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CHOLERA BACTERIOPHAGES REVISITED

Studies on phages of *Vibrio cholerae* O1 have been of historical interest. The phages had been used earlier for the confirmatory diagnosis of *V.cholerae* O1 infection. They are still being used for the differentiation of classical and ElTor biotypes of *V.cholerae* O1. In the past, *Escherichia coli* phages had been used to detect non-enteropathogen-specific fecal contamination in water[1]. In countries where cholera is endemic, *V.cholerae* O1 bacteriophages (*ie*. vibriophages) have been detected in sewage water and served as strain markers[2] and for typing of *V.cholerae* classical, O1 and O139 strains[3-6]. In countries where cholera exhibits a seasonal behaviour characterized by fluctuations in incidence[7], environmental surveillance can play an important role in cholera control. Asymptomatic infection with *V.cholerae* O1 occurs much more frequently than do active cases[8] and surveillance by detecting *V.cholerae* O1 bacteria and vibriophages in sewage water may be a feasible means of predicting outbreaks of cholera before a significant number of cases occur. It has been reiterated that the presence of vibriophges in the sewage water is related to the number of cholera cases. A study conducted in South America favoured the use of presence of phages in sewage water as a potential predictor of outbreaks of cholera disease[9].

Historical Context and Phage Therapy Research

Phage therapy is the recent development in the field of phage research. The discovery of antibiotics is considered as one of the most important achievements in the history of medical science. However, the emergence of multi antibiotic resistant bacteria is one of the most critical problems of modern medicine. Prior to the discovery and widespread use of antibiotics, it was suggested that bacterial infections could be prevented and/or treated by the administration of bacteriophages. The bacteriophage was discovered by Felix d'Herelle, in the early part of the 20th century and the name bacteriophage was proposed to imply that phages eat or devour bacteria[10]. The successful use of phages for treating staphylococcal skin infection was reported in France[11]. Ernest Hankin, a British bacteriologist, reported the presence of marked antibacterial activity against *V. cholerae* in the waters of the Ganges and Yamuna rivers in India[12]. He suggested that an unidentified substance (which passed through fine porcelain filters and was heat labile) was responsible for this phenomenon and for limiting the spread of cholera epidemics[12]. Almost twenty years after Hankin's observation, Frederick Twort from England, gave a hypothesis that it may be a virus[13]. During 1925, d'Herelle's report of treating

four cases of bubonic plague with antiplague phage drew attention towards phage therapy. He latter visited India and worked on phage therapy of plague at the Haffkine Institute, Bombay (Mumbai) which led to the establishment of the Bacteriophage Inquiry in India under the Indian Research Fund Association and the initiation of phage therapy, especially for the cholera epidemics occurred during religious festivals and pilgrimages[14].

The disease cholera caused by *V. cholerae* is an ideal test case for therapy with phages. The enteric pathogen is confined inside small intestine, liberates cholera toxin, the mode of transmission and epidemiological characteristics of the disease are well known and effective vaccines are still not available. But the phage therapy for cholera is not clearly established in the treatment of patients or as a preventive measure as d'Herelle had hoped earlier[15]. It was observed that the severity and duration of cholera symptoms and the overall mortality from the disease were reduced in patients given cholera-specific phage by mouth[16]. In late 1960s, the World Health Organization (WHO) set up an international trial of phage therapy for cholera in Dhaka. This trial was designed according to the widely accepted international standards and conducted with the support and under the review of the National Institutes of Health, USA. In several WHO sponsored studies in East Pakistan (now Bangladesh) in the 1970s, bacteriophage therapy was compared with tetracycline as the therapeutic agent. It was reported that very high dose phage therapy was comparable to tetracycline in reducing the excretion of vibrios in the stool[17, 18].

Prophylaxis and Treatment

The therapeutic phage preparations were important products of the pharmaceutical industry in the Eastern Europe since 1952 and in the erstwhile Soviet Union since 1923. In these countries, therapeutic phage research and development was centered at the Hirszfeld Institute of Immunology and Experimental Therapy of the Polish Academy of Sciences, Wroclaw, Poland; and on a much larger scale at the Eliava Institute of Bacteriophage, Microbiology and Virology of the Georgian Academy of Sciences, Tbilisi, formerly the Soviet Republic of Georgia. The therapeutic phages were also produced in the USA. William Smith and his colleagues reported the successful use of phages to treat experimental *E.coli* infections in mice[19]. There are reports of the utility of phages in preventing and treating experimental disease in mice and guinea pigs infected with *Pseudomonas aeruginosa* and Acinetobacter[20-22]. Bogovazova *et al*[23] evaluated the efficacy of bacteriophages in the treatment of infections caused by *Klebsiella ozaenae, K.rhinoscleromatis* and *K.pneumoniae*. Phage therapy was applied to a variety of infections like bacterial dysentery[24,25], wound[26], gastrointestinal tract infections[27]; infections of skin and nasal mucosa[28] and salmonellosis[29] caused by other etiologic agents like Shigella, Salmonella, Proteus, Staphylococcus, Streptococcus.

Safety Profile and Limitations

Phages are similar to antibiotics as they have remarkable antibacterial activity. All phages are specific *ie* they react to only their targeted bacterial host and not to human or other eukaryotic cells. For example, phages specific to *V. cholerae,* always lyse *V. cholerae* and will not lyse Shigella, Salmonella or *E. coli* bacteria. This is a clear contrast to antibiotics which target both pathogenic microorganisms and normal microflora. As a result, the microbial balace in the patient is disturbed and may lead to serious secondary infections. Several reports about the use of phages in clinical settings have come from many countries especially the USSR and Eastern Europe; virtually all of them supported favourably the prophylactic and therapeutic use of phages. In all cases, phage therapy appeared to be safe and there have been virtually no reports of serious complications associated with the use of lytic phages in humans[30]. However, despite favourable reports, the phages are not commonly used prophylactically or therapeutically throughout the world and their efficacy is still a matter of controversy. One limitation is the high specificity of phages against targeted bacterial species. Phage susceptibility is necessary before administered and polyvalent phage cocktails lyse the majority of strains of the etiological agents. Today, interest in this subject has regained and phages as therapeutic agents seem to have the effect of diminishing the chances of selecting multi drug resistant bacteria in clinical trials of phage therapy.

Phage Typing Study

From early days, one major practical use of phages was for bacterial identification through a process called phage typing – the use of patterns of sensitivity to a specific battery of phages to precisely identify microbial strains. This technique takes advantage of the specificity of phages to their hosts and is still in common use around the world. Phages adsorb to specific receptor sites on the bacterial cell wall. In gram-negative bacteria, the receptors have

been identified as protein and lipopolysaccharide components of the outer membrane layer surrounding the peptidoglycan. A particular phage or group of phages will adsorb to specific site and different phages will adsorb to different sites. Thus, on the surface of a given bacterial cell a variety of different receptors are present; each type being represented in a large number of copies.

Despite more than a century of study, cholera remains an important cause of morbidity and mortality and still presents a devastating global problem. Strategies for the prevention and control of an infectious disease like cholera depend on understanding the origin, transmission, and other characteristics associated with the epidemic and its spread. Among the several typing methods, phage typing is one of the important and useful methods for the identification and differentiation of *V.cholerae* strains.

The use of bacteriophages as a method of strain differentiation has contributed greatly to the understanding of the epidemiology of the disease cholera. The first vibrio phages were identified by d'Herelle (1926)[31], and subsequently several distinct types of bacteriophages acting on *V.cholerae* had been described[32-34]. Majority of early studies were directed towards the use of cholera phages for treatment or prophylaxis rather than strain discrimination. A study was initiated on the cholera phages and phage typing in Calcutta in 1955. During the initial study of the classical biotype of *V. cholerae*, over 600 phages were screened which could be divided into four antigenically distinct groups. Four of these phages, isolated from the stools of cholera patients were included in the phage typing scheme for classical cholera. The replacement of the classical biotype by ElTor biotype rendered this scheme obsolete since the classical biotype disappeared from India during 1960s. The last case of classical cholera isolated at the National Institute of Cholera and Enteric Diseases (NICED), Kolkata was from Baroda in India in the year 1980. The internationally recognized phage typing scheme of Basu and Mukerjee (1968)[4], includes five phages (I, II, III, IV, and V) by which *V.cholerae* O1 biotype ElTor strains can be differentiated into six different phage types. Phage typing has been routinely performed on the basis of this scheme at the NICED, Kolkata since 1968. With the passage of time, the scheme was restricted to only two phage types – phage type 2 and 4. Additionally, increasing number of untypeable strains (10-11%) were also being encountered with the existing phages of Basu and Mukerjee. These limitations and the restrictions of this scheme led to the development of a new phage typing scheme at the NICED with the help of newly isolated phages for *V.cholerae* O1. The new scheme comprised five lytic phages different from each other and also from the phages of Basu and Mukerjee. A total of 1000 strains of *V.cholerae* O1 biotype ElTor from different sources were included for phage typing by this scheme. Almost 100 per cent strains were found to be typeable and the strains could be clustered into 27 types. The scheme was found to be highly effective and could be widely adopted for phage typing of *V.cholerae* O1 biotype ElTor, particularly in outbreaks originating from single source of infection .

Until 1992, *V.cholerae* O1 was considered the sole causative agent of the cholera. At the end of 1992, the scenario of cholera that had existed so far changed because of the emergence of a new etiologic serogroup of *V.cholerae*, which is known as O139 Bengal. The emergence of toxigenic *V.cholerae* O139 led to the development of an effective phage typing scheme for this organism. A total of five newly isolated phages lytic to *V.cholerae* O139 strains, differing from each other and also from O1 phages, were included in this scheme. A total of 500 *V.cholerae* O139 strains were evaluated with this scheme for their phage types, and almost all strains were found to be typeable. The strains were clustered into 10 different phage types, of which type 1 (38.2%) was the dominant type, followed by type 2 (22.4%) and 3 (8%). This scheme comprising of five newly isolated phages would be another useful tool in the study of the epidemiology of cholera caused by *V.cholerae* O139[6]. The NICED which was recognized as a WHO Collaborating Centre for Diarrhoeal Diseases Research and Training, operates today as a reference laboratory and receives strains of *V.cholerae* from all parts of India and abroad for biotyping, serotyping and phage typing. In India, constant monitoring using phage typing of the isolated strains of *V.cholerae* O1 is very important. Any noticeable change in this phenotypic marker may raise the suspicion of emergence of a new clone. The phages may play an important role as a potential predictor of outbreaks of the disease, which serves as an early useful signal for monitoring control measures of cholera.

Conclusions

The NICED, Kokata is engaged in research on cholera phages. The other groups are working on phage typing of other organisms in Russia and Canada[35-37]. All isolates of *V.cholerae* from different parts of the country are being sent to NICED, Kolkata for confirmation, serotyping, biotyping and phage typing. A total of ten O1 and five

V.cholerae O139 phages have been included in two separate schemes maintained at NICED. These phages were supplied to different institutes for research purposes from time to time. American Type Culture Collection (ATCC) – An international depository of phages has given an account number to this institute. In India, the predominant serotype was Ogawa. In 1992-93, the newly emerged strains of *V.cholerae* O139 were similar to *V. cholerae* O1 except in serology. The only other marker to differentiate between the two serovar was reactivity towards O139 specific phages. O1 phages do not react with O139 strains and O139 phages do not react with O1 strains.

There is a need to continue research in this area, which offers us a basic idea as a tool of identification with the help of this phenotypic marker, which is useful for all developing countries. The recent emergence of multi drug resistant pathogenic bacteria is a very serious problem. The phages especially the cholera bacteriophages may be helpful as therapeutic agents for treating infections. The effective approach and additional rigorous studies are urgently required in this field of research.

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This write-up has been contributed by Dr. B. L. Sarkar, Senior Research Officer, National Institute of Cholera and Enteric Diseases, Kolkata.

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