vaccine for cholera.

Effect of Laboratory Subculturing

It is generally observed that the virulence of pathogenic strains of classical vibrios and the amount of enterotoxins produced by them is reduced during laboratory maintenance[41]. Animal passage of laboratory subcultured strains enhances both virulence and the level of toxin production. Roy *et al*[42] and Das and Das[43] reported that during laboratory maintenance the capability of dark repair of UV-induced DNA damage, phage sensitivity and the stability of alkaline phosphatase were reduced along with the reduction of toxinogenicity of strain 569B. These properties also could be restored by animal passage of the strain. It has been reported that most *V. cholerae* strains have an active gene analogous to the trans-acting positive regulatory factor but the level of ctx activation by this factor varies in different strains. Whether laboratory subculturing of hypertoxinogenic strains perturbs the activity of the regulatory gene is not known.

Cell Surface of *V. cholerae*

Several model systems simulating the important features of the interaction between the bacterium and the epithelial surface have been proposed. However, neither the host nor the microbial factors involved in colonization have been identified. Studies on surface architecture of *V. cholerae* was thus considered useful for the better understanding of the cell-cell interaction. Furthermore, excretion of cholera toxin from its cellular location is unique to *V. cholerae* since cholera toxin synthesized in *E. coli* cells was not excreted. In contrast when the *E. coli* heat labile toxin was synthesized in *V. cholerae* cells the product was exported out[44]. These observations suggested differences in the cell surface architecture of these two organisms. Recent studies have shown that the cell surface of *V. cholerae* is much more permeable than that of *E. coli* [45, 46].

These cells are highly sensitive to a wide variety of chemicals, in general, and hydrophobic compounds and neutral and anionic detergents, in particular. Segments of the outer membrane of these cells contain a lipid bilayer and has a relatively less negative charge associated with the polysaccharide moiety of lipopolysaccharide (unpublished observation). These cells are equally sensitive to penicillin G and ampicillin unlike other gram negative organisms which are resistant to penicillin. A beta-lactam induced 12,000 dalton outer membrane protein has been identified which presumably plays a significant role in conferring beta-lactam resistance to these cells (unpublished observation). Recently a lipid A mutant of *V. cholerae* has been isolated which is endotoxin negative. Most mutants in lipid A described so far in other organisms have defects in the polar substituents of lipid A. The *V. cholerae* lipid A mutant is perhaps the first to have specific blocks in the acylation of lipid A.

This review has focused on some of the major results achieved in cholera research in India since its inception in the early fifties. Emphasis has been given to describing early breakthroughs and on those current molecular genetic studies which have strengthened the early phenomenological findings. Thus, it has not been possible to cover all aspects of studies on cholera carried out in different laboratories of the country. If any relevant major finding has escaped my attention, it has happened unintentionally and I beg to be excused for the mistake.

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The Beginnings of American Research on Cholera

In the years preceding the Bangkok outbreak, research on cholera was proceeding only in a desultory fashion in the United States and elsewhere in the West. Working on a grant from the Army, William Burrows was pursuing the false trail of endotoxins in Chicago. In 1952, he was engaged in a collaborative study with Charles Lankford of the University of Texas in Austin, who had been asked by the Army microbiological laboratory at Fort Detrick (set up during the war to study preventive measures against germ warfare) to study the cholera vibrio, because so little was known about it. The stock of vibrio cultures they had were genetically degraded and therefore they travelled (under the auspices of the World Health Organization) to India to get freshly isolated cultures from cholera patients in the Nilratan Sircar Hospital (of S. N. De's medical college) in Calcutta. When Lankford returned to Texas, he made a decision to carry out an investigation that was going to have a most important influence on the eventual purification of cholera toxin by a pupil of his, Richard A. Finkelstein.

Excerpted from *Cholera: The American Scientific Experience, 1947-1980* by W. E. van Heyningen and J. R. Seal.