

STUDY OF POLIOVIRUS ISOLATES BY OLIGONUCLEOTIDE FINGERPRINTING

**THESIS SUBMITTED
UNIVERSITY OF BOMBAY**

**FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN
MICROBIOLOGY**

**SUBMITTED BY
ALPANA P. KHIRWADKAR**

**UNDER THE GUIDANCE OF
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BOMBAY**

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A

Thesis Submitted

To The

University of Bombay

For The

Degree Of Doctor Of Philosophy

In

The Faculty Of Science
(Microbiology)

by

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This thesis and the research efforts made are most humbly and respectfully dedicated to the millions of unfortunate souls whose all cherished ambitions of prosperity and healthy future were most cruelly crushed in the bud due to the dreaded disease 'Polio'.

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PREFACE

Paralytic poliomyelitis emerged as a horrifying scourge to the mankind at the beginning of this century. Paralytic afflictions of the central nervous system characterized the disease finally resulting in severely crippled survivors. The disease spread to distant areas and the epidemics increased in severity and magnitude over the next fifty years. Great strides made towards finding a solution over this pernicious disease finally culminated in introduction of inactivated poliovaccine (Salk) in 1954 and oral poliovaccine (Sabin) in 1961. Systematic use of the vaccine resulted in a spectacular decline in the incidence of the disease in America and the other developed nations. Its virtual disappearance from the large geographic areas provided an incentive for the World Health Assembly to embark on a programme in 1988, targetted to the global eradication of poliomyelitis by the year 2000.

Paralytic poliomyelitis is, however, still endemic in the developing countries of Asia and Africa. India together with its neighbouring countries, Pakistan and Bangladesh in particular faces a far more greater challenge as two third of the total poliomyelitis cases reported worldwide belong to these countries. In 1991, India formulated a National Plan of Action with strategies aimed towards reinforcement of vaccination programmes and strengthening the disease surveillance. Currently polioeradication is a nationwide drive requiring efforts from all sections of the community.

This thesis is a small contribution in these efforts.

REVIEW OF LITERATURE

PART 1

Historical Background

Acute anterior poliomyelitis has originated probably before the recorded history of man. The earliest evidence has been traced to an Egyptian relief (about 1300 B.C.) showing a young man with a withered, shortened limb typical of paralytic poliomyelitis. Hippocrates described paralysis that afflicted patients predominantly in summer and autumn. During the archeological excavations in Southern Greenland, skeletons from fifteenth century were discovered which showed bone deformities reminiscent of those typically associated with severe poliomyelitis¹. The examples ascertain that occasional cases of poliomyelitis have occurred throughout the history of mankind.

The first scientific description of poliomyelitis as a distinct clinical entity was recorded by Heine in 1840². Studies of Charcot et al (1870) defined the association of specific neuronal and inflammatory reactions in the central nervous system with this ailment³. Affliction of forty four persons in Stockholm, Sweden in the year 1889 is the first record of poliomyelitis epidemic in the modern times. The outbreak was described in detail by Medin (1891), who drew attention to the highly infectious nature of the disease⁴. Since this report the disease was also referred as 'Heine - Medin disease'. Increasing number of poliomyelitis epidemics were reported in Europe and North America during the last decade of the nineteenth century⁵⁻⁷.

The devastating sight of usually young and often severely crippled survivors of the poliomyelitis epidemics soon drew attention of the health

workers. The enteric route of infection in man was suggested in 1898⁸. Nevertheless, the general assumption that the infection spreads via respiratory route prevailed for a long time⁹. In 1909, Landsteiner and Popper succeeded in experimentally transmitting the disease to monkeys by inoculation of a bacteria free extract made from the spinal cord of a fatal case and thus established the viral etiology of poliomyelitis¹⁰. Serial transmission of the disease in monkeys was later achieved by Flexner and Lewis¹¹.

In 1916, the worst ever poliomyelitis epidemic known in the history occurred in New York, USA, afflicting more than 27000 persons. The case fatality rate was as high as 22 percent. Desperate acts reminiscent of plague epidemics were reported¹². This epidemic provided great impetus to research on poliomyelitis, particularly in USA. The initiative of President Franklin Roosevelt (1932 - 1945), himself a victim of paralytic poliomyelitis, resulted in the establishment of the National Foundation of Infantile Paralysis in 1938.

Pathogenesis and Disease

The term "Poliomyelitis" ('Polios' meaning gray and 'Myelos' meaning marrow in Greek) referred to the characteristic lesions found in the spinal cord of the afflicted cases. Studies of Sabin and Ward established that poliomyelitis is an infection of the alimentary tract. Oral route was found as the portal of entry and faecal matter the main mode of spread¹³.

The ingested virus implants readily in the mucosa of the throat and intestine. On multiplication in mucosa, the newly synthesized virus is released into the intestinal lumen. The virus then makes its way to the blood stream via the

local and systemic lymph nodes. Viremia coincides with a period of 'minor illness' showing symptoms such as headache, vomiting, fever, flushing pains and restlessness. Infection subsides at this stage in majority of the infected persons. In very rare instances (1:100 to 1:1000 of the total infections), the virus invades the central nervous system and the disease progresses to the paralytic phase. In the central nervous system, poliovirus replicates in the anterior horn cells of the spinal cord; reticular formation, vestibular nuclei and deep cerebellar nuclei of the brain stem; and along the precentral gyrus in the motor cortex. In severe cases the posterior horn and the dorsal root ganglia of the spinal cord are also affected. Destruction of the invaded nerve cells result in flaccid paralysis^{14,15}. Depending upon the anatomical localization of the lesions and the muscle groups paralyzed, poliomyelitis has been classified into three types viz. spinal, bulbar and bulbospinal. The spinal form involves the muscles of legs, arms, back, intercostal muscles and the diaphragm in decreasing order of frequency. In the bulbar form, the cranial nerves, respiratory and circulatory centres are affected. Fatality rate is high in the bulbar and bulbospinal poliomyelitis¹⁶. The clinical manifestations of poliovirus infection thus range from inapparent subclinical one on the one hand to the fulminant paralysis and death on the other. The infected individuals transmit the virus to the susceptible persons¹⁶.

Prevaccine Epidemiology

In the beginning of the nineteenth century, paralytic poliomyelitis was rare and largely restricted to infants, while its epidemics were unknown. During the end of the century, however, epidemics of increasing magnitude began to appear involving even older age groups. Soon after the Stockholm episode in 1889, epidemics of poliomyelitis were reported in Vermont (USA, 1894), Philadelphia (USA, 1898), Sainte-Gemmes (France, 1898), Australia

(1904), Sweden (1905), Norway (1905), New York (USA, 1907), Alaska (USA, 1908), Minnesota, Columbia and Alberta (USA, 1910). Severe epidemics were also reported in Canada (1921), Japan (1936), USSR (1934) and in the European countries. The disease, since then became a periodic menace with increasing rapidity in these regions. Poliomyelitis also assumed an epidemic form in tropical and subtropical regions like Puerto Rico (1942), Costa Rica (1944), Malta (1942) Mauritius (1945), India (1947), Ceylone (1948), Belgium Congo (1950); and thus spread almost worldwide^{5-7,17}.

Transition of the disease from relatively endemic to an epidemic one and the shift in the incidence towards higher age groups was presumed to be due to improvements in public sanitation and hygiene¹⁸. The epidemiologic pattern of the disease also differed depending upon the socioeconomic conditions in different parts of the world. In general, in the underdeveloped areas with low standards of living, poliovirus infection was ubiquitous. Most children were exposed to the virus infection early in the childhood. Poliomyelitis cases were confined to the youngest age group and epidemics occurred rarely. Serologic surveys of these populations revealed that almost 90 percent of the children above the age of three years had serologic evidence of poliovirus infection¹⁹. On the other hand, in the better sanitated areas of the world, improvements in general hygiene diminished the chances of contact with the virus at an early age, resulting in built up of a large number of susceptible individuals in the community. Introduction of a virulent strain in such communities resulted in progressive epidemics involving even older age groups²⁰.

Development of Poliovaccines

The Landsteiner and Popper's discovery of poliomyelitis virus was followed by efforts to achieve the prevention of the disease through artificial immunization. Initial attempts to actively immunize against poliomyelitis employed suspensions of infected monkey spinal cord in which the poliovirus had been inactivated by physical or chemical methods²¹⁻²³. Inactivation was, however, often incomplete. The risk of post vaccinal paralysis loomed almost as large as the danger of the naturally acquired disease^{24,25}.

Lenette and Gorden (1940) and Kessel et al (1946) provided evidence for the existence of more than one immunologic types of poliomyelitis virus^{26,27}. A collaborative study organized by the Committee on Typing of Poliovirus (1951) classified all the strains of poliomyelitis virus into three distinct immunologic types, namely poliovirus type 1, type 2 and type 3. This study also revealed that poliovirus type 1 strains were most regularly responsible for epidemics, while the type 3 strains were encountered only infrequently. Poliovirus type 2 strains were recovered from the sporadic cases and were the cause of only a single epidemic until then²⁸. It was thus clear that a successful vaccine must be capable of producing immunity against the three serotypes of poliomyelitis virus.

A noble piece of work came in 1949 when Enders et al discovered that the poliomyelitis virus can be propagated in vitro in non nervous tissues of human and monkey origin. This discovery by readily making available large quantities of poliomyelitis virus resolved an important step in the development of an antipoliomyelitis vaccine²⁹.

The concept of attenuation of virulent organisms for preparation of antidotes (vaccines) was developed by Jenner and Rockefeller foundation workers^{30,31}. By 1940's attenuated viral vaccine was available for immunization against yellow fever. Attenuation was defined as alteration of the virulence of a pathogenic micro-organism by passage through another host species decreasing virulence of the organism for the native host and increasing it for the new host.

Max Theiler was the first to report attenuation of poliovirus. A poliovirus type 2 strain following fifty rapid passages in mice no longer produced symptoms of poliomyelitis in intracerebrally inoculated rhesus monkeys³². Similar attempts by Bodian et al (1950) and Melnick et al (1951) to attenuate poliovirus type 2, however, failed^{33,34}. Nevertheless, Koprowski et al (1952) and Cabasso et al (1952) succeeded in attenuating a poliovirus type 2 strain by repeated passages in cotton rats and in chick embryo^{35,36}.

Enders et al (1952) reported that propagation of a highly virulent poliovirus strain in human non nervous tissue gave rise to virus progeny of markedly reduced neurovirulence in monkeys³⁷. Oral administration of large doses of this strain, however, failed to produce antibodies in monkeys. In contrast, antibody response was readily demonstrated when these strains were fed to the chimpanzees. It was concluded that the chimpanzees can be used as another indicator for the selection of attenuated strains³⁸. Further studies led to the finding that there existed an inverse relationship of the primates with regard to susceptibility of their nervous system and alimentary tracts to infection with poliovirus. The lower motor nervous of the monkeys are most susceptible to poliovirus infection than those of chimpanzees and

Fig 1

**Live oral poliovirus vaccine
Derivation of attenuated strains**

<u>Isolate</u>	<u>Derivation</u>
<u>Poliovirus type 1</u>	
P1/Mahoney/41 Fecal isolate, healthy children	14 # in vivo (monkeys) 2 # in vivo (monk testicles)
P1/Mahoney/Monk14T2	24 # in vitro (monk testicles) 18 # in vitro (monk kidney) 10 alternate # 5 in vivo (monk skin) 5 in vitro (monk kidney)
P1/Mahoney/LS-c	5 # in vitro (monk kidney) 3 # plaque purifications 2 # in vitro (monk kidney preparative)
P1/LS-c 2ab/KP2	Sabin vaccine strain
<u>Poliovirus type 2</u>	
P2/P712/56 Fecal isolate, healthy child	4 # in vitro (monk kidney) 3 plaque purifications 1 # oral in chimpanzee 3 plaque purifications 2 # in vitro (monk kidney preparative)
P2/P712/ch,2ab/KP2	Sabin vaccine strain
<u>Poliovirus type 3</u>	
P3/Leon/37 Fetal case of poliomyelitis isolate from brain & cord	21 # in vivo intracerebral 8 # in vitro (monk testicle) 37 # in vitro (monk kidney) 3 plaque purifications 3 # in vitro (monk kidney preparative)
P3/Leon/12 a1b/KP3	Sabin Vaccine strain

humans. Whereas the intestinal tracts of the humans are the most susceptible followed by the chimpanzees and then of the monkeys³⁹. Cynomolgus monkeys were selected as a model for determining neurovirulence of the selected mutants.

Sabin's work on attenuation of poliovirus began in 1952. He found that rapid passages of the virus at high multiplicity of infection in cell cultures was crucial for the emergence of the avirulent variants. Sabin propagated poliovirus strains of the three immunologic types in cynomolgus monkey kidney cultures to permit the selection of strains that were no longer paralytogenic for cynomolgus monkeys by intrathalamic inoculation. These strains could still effectively produce an immunogenic infection by the oral route⁴⁰. It was, however, soon discovered that such intrathalamically avirulent poliovirus variants could be highly paralytogenic, even in small doses, when placed directly into the anterior horn of the spinal cord of cynomolgus monkeys⁴¹. These observations led to the development of an elaborate neurovirulence test for the evaluation of candidate attenuated strains for vaccine production for human use⁴². The Sabin strains were further passaged and the progeny that showed the lowest spinal neurovirulence in monkeys were finally selected. The history of the attenuated strains selected by Sabin for the use in live oral poliovirus vaccine was published by Boulger and Sabin in 1973⁴³. A summary of this is presented in Figure I.

A parallel series of study was performed for the standardization of inactivation procedure for the vaccine production. The principle methods employed were heat, ultraviolet irradiation and chemicals (formalin or phenol)⁴⁴⁻⁴⁶. The formalin inactivated poliovirus remained the vaccine of

choice since its power of evoking antibody response equalled to any other and overinactivation did not result in denaturation (loss of antigenicity). Salk and his associates accomplished inactivation of the representative strains of each poliovirus type using formaldehyde at suitably controlled pH, temperature and duration in 1953. Inactivation was specifically controlled to render the virus noninfectious while maintaining its capacity to produce antibodies when injected intramuscularly⁴⁷. Inactivated Poliovirus Vaccine (IPV) also known as the Salk Vaccine was evaluated in the USA for prophylactic effectiveness in a very large field trial involving over one million children from forty four States. In children injected with three doses, the vaccine proved 68 percent effective against poliovirus type 1, 100 percent against type 2 and 92 percent against type 3 without any adverse side effects⁴⁸. Follow up studies demonstrated that booster doses would be required at frequent intervals to maintain the immunity. Greatly fortified by the results of this trial, the American authorities granted licenses for the manufacture of IPV for mass utilization in the United States⁴⁹. An immunization schedule of three intramuscular injections at two weeks interval, starting at six months of age, followed by a booster after an interval of seven months was suggested to provide adequate protection against poliomyelitis.

Introduction of IPV in 1955 was followed by a sharp decline in the incidence of paralytic poliomyelitis in the United States. The incidence declined from 33,000 cases (25 cases per 100,000 population) in 1954 to as few as 5894 cases (5 cases per 100,000 population) in 1957⁵⁰. With the success of IPV in the United States many other countries including Canada, Hungary, Belgium and The Netherlands, reeling under heavy onslaught of poliomyelitis, opted for IPV vaccination.

Unfortunately a disastrous episode occurred in 1956. About 260 cases of paralytic poliomyelitis were reported in Cutter, USA, following the administration of certain production lots of IPV. Although incomplete inactivation of the poliovirus in these batches was detected retrospectively, this incident (known as the Cutter incident) set a wave of reluctance among people to accept IPV for immunisation⁵¹. Moreover, IPV immunized individuals who were subsequently exposed to polioviruses developed gastro intestinal infections (not paralytic disease) as readily as did the unvaccinated individuals similarly exposed⁵²⁻⁵⁴. IPV was therefore unable to stop the circulation of wild poliovirus in the vaccinated community and the emergence of outbreaks among the nonimmunized subjects⁵⁵. It was concluded that although IPV offered protection to those immunized, it was unlikely to impede transmission of poliovirus in the community.

Meanwhile, each of the three attenuated poliovirus strains selected by Sabin were tested on large number of monkeys (Nos. 9000) and chimpanzees (Nos. 150) to confirm their non-neurovirulent character. The first human studies volunteered by Sabin's wife, daughters and their three playmates warranted the high immunogenicity and safety of the Sabin's vaccine strains⁵⁶. In July 1957, the World Health Organization recommended field trials for evaluation of safety and efficacy of the live attenuated poliovirus vaccine strains. The large scale field trials performed at Mexico, Singapore, Czechoslovakia and the USSR, involved sequential administration of a single dose (10^5 TCID₅₀) of each of the three live attenuated poliovirus vaccine strains (Sabin) in monovalent form. All the three strains were found to multiply extensively in the intestinal tract of the vaccinees irrespective of their prior inoculation status with IPV. Contrary to IPV, resistance of the intestinal tract of these vaccinees to reinfection with poliovirus was demonstrated. Dissemination of vaccine virus to children at

play or at home via faecal contamination facilitated cross immunization of the contacts. This was viewed as an advantage of the oral live attenuated vaccine. No evidence of any illness or complications involving central nervous system associated with the multiplication of the vaccine virus was observed. This was regarded as a valid evidence for safety of the vaccine⁵⁷.

Approximately four million children under fifteen years of age participated in the field trial performed in USSR in 1959. About 50 to 60 percent of the indicated groups in each region were vaccinated and the remainder were available for judging the effect of spread of the vaccine strains. An unprecedented reduction in the incidence of poliomyelitis in the entire study population was demonstrated. The usual seasonal high incidence peak also failed to appear. The presidium of the USSR Academy of Medical Sciences approved the findings and the Ministry of Health issued orders for mass vaccination against paralytic poliomyelitis using live attenuated oral poliovirus vaccine (Sabin)⁵⁸.

With the emerging success of the Oral Polio Vaccine (OPV) in USSR, most of the countries using IPV, replaced it with OPV in their anti poliomyelitis immunization programme. OPV was introduced in USA (in 1962), United Kingdom, West Germany, France, Yugoslavia, South Africa and Japan.

Sequential administration of the three poliovirus vaccine types to the entire population at an appropriate time interval proved to be a major practical problem. Attempts to produce a trivalent vaccine using equivalent amount of the three serotypes revealed interference of the type 2 poliovirus with the antibody response to types 1 and 3⁵⁹. The study conducted in Canada in 1961 was the first trial in which the current and most widely used

formulation of trivalent oral poliovirus vaccine (TOPV) containing $10^{6.0}$, $10^{5.0}$ and $10^{5.5}$ TCID₅₀ (10:1:3 combination) of poliovirus types 1,2 and 3 respectively was evaluated. A single dose resulted in optimum antibody response to all the three types. The balanced preparation was licensed for use in Canada in 1962⁶⁰. TOPV was introduced in USA in 1965.

Alternate Vaccine Strains: Other investigators also attempted the development of attenuated strains for the preparation of live attenuated poliovaccine. Strains developed by Koprowski et al and Cox et al were ready for testing at the time when WHO Expert Committee recommended field trials using the live attenuated poliovirus strains^{61,62}. These strains were tested in many large field trials in United States, Latin America, West Germany, Poland, Switzerland and Belgium Congo. However they were found to be much less attenuated than the strains developed by Sabin. These strains did not meet the requirements of the WHO Expert Committee and were discontinued from further use⁶³.

Induction Of Protective Immunity By Poliovirus Vaccines

The efficacy of IPV and OPV in controlling poliomyelitis was amply illustrated in the field trials and during their routine use in several countries. Both the vaccines were effective in evoking adequate immune response but they achieved success in different ways.

Immunity induced by natural poliovirus infection : Natural infection of poliovirus induces both the systemic and secretory antibody response⁶⁴. The role of cell mediated immunity and interferon in poliovirus infection has not been established in unequivocal terms. The virus neutralizing antibodies appear as IgM followed by IgG during the first week of infection. Shortly after

the appearance of IgG antibody, IgM begins to decline⁶⁵. Multiplication of poliovirus in the intestinal lumen sensitizes the IgA committed plasma cells in the intestinal lymph follicles and stimulates mucosal immune response. The resulting B lymphoblasts bearing IgA on their cell surface migrate through the thoracic duct and the peripheral circulation before localizing in the lamina propria of the gut and submucosal regions of the bronchial tissue. During their course they also seed the stroma of the mammary glands, mucosal tissues of the pharynx and buccal cavity. Secretory IgA production thus occurs in nasal, duodenal secretions and in breast milk. IgA is present as a firmly adherent layer on the surface of the mucosal epithelial cells lining throat and intestine thus shielding them from virus penetration⁶⁶. The immunity induced by wild poliovirus infection is almost lifelong and affords solid protection against the disease caused by homotypic infection.

Immunity induced by IPV : The IPV vaccine formulation consisted of 20, 2 and 4 D antigen units of poliovirus type 1, type 2 and type 3 respectively. Parenteral immunization of a single dose of IPV induced humoral antibody response. However due to absence of virus multiplication in the gut, IPV, failed to elicit the secretory IgA response^{66,67}. The serum antibody levels reach plateau in two weeks, and declined gradually over a very long period. Persistent immunologic memory after a single dose of IPV was demonstrated by Salk^{68,69}. New formulations of IPV with higher concentrations of antigen viz; 40, 8 and 32 D antigen units of poliovirus type 1, type 2 and type 3 respectively, was developed in 1978⁷⁰. The new enhanced potency vaccine formulation (E-IPV) elicited high levels of serum antibody by two doses. However, no enhancement in gut immunity could be demonstrated⁷¹.

The advantages and limitations of IPV are summarised below.

	Advantages	Limitations
1.	Confers high humoral immune response	Booster doses are required to maintain detectable antibody levels.
2.	Absence of living virus excludes potential for mutation and reversion to virulence.	Absence of intestinal immunity.
3.	Beneficial in immunodeficient or immunocompromised individuals.	Community protection requires very high vaccination coverage.
4.	Can be combined with DPT vaccine to form a quadruple vaccine preparation DPT-P.	High cost.

Immunity induced by OPV : Immune response to OPV parallels to that of natural infection⁷². The selective advantage of OPV over IPV is its ability to induce secretory IgA antibodies. Because of the sufficient immunity at the primary site of virus multiplication, the vaccinee ceases to be part of the virus transmission chain⁷³.

	Advantages	Limitations
1.	Confers high humoral and intestinal immunity.	Three doses are required to confer adequate immunity.
2.	Blocks the spread of wild virus rapidly during the epidemic conditions.	Intestinal infections due to other organisms interfere with the 'take' of the vaccine.
3.	Oral administration is easier to accomplish.	Risk of reversion of attenuated strains.
4.	Relatively inexpensive.	Poor thermostability.

Control of Poliomyelitis

Control in countries using OPV : Increasing use of OPV in several countries heralded a marked reduction in the incidence of paralytic poliomyelitis. USA relied almost completely on the use of OPV since its introduction in 1962, and succeeded in extending immunization to over 60 percent of the population by 1970⁷⁴. About 700 fold reduction in the incidence of poliomyelitis as compared to the prevaccine years was recorded in 1968 (0.5 cases per 100,000 population). Since then, only two outbreaks in the pockets of poorly immunized subpopulations were reported in USA. One of the two outbreaks occurred in 1972 among students of a boarding school in Connecticut. Eleven cases of poliomyelitis were observed among the 128 inmates, out of which 10 had not received the poliovaccine. The second outbreak was reported in 1979 (13 cases in US, 2 cases in Canada) among members of a community who refuse vaccinations on religious beliefs. The cases were scattered among the 75000 people of this community, resident in 23 states. Despite multiple contacts with the general public, no paralytic poliomyelitis cases were identified outside the affected religious group⁷⁵. A total of 187 paralytic poliomyelitis cases were reported in USA during the ten years period (1969-1979). Out of these, 40 (18%) were identified as epidemic, 32 (10%) as endemic, 22 (10%) as imported and 86 cases (47%) as oral poliovaccine associated. The overall frequency of vaccine associated cases was estimated as one case per 2.6 million vaccine doses distributed. It was however, found to be more often associated with the first dose (1 case per 520,000 doses distributed) than with the subsequent doses (1 case per 12.3 million doses distributed)⁷⁶. Virological investigations provided evidence that since 1980, there has been no endogenous reservoir of wild poliovirus in USA and a true break in the chain of transmission had been achieved⁷⁷. Less than 10 cases of paralytic

poliomyelitis were reported annually since 1980 in USA which were either due to importation of wild poliovirus or were associated with vaccination.

In Canada, poliomyelitis outbreaks were reported in cycles of 2 to 4 years during the prevaccine era. IPV was introduced in the National Immunization Programme in 1955 and OPV in 1962. The two provinces viz; Ontario and Nova Scotia continued the use of IPV. Both types of vaccines proved successful in controlling poliomyelitis in Canada. Only 34 cases were reported during the period from 1970 to 1979. Since then less than five cases occurred annually⁷⁸.

In Europe; the countries, Austria, Belgium, Czechoslovakia, Denmark, Eastern Germany, Ireland, Norway, Portugal and Switzerland which had recorded more than 1000 cases annually during the prevaccine years were successful in controlling the disease as early as in 1965, to less than 10 cases per year⁷⁴. In 1988, out of the 32 countries of the European region, 25 countries reported zero polio cases and only 213 cases were reported from the remaining countries⁷⁹.

Control in countries using IPV : The Netherlands, Sweden, Finland, Iceland and two provinces of Canada continued the exclusive use of IPV. These countries achieved extra ordinary success in controlling poliomyelitis. High degree of success has also been attributed to their high economic standard. These countries are mainly small, scarcely populated and with very well developed health care infrastructure which ensures immunization of the entire population. In Finland, systematic search failed to detect any poliovirus even from the sewage samples since 1963⁸⁰. In Netherlands, however, few local outbreaks among the religious groups who refuse immunization were reported⁸¹.

Poliomyelitis In Developing Countries

Information concerning the incidence of paralytic poliomyelitis in the economically underdeveloped countries remained obscure for a long time due to under reporting. Attention was focussed on the developing countries, when Nicholas et al (1976) and Ofosu-Amah et al (1977) conducted lameness surveys among children in Ghana. The surveys determined the prevalence rate of paralytic poliomyelitis to be 5-7 cases per 1000 children and an annual incidence rate as 23 to 28 cases per 100,000 population. This incidence was almost thirteen times more than what was reported through the routine surveillance system^{82,83}. Since then surveys for the residual paralysis due to poliomyelitis were carried out in many other developing countries. Surveys from Burma, Egypt, The Philippines, Thailand, India, Indonesia, Malawi, Ivory Coast, Brazil determined that the annual incidence of poliomyelitis in these countries had been as much as or even greater than that observed during the prevaccine era in the developed countries⁸⁴.

Immunization coverage in the developing countries was estimated to be less than 5 percent until 1974. To address the problem, in the developing countries, the World Health Organization (WHO) established an Expanded Programme on Immunization (EPI) in 1974, with the goal of extending immunization services to all the children by 1990⁸⁵. EPI provided an initiative to commence immunization in most of the developing countries. In view of the proved advantages, OPV was recommended as the standard EPI antigen for control and eradication of paralytic poliomyelitis. During the initial years of immunization, inadequate system of information collection and reporting in most of these countries made it difficult to establish a numerical correlation between immunization coverage and reduction in incidence of paralytic poliomyelitis. However, in general a declining trend

was observed. In 1980, a randomized house to house survey in Morocco revealed the rate of residual poliomyelitis paralysis in 10-14 years old children to be highest (6.5 cases per 1000 children) and in those under 5 years to be lowest (1.5 cases per 1000 children). These data probably reflected the effect of increasing vaccination coverage in the years since 1974⁸⁶. Surveys from Brazil and Egypt reported similar findings^{87,88}.

The annual world summary of poliomyelitis reported by WHO for the period from 1975-1984 indicated that the relatively smaller developing countries such as Israel, Mauritius, Taiwan, Thailand, Bhutan, Gambia succeeded in achieving high immunization coverage (more than 80 percent) and control over paralytic poliomyelitis. A few most populous countries, however, experienced very high endemicity of the disease. Nigeria, Niger, Zaire and Mali alone accounted for more than 50 percent of the cases reported from the entire African Region. Similarly, maximum number of cases from the American region were reported from Brazil, Mexico, Columbia and Peru. About 1500 to 2500 cases were reported annually in these countries. In the South East Asian region, India alone reported 14000 - 18000 cases annually, accounting for about 90 percent of the total number of the regional cases⁸⁹. Vaccination programmes in these countries reached only to a small proportion of the susceptible children. High population density combined with poor sanitation and hygiene were favourable to the continuing transmission of poliovirus.

Ramos Alvarez and Sabin had recognized the problems of poliomyelitis in economically poor countries. They attempted an alternative strategy involving mass vaccination campaigns instead of year round vaccination in Mexico (1960) and Cuba (1962) which had proved highly effective in controlling poliomyelitis^{90,91}. In 1980, Brazil adopted this strategy which involved immunization of all the children under five years of

age with two doses of OPV given one month apart, irrespective of their previous immunization status. A precipitous drop from 2.1 cases per 100,000 population in 1980 to 0.1 case per 100,000 population in 1981 was reported⁹². Similar campaigns in Mexico (1982 onwards) and Dominican Republic (1983 onwards) reduced the number of poliomyelitis cases in the South American region to a negligible level. In 1984, the reported incidence for this region fell below 0.1 cases per 100,000 population. A plan for eradication of wild poliovirus from the South American region was established in 1985⁹³.

In 1986, however, an outbreak involving more than 350 cases of paralytic poliomyelitis of poliovirus type 3 etiology occurred in north-east province of Brazil. Supplementary mass vaccination campaigns with TOPV had little effect in controlling the spread of the virus. Low levels of neutralizing antibodies against poliovirus type 3 in the vaccinees of this region were determined retrospectively. An alternative formulation of TOPV containing twice the concentration of type 3 poliovirus ($10^{5.8}$ TCID₅₀) was evaluated. Seroconversion rates to poliovirus type 3 with the new formulation were 2.7 times higher in comparison to the standard vaccine formulation. The epidemic declined in all the nine states. Failure of the conventional TOPV to halt the spread of wild virus was retrospectively traced back to the lower antibody response to type 3 vaccine component⁹⁴.

Lower antibody response even with three or more doses of TOPV was also observed in other developing countries (viz Gambia, South Africa, Nigeria and India)⁹⁵⁻⁹⁸. Collectively the data revealed that an average of 90 per cent of children seroconverted to type 2 whereas only 73 per cent (C.I. 36-99) and 70 per cent (C.I. 40-90) seroconverted to type 1 and type 3 respectively after completion of the primary immunization schedule. The

lower seroconversion rates in developing countries have been ascribed to various factors. These include loss of potency due to exposure of the vaccine to heat in warm climate countries; presence of concurrent enteric infections which are highly prevalent in the developing countries; nutritional status of the infants, passively acquired maternal antibodies in breast milk and interference due to poliovirus type 2⁹⁹. Sabin reemphasized the use of mass immunization strategy for the rapid elimination of paralytic poliomyelitis from the developing countries¹⁰⁰.

Poliomyelitis in India

Paralytic poliomyelitis is highly endemic in India ever since its first epidemic was reported in 1947 at Car Nicobar in the Andaman group of Islands of the Indian Union¹⁰¹. The OPV immunization services were introduced in the wake of one of the major epidemic in Andhra Pradesh in 1961¹⁰². Baseline sample lameness survey conducted in 1981 in 14 states of India detected an average annual incidence rate of 20 to 25 cases per 100,000 population. Based on these data it was estimated that about 140,000 to 170,000 cases of poliomyelitis occurred annually in India¹⁰³. This incidence was highest reported by any country in the 1980's and represented more than 50 per cent of the global cases.

EPI was launched in India in 1979. The national immunization coverage level with the third dose of OPV was estimated to be around 50 percent in 1986. Immunization services and the surveillance systems were intensified with the start of Universal Immunization Programme (UIP) in 1985-1986. Its impact was observed since 1988. A decline in the incidence from 28,350 in 1987 to less than 10,000 was reported in 1990¹⁰⁴. A review undertaken in 1992 by the national and international experts in two randomly

selected districts from five different states (Haryana, Madhya Pradesh, Tamil Nadu, Maharashtra and Orissa) determined that OPV 3 coverage reached to about 90 percent in Bombay and Madras and it ranged from 57 percent to 81 percent in other districts. The incidence rate in the range of 0.6 to 3.1 cases per 100,000 population was reported in these states¹⁰⁵.

Epidemics in the Poliocontrolled Regions

During the last decade, number of poliomyelitis outbreaks were also reported in countries which had controlled the disease to zero cases and had high levels of routine vaccination coverage. In Netherlands 178 cases of paralytic poliomyelitis were reported in 1978 in a religious community refusing vaccination¹⁰⁶. After absence of any endemic cases for fourteen years, an outbreak involving 71 paralytic cases was observed in the same group in 1992⁸¹. Although cases were confined only to the unvaccinated members, these outbreaks pointed out that clusters of unvaccinated individuals may be at risk even in populations with excellent immunization coverage. In Finland, after 20 years of freedom from poliomyelitis and with more than 97 per cent of immunization coverage, an outbreak (10 cases) caused due to poliovirus type 3 infection occurred in 1984. It was estimated that at least 100,000 persons in the general population were infected. Out of the 10 afflicted, 7 had received 3 to 5 doses of IPV. An antigenically drifted poliovirus type 3 wild strain was identified to be a precipitating factor in the outbreak. About 80% of the serum samples collected before the outbreak demonstrated a titre greater than 1:4 for type 3 strain (saukett E) used in IPV, whereas only 39 percent of the sera had a titre greater than 1:4 against the epidemic strain. The data reflected the ability of antigenically altered strain to escape the vaccine derived immunity¹⁰⁷. Similar episode was reported in Israel. Fifteen cases mostly in young adults (above the age

of 15 years) were reported in 1988 after six years of reporting less than 5 polio cases and immunisation coverage (4 doses) exceeding 90 percent. Out of the 15, 9 had received 3 to 5 doses of IPV. An age linked fall in neutralizing antibody levels was demonstrated in these patients. Differential pattern of seroresponse was also observed in the serosurveys suggesting involvement of an antigenically drifted strain comparable to that of Finland epidemic¹⁰⁸.

While the above incidents occurred in populations immunized partly or exclusively with IPV, break-throughs were also reported in OPV vaccinated populations. After documentation of zero polio cases and attainment of over 80% of vaccination coverage (OPV), outbreaks were reported in Taiwan (1981, 1031 cases), Gambia (1986, 305 cases), Oman (1988, 118 cases), Brazil (1986, 350 cases), Malaysia (1992), Jordan (1991), Bulgaria (1992, 43 cases) and Namibia (1993, 27 cases)¹⁰⁹⁻¹¹⁴. Retrospective studies demonstrated that the etiologic agents in majority of these outbreaks had migrated from other polioendemic regions. Previous success of the immunization programme in reducing the spread of wild poliovirus strains, suboptimum efficacy of OPV, delay in completing the primary immunization series until 7 months of age and pockets of unimmunized children probably resulted in accumulation of susceptible population to sustain an outbreak¹¹¹. These outbreaks pointed out that until wild poliovirus is eradicated globally, every poliofree country will be at risk of importation of wild poliovirus from the polioendemic reservoirs.

Eradication of Poliomyelitis:

Data on the incidence rates of poliomyelitis over the 15 years period from 1974 to 1988 had suggested a declining trend, especially since 1982.

In 1988, EPI documented that 67 per cent of children worldwide received full course of poliovaccine before twelve months of age. Among the six provinces reporting to WHO, the Region of Americas reported a decline in the incidence from 920 cases reported in 1986 to 344 cases in 1988. Out of the 47 countries, 34 countries reported zero polio cases. In the European Region, 25 countries out of the 32 reported zero polio cases and a total of 213 cases were reported from the remaining 7 countries. In the Western Pacific Region, a total of 1752 cases of poliomyelitis were recorded. These were mainly from China, Lao People's Democratic Republic, Phillipines, Vietnam & Cambodia. In the Eastern Mediterranean Region, 2317 cases were reported in 1988, of which 75 per cent were from the three countries Afganistan, Egypt and Pakistan. The South East Asia Region reported 22567 cases in 1988. Cases reported from India represented more than 90 per cent of the regional total⁷⁹.

It was thus observed that the poliomyelitis had been endemic only in few countries, where as the disease virtually disappeared in major part of the world. The progress of EPI and the success achieved in the American, European and the Western Pacific Regions resulted in optimism about the eradication of poliomyelitis. In May 1988, the forty first World Health Assembly committed WHO to the global eradication of poliomyelitis by the year 2000¹¹⁵.

The broad objectives of this eradication initiative are to achieve, by the year 2000 :

- no case of clinical poliomyelitis associated with wild poliovirus, and,
- no wild poliovirus identified worldwide through sampling of communities and the environment.

The quest for achieving control over poliomyelitis also led to the vast knowledge of the molecular nature of poliovirus and its pathogenicity. Advancements of new technologies during the last decade paved the way to the elucidation of the chemical and three dimensional structure of the poliovirion. The fingerprint of the genetic map was established through the sequence analysis of viral RNA and viral proteins. Construction of infectious cDNA clones and the in vitro synthesis of viral RNA with high specific infectivity opened new possibilities for biochemical and genetic studies. An important outcome was in understanding the fundamental properties of the virus including its assembly, immunogenicity and neurovirulence.

Advances in the molecular biology of poliovirus were quickly followed by their practical applications. Use of techniques such as oligonucleotide fingerprinting, partial genomic sequencing, polymerase chain reaction, restriction fragment length polymorphism and probes such as monoclonal antibodies permitted the accurate identification of poliovirus strains isolated from patients or from the environment. The following part of the review includes a brief account of these advances and their significance in achieving the WHO target of the global eradication of poliomyelitis.

PART- 11

Characteristics of Poliomyelitis Virus

Classification : Poliovirus is taxanomically classified as a member of the family picornaviridae and subfamilial genus enterovirus. The family picornaviridae, proposed in 1963 by the International Enterovirus study group, comprises of the small, ether resistant, polyhedral, RNA containing viruses of man and animals¹¹⁶. The picornaviruses are classified into four genera on the basis of their stability under mild acidic conditions. Those which retain their structural integrity and infectivity at pH 3.0 include the enteroviruses and the cardioviruses. Those unstable at pH 3.0 include the rhinoviruses and the aphthoviruses. The four genera are further distinguished by their buoyant density in cesium chloride solutions¹¹⁷.

The genus enterovirus includes polioviruses (3 serotypes), coxsackieviruses, group A (23 serotypes), group B (6 serotypes), echoviruses (32 serotypes), human enteroviruses (5 serotypes, 68 to 72) and human hepatitis A virus¹¹⁸. Members of nonhuman enteric viruses (37 serotypes) are also included in the enterovirus genus.

The cardioviruses are the murine viruses. They include columbia SK virus, encephalomyocarditis (EMC) virus, Maus Elberfeld (ME) virus, MM virus and mengovirus.

Rhinoviruses, the most important etiologic agents of common cold include 89 serotypes.

Aphthoviruses infect the cloven footed animals and rarely humans. They include 7 immunotypes and 53 subtypes of foot and mouth disease virus.

Composition : Poliovirus is a roughly spherical particle of 28-30 nm diameter, enclosing a single stranded positive sense RNA molecule¹¹⁹. It has a buoyant density of 1.33 g/cm³ in CsCl and sedimentation co-efficient of 156S¹²⁰. The native virion has a molecular weight of 11.5×10^6 daltons. Approximately 25 percent (w/w) is RNA (2.6×10^6 daltons); 50 percent is protein comprising of four types of polypeptide chains (5.9×10^6 daltons) and 25 percent is an aqueous environment¹²¹. The virion contains about 5800 monovalent cations (K^+ and Na^+ in a ratio of 5:1) and a few polyvalent cations (Mg^{++} , Putrescine⁺⁺ and spermidine⁺⁺⁺). They account for the neutralization of the negative charge carried by the phosphate groups of the RNA¹²². The virion contains no carbohydrate or lipid layer. However, recently, a myristate group and a molecule of a 16 carbon lipid, possibly sphingosine linked to the poliovirus capsid protein VP4 has been detected¹²³.

Structure : Poliovirus was successfully crystalized in 1955¹²⁴. X-ray diffraction analysis of the crystals revealed that the poliovirion is made up of 60 identical asymmetric structural units of 6.0 to 6.5 nm diameter arranged in an icosahedron¹²⁵. Electron microscopic studies confirmed the findings¹²⁶. Polyacrylamide gel electrophoresis (PAGE) of purified virus detected presence of four distinct polypeptides¹²⁷. These were named VP1 through VP4 in the order of increasing mobility in the SDS-PAGE system¹²⁸. Molecular weights of these proteins were estimated as 33500, 30100, 26400 and 7400 daltons, respectively¹²⁹. PAGE profiles of capsid

proteins also revealed the presence of an additional protein component, VPO (MW37500). It was detected as the uncleaved precursor of capsid proteins VP2 and VP4 which often remains intact as one or two copies in the mature virion¹³⁰.

Capsid protein VP1 was found to be the most basic polypeptide and VP3 the most acidic. Isoelectric focussing studies indicated a potential for strong interaction between VP1 and VP3 proteins¹³¹. Specific chemical modification of the capsid proteins in intact and disrupted virions using radioactive iodine (specific for tyrosine residues); iodoacetamide (specific for cysteine residues) and acetic anhydride (specific for the terminal lysine residues) provided evidence for the relative positions of capsid proteins. VP1 molecules were readily labeled in both intact and disrupted virions, VP2 and VP3 were labeled less but still significantly in intact virions, and VP4 could be labeled only in disrupted virions. These observations were taken to suggest that VP1 occupies most, VP2 and VP3 some and VP4 no part of the external capsid surface¹³².

Chemically induced crosslinking further provided evidence for the immediate neighbourhood of VP1 and VP3¹³³. Ultraviolet irradiation of intact virion induced VP4-genomic RNA cross linkages confirming internal location of the VP4 polypeptide in the capsid assembly¹³⁴. Stepwise destabilization and degradation of the virion using physical and chemical agents suggested that VP1 and VP3 cluster in the form of (VP1 - VP3) trimers and VP2 and VP4 probably lie in the form of (VP2 - VP4) pentamers¹³⁵. The relative localization of the structural proteins in the virion structure, however, could not be unequivocally determined by these methods.

Hogle et al (1985) elucidated the three dimensional structure of poliovirus by X-ray crystallography at 2.9Å⁰ resolution. The three dimensional conformation of the capsid proteins VP1, VP2 and VP3 was found to be remarkably similar to one another. Each of these proteins consisted of a common structural motif (core), with variable elaborations. Each core is composed of an eight stranded antiparallel beta barrel with two flanking helices. In contrast, VP4 is a short two stranded antiparallel beta sheet. The closed end of the barrel of VP1 is located near the five fold axis and those of the VP2 and VP3 barrel alternate around the three fold axis of the icosahedron. The closed ends of the VP1 barrel exposed along the five fold axis form five fold peaks. The uppermost three loops of the barrel (amino acid residues 96-194, 245-251 and 142-152) of VP1 are exposed at the summit. The top loop (amino acid residues 96-104) twists out from the surface such that it is particularly well exposed to the external environment. The outward tilt of the closed ends of the VP2 and VP3 barrel along the three fold axis are less pronounced. Only the outermost two loops (amino acid residues 72-75 and 242-244 in VP2 and residues 75-81 and 196-206 in VP3) are exposed¹³⁶.

Antigenicity : The antigenic sites (epitopes) of poliovirus were recognized by the isolation and genetic characterization of mutant viruses resistant to neutralizing monoclonal antibodies. The immunodominant poliovirus neutralization epitope was detected within amino acid residues 93-104 of VP1¹³⁷. This site correlates with the exposed loop of the VP1 around the five fold axis¹³⁶. Monoclonal antibodies directed against trypsin treated poliovirus (trypsin disrupts the major antigenic site) helped to recognize the subsidiary antigenic sites. Three such sites have recently been identified. The structures of these sites are more complex involving amino acid residues from VP1, VP2 and/or VP3 capsid proteins^{138,139}.

Genome : The genome of poliovirus is a single stranded molecule of RNA. The genomic RNA is directly utilized as mRNA upon infection¹⁴⁰. The viral RNA has a sedimentation co-efficient of 35S and a molecular weight of 2.6×10^6 daltons¹⁴¹. Poliovirus RNA is approximately 7500 nucleotides long, containing nearly equivalent molar amounts of adenylate (30%), cytidylate (24%), guanylate (22.5%) and uridylate (23.5%)^{142,143}. At its 5' end the genomic RNA is covalently linked to a small viral coded protein, VPg (virus protein genomic),¹⁴⁴. The 3' end comprises of a stretch of adenine residues known as the poly A tail¹⁴⁵. The genome has a long open reading frame encoding a single polyprotein (MW 246,000 daltons), which is proteolytically processed to produce functional viral polypeptides¹⁴⁶. About one third of the genome towards the 5' end codes for the capsid proteins. The remaining portion codes for proteins required for viral RNA replication (RNA polymerase), translation, processing of viral proteins (proteases), virion assembly and reorganisation of cellular activity.

Structural Organization Of Polioviral RNA

5' non coding region : Search for 'cap' structure in RNA of poliovirus, led to the identification of a small viral coded polypeptide VPg covalently linked to the 5' end terminal nucleotide. VPg is composed of 22 amino acids and has a molecular weight of 2400 daltons¹⁴⁷. No significant change in the infectivity of the RNA was observed when the RNA was not linked to VPg, suggesting that VPg is not required during the early stages of infection¹⁴⁴. Recent observations have shown that VPg plays a role in poliovirus RNA replication. A step other than initiation of RNA synthesis during the poliovirus replication, was found to be interrupted by lethal mutations in VPg¹⁴⁸.

The ninth AUG codon located at nucleotide 743 serves as the initiation site for protein synthesis¹⁴⁹. Poliovirus genome, thus, contains an untranslated stretch of 742 bases at the 5' terminus (5' UTR). The 5' UTR of poliovirus RNA is unusually longer than that found in most viral and eukaryotic mRNA molecules. The first 620 bases of 5' UTR show high sequence homology among the enteroviruses and rhinoviruses. The marked conservation suggested that it serves important functions in poliovirus life cycle¹⁵⁰.

A model of secondary structure proposed for the first 620 bases of 5' UTR predicted that this region has a potential to fold into 7 structural motifs (or domains) with primary and secondary structural elements. The first 100 bases are involved in forming the domains I and II. This region interacts with viral coded RNA polymerase (polypeptide 3 CD), and function in initiation of negative strand RNA synthesis. The sequences between bases 125 to 620 (domains III to VII), termed as internal ribosome entry site (IRES) direct cap independent initiation of protein synthesis. Poliovirus RNA containing deletions in the IRES failed to initiate protein synthesis¹⁵¹⁻¹⁵³. 5' UTR also control elements involved in viral neurovirulence. Its contribution in neurovirulence has been described in detail separately.

3' Non coding region : The polyprotein synthesis is terminated at nucleotide 7361. There are 73 nucleotides between the termination codon and the poly A tract at the 3' end of the poliovirus RNA (3' UTR). The 3' UTR is unusually short as compared to those in the eukaryotic mRNAs. It also does not contain the AAUAAA hexanucleotide sequence that characteristically occurs within first 20 bases of poly (A) in all 3' untranslated regions of the eukaryotic mRNA¹⁵⁴. The 3' end noncoding

sequences are involved in binding the viral RNA polymerase complex during the initiation of positive strand RNA synthesis¹⁵⁵.

Poly A tract : Like many eukaryotic mRNA's poliovirus RNA has a terminal stretch of poly (A) at the 3' end. The length of the poly (A) tract in a given viral RNA population is usually heterogenous (40 to 100 bases). Enzymatic shortening of the poly (A) tract in polioviral RNA resulted in decrease in its specific infectivity. Poly (A) tract of poliovirus probably plays a role comparable to that of the poly (A) found in eukaryotic mRNA's¹⁵⁶.

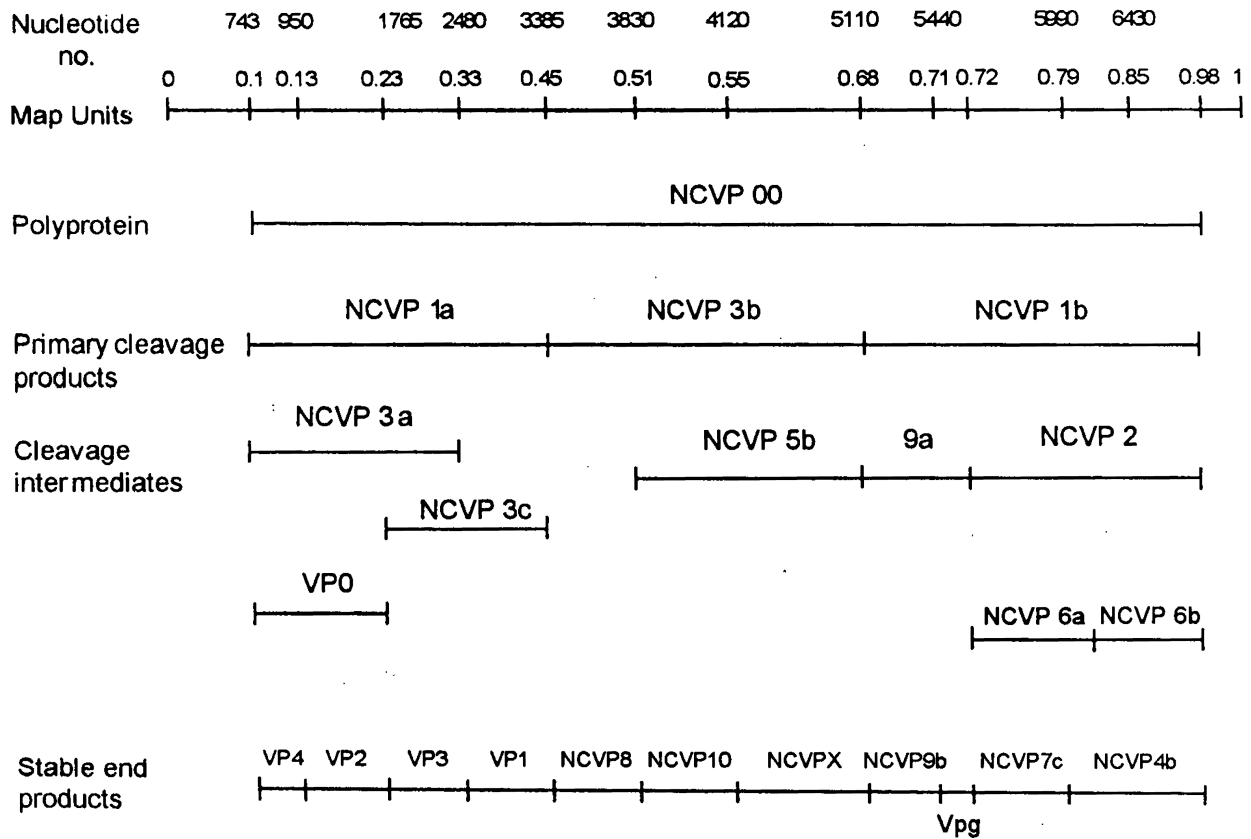
Features of the coding region

The polioviral open reading frame starting from nucleotide 743 upto nucleotide 7361 allows synthesis of a large polypeptide (2200 amino acids) which is post translationally processed to produce the virus polypeptides. About 12 to 15 'mature' functional proteins have been detected during the poliovirus growth cycle. The proteins were distinguished by their electrophoretic mobility in polyacrylamide gels. The capsid proteins of poliovirus were designated as viral proteins (VP1 to VP4) and the others as 'non capsid viral proteins' (NCVP1 to NCVP10). The polypeptides detected since the above nomenclature came into effect, have been named as NCVP1a, NCVP 1b and so on. The precursor proteins are called NCVPO and NCVPOO¹⁴².

The gene order of poliovirus RNA was deduced by (a) tryptic peptide mapping,¹⁵⁷⁻¹⁵⁹ (b) pactamycin and hypertonic block mapping,^{160,161} and through (c) genetic recombination experiments¹⁶². The 'genomic map' of poliovirus is summerized in Fig. 1:

Fig. 1

The Genome Map Of Poliovirus



Cleavage of virus specific polyprotein (NCVPO) is accomplished in three steps. The nascent polyprotein chain, while still attached to ribosomes, undergoes the primary cleavage resulting in three precursor proteins, NCVP 1a, NCVP 3b and NCVP 1b. These three large polypeptides are gradually processed to stable 'mature' products. The NCVP 1a gives rise to the capsid proteins VPO, VP1 and VP3. VPO is the uncleaved precursor of the capsid proteins VP4 and VP2. It undergoes cleavage at a late stage of the virion morphogenesis (the final cleavage event). The precursor protein NCVP 3b leads to the formation of proteins NCVP 8, 10 and X. The function of these proteins are less clear. It has been proposed that protein X either harbours protease activity or it copurifies with a protease¹⁶³. It was suggested that cleavage of VPO is carried out by one of the NCVP 3b proteins. Other functions assigned to these proteins include guanidine sensitivity and induction of cellular reorganization. The precursor NCVP 1b generates proteins VPg, NCVP 9b, 7c and 4b having replicative and proteolytic functions. Proteins NCVP 1b, 2 and 4b show RNA polymerase activity *in vitro*. Protein 7c has been implicated as the main virus specific protease. An alternative cleavage pathway of NCVP2, yielding 6a and 6b instead of 4b and 7c is likely to be preferred by certain strains¹⁶⁴.

Genetic Basis of Attenuation

Differences in the RNA genomes of oral poliovirus vaccine strains (Sabin) and their neurovirulent parent viruses were first investigated by ribonuclease T1 oligonucleotide fingerprinting method. It was estimated that the type 1 vaccine strain (Lsc 2ab) and its neurovirulent parent Mahoney differ at approximately 35 nucleotide positions¹⁶⁵. Complete nucleotide sequencing of the genomes of P1/Sabin and P1/Mahoney revealed that

these two viral RNA's actually differ by 55 nucleotide substitutions. These mutations are scattered throughout the viral genome and result in 21 amino acid replacements¹⁶⁶. The genomes of P3/Sabin and its neurovirulent parent P3/Leon showed only ten point mutations¹⁶⁷. As the progenitor of the Sabin type 2 strain has unfortunately not been preserved, similar sequence comparison was not possible. As an alternative, the sequences of the vaccine strain (P712) and a type 2 neurovirulent revertant isolated from a vaccine associated paralytic poliomyelitis case were compared. The two strains differed at 23 nucleotide positions¹⁶⁸.

Genetic recombination in poliovirus was known since 1962¹⁶⁹. The recombinants recovered from cells coinfecting with vaccine strain and its neurovirulent parent virus were tested for neurovirulence in cynomolgus monkeys by intracerebral inoculation. It showed that the major attenuating mutations in the P1/Sabin are present in the regions covering nucleotides 1 to 1122 and from 3664 towards the 3' end of the viral RNA¹⁷⁰. It was soon possible to construct viral recombinants at the desired crossover sites by exchanging DNA restricting fragments among different cDNA clones of poliovirus and transfecting the recombinant molecules to cultured cells. Analysis of type 1 recombinants in which small segments of the 5' noncoding region were exchanged indicated that a single base change at the nucleotide 480 from adenine (A) in P1/Mahoney to guanine (G) in P1/Sabin formed the major attenuating mutation in poliovirus type 1 Sabin Vaccine Strain¹⁷¹. It has recently been demonstrated that Sabin type 1 strain can apparently revert to neurovirulence by introduction of only two to three mutations at nucleotide 525, 6203, 7441, in addition to residue 480¹⁷².

Out of the 10 probable attenuating mutations of poliovirus type 3, the large part of the attenuated phenotype was attributed to only two mutations. Nucleotide 472 in the 5' UTR and 2034 in capsid protein VP3 were finally identified. The 472 site (cytosine in P3/Leon to uridine in P3/Sabin) in the 5' UTR is equivalent to site 480 of poliovirus type 1. A base change at 2034 induces a change from serine in P3/Leon to phenylalanine in P3/Sabin at amino acid residue 91 of the capsid protein VP3¹⁷³. Recently, a third mutation at nucleotide 2493 resulting in amino acid change from isoleucine to threonine at sixth amino acid in the capsid protein VP1 has been identified to have an attenuating effect¹⁷⁴.

In type 2 strain, the major attenuating mutations were identified at nucleotide 437 or 481 in the 5' UTR and at 2903 in the coding region. Mutation at the base 2903 resulted in a change of the amino acid isoleucine in P2/reverant to valine in P2/Sabin in the polypeptide VP3^{175,176}. These findings have recently been confirmed by attenuating wild virus by a single step of site directed mutagenesis¹⁷⁷.

It was striking that all the strains selected by Sabin possessed the major attenuating site in the same short region of the 5' end of the genome. This site is predicted to be involved in the formation of a complex stem loop structure in the domain V of the 5' UTR¹⁵¹. The A to G base change attenuating P1/Sabin at position 480 resulted in the alteration of the A-U base pair of the Mahoney strain to the allowed but weak G-U base pair in the vaccine strain. Similarly in P3/Sabin, the C to U base change at position 472 resulted in the alteration of C-G base pair to a weak U-G base pair. It is suggested that this mutation changes the spatial structure of the domain V that participates in the assembly of the ribosomes and initiation of protein synthesis and thus weakens its interaction with the cellular initiation factors. As a result, the level of viral protein synthesis decreases, the yield of virus

is reduced and it becomes less competitive compared to the revertant¹⁷⁸⁻⁸⁰.

The effects of attenuating mutations in the coding region are less clear. It has been suggested that a base change at site 2034 in poliovirus type 3 acts by destabilizing interactions between adjacent protomers in the virion¹⁸¹. Macadem et al (1991) presented the evidence that its effect was to prevent assembly of the protomers¹⁸².

Characterization of Poliovirus Field Isolates:

Poliovirus attenuation was accompanied by alteration of a certain phenotypic characters. These characters served as markers to differentiate the vaccine strains from the virulent progenitor strains. Following are the important markers associated with the Sabin's attenuated strains.

- a) Reproductive capacity temperature marker (RCT) - The naturally virulent prototype poliovirus strains possessed the capacity to multiply in tissue culture at 37° C as well as at 40° C. The growth of attenuated strains was completely inhibited at the supraoptimal temperature¹⁸³.
- b) Delayed growth marker (d) - Attenuated strains had markedly reduced ability to produce plaques under agar overlay containing low concentration (0.1 percent) of bicarbonate, as compared to the virulent parent viruses¹⁸⁴.
- c) MS marker - Attenuated strains also had a reduced capacity to produce plaques on MS cell line (tissue culture line originally

derived from the renal epithelium of monkey) than the virulent viruses¹⁸⁵.

- d) E marker and AI marker - The differential adsorption and elution of virulent and attenuated strains on DEAE cellulose column or on aluminium hydroxide gel formed the basis for E marker and the AI marker tests^{186,187}.

During the vaccine development phase, number of safeguards were obvious prerequisite to the release of live attenuated vaccine for the general use. The most important was to ascertain that the attenuation of the vaccine virus remains unchanged during the course of repeated passages in the vaccinees and their contacts. This raised the necessity for characterization of the polioviruses isolated from the vaccinees. The lowest spinal neurovirulence in monkeys constituted the only available laboratory yardstick for measuring the attenuation. It was however prohibitively expensive and difficult to perform on large number of field isolates. The in vitro tests on genetic markers thus gained practical significance.

The RCT and d markers showed maximum correlation with the monkey neurovirulence and were applied widely to study field isolates¹⁸⁸⁻¹⁹⁰. These markers were, however, often not stable after human intestinal passages. The marker tests thus failed to unequivocally determine whether the poliovirus isolates were attenuated or virulent. This stimulated the search for other characteristics for strain differentiation. Intratypic antigenic differences among poliovirus strains were shown by Wenner et al (1956)¹⁹¹⁻¹⁹³. The difference between the rate of reaction of an antiserum with its homologous and heterologous antigens provided the basis for McBride's 'kinetic neutralization test' devised for the identification of vaccine related or wild virus isolates (1959)¹⁹⁴. Wecker (1960) developed a

plaque reduction method involving antiserum incorporated into the agar overlay¹⁹⁵. Nakano and Gelfand (1962) modified the Wecker's method and used two predetermined dilutions of a specific antiserum in the agar overlay instead of only one¹⁹⁶. McBride's and modified Wecker's tests were widely used to study field isolates. The accumulated results indicated that vaccine strains on extensive multiplication in the human intestinal passages have a tendency to shift to 'non vaccine like' character¹⁹⁷.

The safety and stability of Sabin's attenuated strains on the other hand was proved beyond doubt through the use of millions of doses of OPV worldwide. Poliomyelitis incidence in most of the developed countries declined to only few cases per year within four to five years use of the vaccine. Control of the disease seemed to be a reality. Virological data was, however, needed to prove the minimal or non existent circulation of wild poliovirus. More importantly, the small number of paralytic poliomyelitis cases in these countries needed correct diagnosis to determine if the disease was caused due to infection by wild virus or the vaccine virus.

In 1979, van Wezel introduced a simple virus neutralization assay involving strain specific polyclonal antisera obtained by cross absorption with the heterologus strains¹⁹⁸. Introduction of monoclonal antibodies in 1975 provided a highly sensitive means of strain characterization. Several laboratories developed monoclonal antibodies to poliovirus which could be utilized in poliovirus characterization¹⁹⁹⁻²⁰¹. A range of wild polioviruses were tested by virus neutralization and single radial diffusion test using a panel of monoclonal antibodies. Some of the monoclones showed absolute differences with different virus strains within each serotype where as other monoclones showed wide cross reactivity. This revealed that certain epitopes available on the virus capsid of the different strains are well conserved for each poliovirus type, while others are relatively strain

specific²⁰⁰. Monoclonal antibodies were identified that reacted specifically with the Sabin strains and consequently with isolates derived from vaccine associated cases providing an indication of their vaccine origin²⁰¹.

Kew et al (1980) used the differences in electrophoretic mobility of viral polypeptides in polyacrylamide gel to identify the vaccine or wild origin of a poliovirus isolate; and also ... the relationship between the wild poliovirus strains²⁰². Mobility differences were observed in each of the major capsid proteins VP1, VP2 and VP3 as well as in the non-capsid proteins of different strains.

These classification criteria chiefly based on the antigenic framework of the virus, although was of potential value in identifying strain origin, they still left a number of isolates as unclassified or intermediate to vaccine and wild poliovirus. Identification was often restricted due to rapid evolution of RNA viral genome during their natural transmission. The recognition of vaccine origin in vaccine associated cases was impeded by the possibility of antigenic drift of the vaccine strain towards non-vaccine like characteristics upon replication in vaccinees. Moreover, the classification also could not provide evidence for the relationship and/or divergence between the wild strains^{197,203}. A radically different approach for strain characterization seemed essential.

De Watcher and Fiers (1972) developed, a two dimensional polyacrylamide gel electrophoresis technique for the separation of RNA fragments (oligonucleotides) obtained by hydrolysis of an RNA molecule with a site specific ribonuclease. The fractionation based on base composition in the first dimension and size in the second permitted effective separation of the RNA fragments. The distribution pattern so obtained was

found to be highly characteristic for the given RNA species²⁰⁴. Frisby et al, (1976) applied this technique to the comparative studies of picornaviruses. Considerable differences were observed in two dimensional separation pattern of the ribonuclease T1 resistant RNA fragments of the representative members of the entero, cardio and aphthovirus subgroups of picornavirus family. Individual members of the different subgroups could also be distinguished by the use of this technique²⁰⁵.

Lee et al (1976, 1979) demonstrated that inspite of high sequence homology between the RNAs of the three poliovirus serotypes their fingerprint patterns differed strikingly^{206,207}. It was soon observed that oligonucleotide fingerprints (maps) could readily distinguish poliovirus strains of vaccine and wild origin. The fingerprint pattern of the isolates from vaccine associated poliomyelitis cases were very similar to the pattern shown by the vaccine strain²⁰⁸. A methodology thus became available for the unequivocal diagnosis of vaccine derived poliomyelitis cases.

Oligonucleotide fingerprinting was soon applied to study large number of field isolates. Fingerprint patterns of the wild strains associated with the same epidemic were found to be very similar, whereas those of the isolates from the epidemiologically distinct epidemics were very different. On the basis of similarity and differences of their fingerprint patterns, wild strains could be classified into genetic subpopulations (genotypes). Fingerprints of the poliovirus type 1 strains isolated from 1972 epidemic of Connecticut (USA) were found to be very similar. They, however, differed from those isolated during the 1979 epidemic among the religious community in USA. The two epidemics caused due to poliovirus type 1 in USA were thus associated with distinct genotypes of the type 1 virus ²⁰⁹. The genomic fingerprint patterns of the 1979 epidemic strains, on the other hand, were clearly related to the isolates of 1978 Netherlands epidemic.

Chronological arrangement of the oligonucleotide fingerprint patterns of these isolates suggested migration of the Netherlands strain to United States via Canada²⁰⁸. Fingerprinting data thus provided a basis to construct migration pattern of poliovirus strains and provided an effective means of tracing the epidemiologic relationship among the circulating poliovirus strains. Evolution of many RNA viruses viz. influenza, foot and mouth disease, vesicular stomatitis, dengue, yellow fever, St. Louis encephalitis and Japanese encephalitis virus over a period of time were also traced by comparison of oligonucleotide maps of their serial isolates²¹⁰⁻²¹⁶.

The lower threshold for the recognition of genomic relatedness by oligonucleotide fingerprinting was determined at a level of 90 per cent base sequence homology. From the average rate of fixation of mutations over the polioviral genome (1 to 2 base substitutions per week), it was estimated that relationship between poliovirus strains separated from the ancestral strain by about 3 to 5 years can be recognized by fingerprinting²¹⁷. To obtain a substantially broader view of the distribution of wild poliovirus type 1 genotypes in nature, Recco Hesse et al (1987) compared a sequence of 150 nucleotides (3296 to 3445) encoding the regions of two functionally distinct proteins VP1 and 2A, of the polioviral genomes. The VP1 region encoded the sequences involved in the constitution of an antigenic determinant, whereas the adjacent 2A was a non capsid protein. As the two functionally distinct protein regions were subject to very different selective pressures, the sequence information provided insights into the genetic relationship among the poliovirus strains isolated from diverse epidemiological background. The partial genome sequence analysis of the poliovirus type 1 strains obtained over a 37 year period from five continents established relationship among them and revealed the complexity of the patterns of wild type poliovirus transmission in nature²¹⁸.

The epidemiologic range of molecular methods for poliovirus identification was further extended to include nucleic acid hybridization method using synthetic oligonucleotide probes. Probes were prepared that recognize either all human enteroviruses or specific poliovirus strains such as isolates derived from Sabin vaccine strains. Recognition is by specific base pairing of the probes to sequences within the genomic RNAs of poliovirus strains. The enterovirus group probe binds to sequences conserved among enteroviruses and polioviruses. While the strain specific probes bind to sequences that are highly variable across and within poliovirus serotypes. The probes can be used in concert with primary isolation and typing with hyperimmune sera and thus permits rapid diagnosis of enteroviruses. Probes specific to several wild poliovirus genotypes have also been prepared, such that important epidemiological information regarding the distribution of specific wild genotypes is obtained through routine diagnostic procedures^{219,220}. Recently, combination of the polymerase chain reaction with reverse transcription (Rt-PCR) provided possibility to produce a large number of DNA copies from RNA and to apply restriction fragment length polymorphism (RFLP) to the study of RNA genomes. Through this approach an assay involving analysis of polymorphism of a 480 bases long nucleotide sequence of the capsid protein VP1 (nucleotide 2402 to 2881) of the poliovirus genome, using Hae III, Hpa II and Dde I restriction enzymes has been devised^{221,222}.

Countdown To Poliomyelitis Eradication

In May 1989, the forty second World Health Assembly (WHA) endorsed the global Plan of Action for the eradication of poliomyelitis²²³. The plan highlighted three major areas requiring intensified efforts for the progress of the eradication programme.

Raising and sustaining high immunization coverage: Vaccination coverage of 80% or more by all the countries was considered a prerequisite for the global eradication of poliomyelitis. Supplemental immunization strategies such as National immunization days; inclusion of OPV at birth in the EPI immunization schedule; moppping up immunization and outbreak response were recommended in the polio endemic regions.

Strengthening of disease surveillance: Effective surveillance was recognized as the key element for monitoring the gigantic task of eradicating poliomyelitis from the entire world. A very well organized surveillance system was considered essential, initially for detecting the wild poliovirus reservoirs in the community, targetting for the supplemental immunization, and later for documenting that zero polio cases have indeed been achieved for successive years. The Plan of Action prepared a well formulated field guide setting uniform surveillance indicators of programme performance. The principle indicators are listed below:

- o A standard case defination for the reporting of paralytic poliomyelitis should be adopted by all countries. A case of poliomyelitis was defined as that occuring in any patient with acute flaccid paralysis (including any child less than fifteen years of age diagnosed to have Gullian - Barre Syndrome) for which no other cause can be identified.
- o Poliomyelitis incidence including the reports of zero cases should be reported at least monthly by each district to the regional and global level.

Countries close to achieving poliomyelitis eradication should establish weekly reporting.

- o In countries where poliomyelitis is endemic or epidemic, faecal specimen from a small number of representative cases should be virologically investigated using the type specific poliovirus antisera. The laboratory investigations in these areas should concentrate mainly on establishing the serotype of poliovirus circulating.

- o In countries where less than 50 cases are reported per year, virus isolation should be attempted in every suspected case. All viruses isolated should be identified using not only poliovirus antisera, but also those prepared against most of the other enterovirus serotypes. The viruses should be investigated further using the appropriate characterization methods and reported as vaccine associated, wild virus/indigenous, wild virus/ imported or unknown other. These countries should establish an expert review committee responsible for the final diagnosis of cases reported as poliomyelitis and for their further classification.

- o The report of the individual case should include, notification of time interval between the onset of paralysis, case reporting, laboratory investigations and the execution of control measures.

There is circumstantial evidence for the survival of poliovirus for several months or several years in sewage water²²⁴. This reinforces the demonstration of the environmental samples to be negative for wild poliovirus, in addition to the negative reports of all the clinical samples for the final documentation of the local elimination of virus. Thus, as the eradication programme progresses, poliomyelitis surveillance will shift its

focus from case reporting to an intensive search for wild poliovirus in the environment.

Strengthening laboratory capabilities : The Plan of Action has recommended that a network of poliomyelitis reference laboratories should be established that are able to isolate and type polioviruses and that are willing to test specimens from one or more countries for diagnostic purposes. The objectives of laboratory investigations in the eradication programme are; a) to establish the etiology of acute flaccid paralysis for the documentation of above mentioned surveillance parameters; b) to monitor the potency of vaccine being used; c) support special epidemiologic investigations and d) research. Polio endemic regions are prompted to undertake epidemiologic research to better characterize the patterns of spread of wild viruses defining rapid, effective outbreak control measures²²³.

In response to the implementation of the Plan of Action at the Regional and National level, the global OPV3 immunization coverage increased to 81% in 1993 with consequent decline in the incidence of paralytic poliomyelitis. A total of 9665 cases of poliomyelitis were reported in 1993, representing 72% decrease from the 34,762 cases reported in 1988 and 37% decrease from 15261 cases reported in 1992. Of the 210 countries/areas, 145 countries reported zero cases of poliomyelitis and 102 countries reported zero cases for at least 3 years in 1993.

Among the six WHO defined regions, the Region of Americas and the Region of Western Europe (except Turkey and few republics of former USSR) have been free of poliomyelitis. The last paralytic case caused due to wild poliovirus in the Americas was recorded in Aug., 1991²²⁵. In the Western Pacific Region, 1214 cases were reported in 1993, of which 99% were from Cambodia, China and Vietnam. A significant decline has also

been reported from South East Asian and African Region. A 54% decline corresponding to 4423 cases in 1993 as compared to 9600 in 1992 was reported in South East Asia Region\$. Fourteen countries in Africa and six countries namely Bhutan, DPR Korea, Maldives, Mongolia, Nepal and Thailand from South East Asia succeeded in recording zero cases.

The Eastern Mediterranean Region however reported a 26% increase (2404 cases) in 1993 compared to 1992 (1901 cases) 75% of the cases were reported from Pakistan which experienced large outbreaks in its four major provinces.

The efficiency of surveillance system is reported to be highest in countries with low levels of poliomyelitis and is low in countries with high level of the disease. WHO has estimated that most cases of paralytic polio are still not reported and about 110,000 cases of poliomyelitis actually occurred in 1993²²⁶.

Less than five years remain before reaching the WHO target date for the eradication of poliomyelitis. While success may seem inevitable, it is far from assured. Poliomyelitis is widespread on the Indian subcontinent which contributes three of every four poliomyelitis cases reported in the World. Declining immunization coverage presages epidemics even in low incidence countries that are currently polio free. Following the global achievement of 80% poliomyelitis immunization coverage among infants in 1990, it has become apparent that global poliomyelitis eradication cannot be achieved through routine immunisation alone. Supplemental immunisation activities will be essential in most countries. High priorities for global poliomyelitis eradication include thorough survey for all the suspected cases and intensifying the immunisation activities in the susceptible regions.

Introduction To Problem

Paralytic poliomyelitis has been endemic in India. It was estimated that in the 1980s more than 200,000 cases of paralytic poliomyelitis occurred annually in our country. The immunization coverage levels with three doses of OPV improved after the inclusion of Universal Immunization Programme (UIP) in 1986 and its impact in Bombay was first observed in 1988. A substantial decline in the incidence of the disease all over the country has been noted since then. However, the incidence rate in the range of 0.6 to 3.1 per 100,000 population reported from different parts of the country is still considered high. While the success in achieving the goal of eradication of poliomyelitis is theoretically guaranteed, it is a herculean task for India. The success will depend heavily upon intensification of the immunization programme supported by the strengthened surveillance systems.

In recent years, technology has advanced to include molecular biology methods for quick diagnosis of infection and identification of the poliovirus isolates. Characterization of the poliovirus strains at the genomic level provided the ability of i) unequivocal identification of the origin of field isolates as wild or vaccine derived; ii) identification of the numerous geographic foci of endemic circulation of wild type poliovirus; iii) detecting the previously unsuspected link between cases in distant communities; iv) determination of the pattern of spread of wild poliovirus from one region to another; v) documentation of cessation of indegenously circulating wild poliovirus strains from a population and the identification of imported strains if any. As the eradication programme in our country progresses, detailed information about the poliovirus isolates will form the basis to properly orient (channelize) the resources of the eradication programme.

The genomic characteristics and the possible variations among the indegenously circulating wild poliovirus strains have not yet been studied. The present study was undertaken to define the genomic characteristics of poliovirus strains circulating in our region. The techniques employed for such studies are; ribonuclease T1 oligonucleotide fingerprinting, partial genomic sequencing, nucleic acid probe hybridization and restriction fragment length polymorphism (RFLP). The oligonucleotide fingerprinting system separates oligonucleotides produced by cleavage of an RNA molecule with a site specific ribonuclease (ribonuclease T1) according to their base composition and size. The large, structurally unique, oligonucleotides resistant to further digestion with ribonuclease T1 separate into patterns or 'fingerprints' that are highly characteristic of the RNA molecule from which they derive. Mechanism of fractionation provides pronounced sensitivity and even a single base change within an oligonucleotide results in its repositioning in the fingerprint. Since the characteristic oligonucleotides originate from all regions of the RNA molecule, a greater spectrum of the viral genome (10%) can be surveyed as against only 2% by partial genomic sequencing or RFLP. The oligonucleotide fingerprinting, thus offers a highly specific means of genomic comparison. Moreover, the fingerprinting analysis does not require prior knowledge of the base sequence information. On the contrary, the exact nucleotide sequence information of the wild poliovirus strains would be necessary for designing the primers for the genomic studies using partial genomic sequencing and RFLP. The oligonucleotide fingerprinting technique was therefore preferred in the present study for the genetic characterization of the poliovirus strains.

As poliomyelitis is highly endemic in our country, the wild poliovirus strains are expected to undergo rapid multiplications in several hosts in a short period of time. In the process, the viruses are expected to accumulate

mutations very rapidly resulting in significant genetic variation among the circulating wild poliovirus strains. As this work formed the first attempt to characterize the poliovirus strains at the genomic level, it was considered essential to be able to demonstrate genetic relationship among the strains. It was postulated that the poliovirus isolates from poliomyelitis cases occurring in a short span of time in a limited area of known high transmission rate would represent genetically related strains. Poliovirus type 1 strains (26 Nos.) isolated from the three epidemiologically important wards of the Municipality Corporation of Greater Bombay during the year 1986 were selected for the present study. Characterization of poliovirus type 1 strains was given priority as poliovirus type 1 was associated with maximum number of poliomyelitis cases in Bombay.

Virus isolates were selected using the following criteria.

- 1) Genetic relatedness among the poliovirus strains would be established with relative ease during high endemicity of the disease.
- 2) There is greater probability to demonstrate if more than one genotype circulated during the high endemicity period.
- 3) Determination of wild poliovirus genotypes which predominated in the polio endemic region will formulate the baseline data for future studies.
- 4) Highest poliomyelitis incidence was noted in 1987 throughout the country. In this context, the 1986 strains tend to gain more epidemiologic significance.

Genomic differences among the poliovirus strains are accompanied by changes in viral proteins. These differences can be detected as shifts in electrophoretic mobilities of viral polypeptides. The poliovirus isolates selected for oligonucleotide fingerprinting were also compared on the basis of electrophoretic separation of the [^{35}S] methionine labeled viral proteins.

Finally, the thesis will serve as a catalogue of oligonucleotide fingerprints which may be used for comparison of genetic characteristics of the strains isolated during successive years. These data will reveal if certain genotypes survived for longer periods, or were replaced by new genotypes or ceased to circulate in the population.

MATERIAL AND METHODS

Cell Culture

HEp-2 (Cincinnati)

The continuous cell line of human epidermoid carcinoma was originally obtained from NIBSC, London. The cell line was maintained using Eagle's Minimum Essential Medium (EMEM) supplemented with 5 per cent foetal bovine serum (FBS). Cells were trypsinized using trypsin solution (TPVG) and the culture flasks were seeded at a cell density of 5.4×10^6 cells (175 cm² flasks), 1.2×10^6 cells (80 cm² flasks) and 0.7×10^6 cells (25 cm² flasks) according to the experimental requirement. Seeded cultures were incubated at 36°C. At 75 per cent confluency (usually after four days), the culture medium was replaced with maintenance medium and cultures were incubated further until used.

HEp-2 cells were used for poliovirus isolation, identification, titration, stock virus preparation and polypeptide analysis.

HeLa S3

The continuous cell line of human epitheloid carcinoma of cervix was obtained from National Facility for Animal Tissue and Cell Culture, Pune. Cells were maintained in growth medium (EMEM) supplemented with 10 per cent sheep serum and subcultured as above.

HeLa cells were used for large scale virus preparation for oligonucleotide fingerprinting experiments.

Virus Isolation

Faecal samples were collected from children suffering from paralytic poliomyelitis during the acute stage of illness. A 10% suspension was made in Hanks' balanced salt solution. The suspension was homogenized by vigorous shaking and clarified by centrifugation at 5000 x g for 30 min. Clear supernatant fluid was treated with 10% (w/v) chloroform to eliminate the microbial contamination. The mixture was allowed to separate by centrifugation. The aqueous layer was collected and 100 U of penicillin and 100 ug of streptomycin per ml of stool suspension were added.

0.2 ml of the processed stool suspensions were inoculated onto HEp-2 cell monolayers grown in 25 cm² TC flasks. Infected cultures were incubated at 35.5°C and were microscopically examined for cytopathogenic effect (CPE) for eight days. Cultures showing CPE were frozen and thawed three times and the culture fluid was collected for identification of the viral agent.

Identification Of Virus Isolates

CPE positive culture fluids were studied for detection and serotyping of poliovirus by virus neutralization test. Equine antisera against poliovirus type 1, type 2 and type 3 were used for the poliovirus identification. Approximately 100 TCID₅₀ of virus (usually a 10⁻⁴ dilution of the culture fluid) was reacted with the equine anti-polio sera (20 units) individually and in combinations so as to facilitate identification even if a mixture of poliovirus was present.

The assays were performed using the microtitration technique. 50 ul of virus suspension and 50 ul of antiserum were reacted in the cups of the 96 well flat bottom tissue culture plates. Plates were incubated at 36°C for 3.0 h. HEp-2 cell suspension (1×10^4 cells in 100 ul) was added to each well and the plates were incubated at 36°C for 7 days. They were observed for cytopathogenic changes on days 3, 5 and 7. Virus type was identified by successful neutralization of the infectivity.

Poliovirus Titration

Viral titers were assayed using a microtitration technique. Serial ten fold dilutions of the virus suspension were prepared in medium (EMEM) containing 2 per cent FBS. 100 ul of each of the selected dilutions of virus (10^{-3} to 10^{-8}) was dispensed in ten replicate wells of a microtitre plate. 100 ul of HEp-2 cell suspension containing 1×10^4 cells was seeded into each well. Plates were incubated at 36°C and were examined for cytopathogenic changes on days 5 and 7. Virus titre was calculated using Karber's formula²²⁷.

Poliovirus Type 1 Strains for Oligonucleotide Fingerprinting

Poliovirus type 1 strains isolated during the year 1986 from Bombay were selected for the present study. Twenty six strains isolated from the three epidemiologically important wards of the Bombay Municipality Corporation were selected from the virus collection at the Enterovirus Research Centre. These included 8 isolates from S ward, 10 isolates from R ward and 8 isolates from M ward. Poliovirus type 1 and type 2 Sabin vaccine strains were used for the reference.

Stock Virus Preparation

A high titre stock suspension of each virus isolate was prepared in HEp-2 cells. HEp-2 cell monolayers in 80 cm² TC flasks were infected at a multiplicity of infection of 10 to 25 TCID₅₀ per cell. The cultures were incubated at room temperature for 30 min to facilitate virus adsorption. Cultures were then replenished with 30 ml of maintenance medium and incubated at 36°C until complete cell destruction was observed (usually 24 h post infection). The culture fluid (virus harvest) was collected and subjected to three cycles of freezing and thawing to release the intracellular virus. The virus harvest was centrifuged at 17000 x g for 30 min in a Beckman JA 21 rotor to remove the cell debris. The resultant stock virus suspensions were titrated and stored at -40°C. These stocks were used as seed virus for preparation of virus in large quantities.

Large Scale Virus Preparation

Twelve flasks (175 cm²) of HeLa cell cultures were infected with the stock suspension of each virus isolate at multiplicity of infection of 10-25 TCID₅₀ per cell. Following the virus adsorption for 30 min at room temperature, 30 ml of maintenance medium was added to the culture flasks. The cultures were incubated at 36°C until complete cell destruction was observed. After three cycles of freezing and thawing the virus harvest was centrifuged at 17000 x g for 30 min and the supernatant was collected.

Virus concentration by PEG precipitation: To the clarified culture supernatant, one fifth volume of 40 per cent polyethylene glycol 6000 and one tenth volume of 23 per cent sodium chloride solutions were added. The

mixture was stirred continuously at 4°C, overnight. Virus precipitate was collected by centrifugation at 12000 x g for 30 min at 4°C.

Virus concentration by ultracentrifugation : The clarified culture supernatant was ultracentrifuged at 132000 x g for 5.0 h at 4°C in a Beckman SW28 rotor to pellet the virus.

Virus pellet was resuspended in 10 ml of TS buffer pH 7.8. Virus suspension was homogenized and sonicated briefly. Insoluble material was removed by centrifugation at 5000 x g for 10 min. The clear suspension was ultracentrifuged at 243000 x g for 3.30 h, 4°C in a Beckman SW 40 Ti rotor to repellet the virus. Virus pellet was resuspended in 400 ul of TSE buffer pH 8.0, treated with 0.1% NP40 and centrifuged briefly in a microfuge. The clarified virus suspension was further purified by density gradient centrifugation.

Rate Zonal Centrifugation In Sucrose Gradient

A 12 ml of continuous sucrose density gradient (15 to 30% w/v) was prepared in a 14 ml polyallomer ultracentrifuge tube (Beckman) using a gradient maker. Concentrated virus suspension (400 ul) was gently layered and the gradient was run at 140000 x g for 2.30 h in a Beckman SW 40 Ti rotor at 4°C. The gradient was fractionated by sequentially aspirating 1 ml of the solution from the top using an Eppendorf pipette. The tip was changed for each fraction collected. Virus content of the fractions was determined by infectivity titration using a small aliquot of each fraction. Those fractions showing high titre were pooled and centrifuged at 243000 x g for 3.30 h. The virus pellet was resuspended in 400 ul of TSE buffer pH 8.0

Equilibrium Density Gradient Centrifugation in CsCl

6 ml gradient of CsCl (10 to 45% w/v) was prepared in a 14 ml polyallomer ultracentrifuge tube using a gradient maker. A preformed gradient was used to achieve early equilibration in the gradient. Concentrated virus suspension (400 ul) was layered and the remaining tube was filled with light mineral oil (half filled tube technique). The gradient was centrifuged at $194000 \times g$ for 16.00 h at 4°C in a Beckman SW40 Ti rotor. Two clear bands were observed in the gradients. In the initial stages, complete gradient was fractionated. The virus band was located by infectivity titration of the gradient fractions. In routine experiments, however, only the virus band was collected by piercing a sterile 22 gauge needle just below the band. The virus fraction was dialysed extensively against TSE buffer pH 8.0.

The usefulness of the different procedures in virus concentration/purification was judged by calculating recovery of the virus at the different steps.

RNA Extraction

Purified poliovirus ($>10^{11}$ TCID₅₀) in 300 to 400 ul of TSE buffer pH 8.0 was digested with pronase E (1 mg/ml) at 37°C for 30 min. Sodium dodecyl sulfate (SDS) and 2 mercaptoethanol (BME) were then added to 1 per cent final concentration. The mixture was heated to 60°C for 90 sec to dissociate the virions. RNA from the aqueous suspension of disrupted virions was extracted by phenol chloroform extraction.

Redistilled phenol was equilibrated with TE buffer pH 8.9 and mixed with chloroform and isoamyl-alcohol in the proportion (25:24:1). Equal volume of phenol chloroform mix was added to the SDS-BME disrupted virion preparation in a 1.5 ml Eppendorf tube. The suspension was mixed gently and centrifuged at 12000 x g for 5 min in a microfuge for separation of the aqueous and organic phases. The aqueous phase was collected in a fresh Eppendorf tube and the phenol chloroform extraction procedure was repeated three times. The last aqueous phase was extracted once with chloroform. The aqueous phase was adjusted to contain 0.2 M LiCl. Finally, 2.5 volumes of ice cold ethanol was added and the tube was chilled to -40°C to effect RNA precipitation. The RNA precipitate was collected by centrifugation at 12000 x g for 20 min at 0°C. The pellet was washed with cold 95% ethanol and dried in vacuo. RNA pellet was dissolved in 10 ul of Tris HCl buffer (40 mM Tris, pH 8.0).

Quantitation of RNA

1 ul of RNA was diluted to 500 ul using Tris HCl buffer. Optical density of the RNA solution was read at 260 nm and 280 nm. Concentration of RNA was determined from the graph prepared by using yeast t RNA as standard.

Oligonucleotide Fingerprinting

Digestion of RNA and labelling of the fragments

The technique described by Kew et al (1984) was used for the digestion of RNA and 5' end labelling of the RNase generated fragments²¹⁶. Viral RNA (1 to 2 ug) was taken in a sterile, siliconized, 0.5 ml Eppendorft tube and dried in vacuo. Dried RNA was resuspended in 1 ul of RNase free

sterile water (Milli Q purified, DEPC treated). RNA was digested with ribonuclease T1. For ribonuclease T1 digestion the following reagents were sequentially added :

- i) 1 ul, 40 mM Tris HCl (pH 8.0) containing 2.5 U of ribonuclease T1 (Pharmacia).
- ii) 1 ul of 40 mM Tris HCl (pH 8.0) containing 2×10^{-3} U of alkaline phosphatase (E. Coli) (Pharmacia).

The reaction was incubated for 2.00 h at 37°C.

The ribonuclease T1 generated oligonucleotides were 5' end labeled with a radiophosphate [^{32}P] using enzyme polynucleotide kinase (PNK).

- iii) While the ribonuclease T1 reaction was incubating, 15-50 uCi (γ - ^{32}P] ATP (BRIT LCP1, Sp act 3000 Ci/ mmole) was lyophilized in a 0.5 ml Eppendorf tube.
- iv) The lyophilysate was dissolved in 50 ul of PNK buffer containing 10 mM K_2HPO_4 - K_3PO_4 (pH 9.5), 10 mM magnesium acetate and 5 mM dithiothreitol.
- v) The PNK reaction mixture was transferred to the tube containing RNase T1 digest.
- vi) 0.5 ul of PNK (Boehringer Mannheim; 10 U/ul) was added to initiate the reaction.

The reaction was incubated at 37°C for 4 to 5 h.

The PNK reaction was terminated by addition of 50 ul of 0.6 M ammonium acetate. 100 ug yeast tRNA was added as a carrier to aid the precipitation step. The oligonucleotides and the carrier RNA were precipitated by mixing with 500 ul of cold (-20°C) 95% ethanol and chilling the tube at (-40°C, overnight). The precipitate was collected by

centrifugation in a microfuge (12000 x g, 15 min, 0°C), washed with 500 ul of cold 95% ethanol (12000 x g, 5 min, 0°C), and lyophilized. Labeled digest was dissolved in 40 ul of electrophoresis sample buffer.

Two Dimensional Polyacrylamide Gel Electrophoresis

The two dimensional polyacrylamide gel electrophoresis technique originally described by de Watcher and Fiers and modified by Lee et al was employed for resolving the radiolabeled oligonucleotides^{204,207}.

First Dimensional Electrophoresis

First dimensional electrophoresis was performed in 8% polyacrylamide gel buffered at pH 3.3, in the presence of 6 M Urea.

First dimensional glass plates (size 13 x 43 cm) were thoroughly cleaned and siliconized using Sigmacoat. Glass plates and spacers (1.25 x 43 cm size, 1.25 mm thickness) were assembled to form a mold. Sides and bottom of the mold were sealed using water resistant sealing tape. The first dimensional 8% polyacrylamide gel solution was prepared by mixing stock solutions as described in Table 1. The gel solution was pipetted into the glass plate assembly (mold) upto about 1.5 cm from the top. A well former was inserted and the gel was allowed to polymerize in an inclined position for 1h. After polymerization, the bottom tape seal and the well former were removed and the assembly was transferred to the electrophoresis chamber. Electrophoresis buffer, citric acid (25 mM, pH 3.3) was filled in the upper and the lower buffer reservoirs. The sample, RNase T1 digest (40 ul) was briefly heated (60°C, 3min) and layered into the sample well. The gel was connected to the upper buffer reservoir by inserting Whatman no 1 filter paper wicks. Electrophoresis was carried out at constant voltage of 480 V

Fig 2

First dimensional electrophoresis in progress

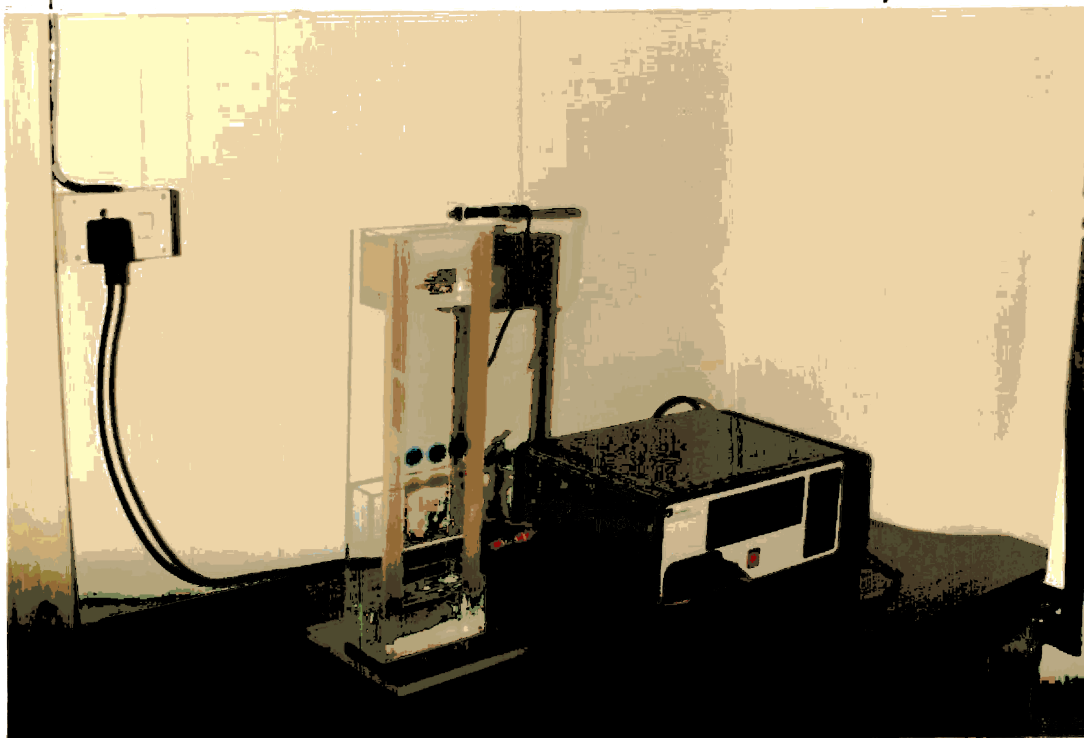


Table -1: Stock solutions for first dimensional gel system.

Component	Volume
Acrylamide : Bis (40:0.5)	18.0 ml
Urea 9 M	60.0 ml
Deionized H ₂ O	8.5 ml
Adjust pH to 3.3 with saturated citric acid	
Degass	
FeSO ₄ 7H ₂ O [0.16% (W/V)]	0.5 ml
Ascorbic acid [1% (W/V)]	2.0 ml
H ₂ O ₂ (30%)	75 ul
Electrode buffer: 25mM citric acid adjusted to pH 3.3 with 10 N NaOH	

and power not exceeding 3.2 W. Electrophoresis was terminated when the bromophenol blue dye migrated to 21.5 cm from the bottom of the sample well. Fig. 2 shows the first dimensional electrophoresis in operation.

Second Dimensional Electrophoresis

The first dimensional glass plate assembly was opened, such that one of the glass plate was removed while the gel was in position on the other plate. A 2.5 cm wide strip containing the migrational lane of the oligonucleotides was cut from the gel using a rotory pizza cutter. The gel strip was trimmed 19.5 cm above and 12.5 cm below the BPB dye marker. The gel strip was lifted using a parafilm as the backing (support) and immersed in 500 ml of second dimensional electrophoresis buffer to remove urea. The gel strip was then placed at the bottom edge of the second dimensional glass plate (35 x 43 cm). The spacers (1.75 x 43 cm, 1.5 mm thick) were adjusted and the second glass plate was aligned to complete the gel mold (assembly). The sides and the bottom of the mold were sealed. About 10 ml of electrode buffer was dispensed into the assembly and it was immersed in a tank of cold water to allow the hydrostatic pressure to express bubbles trapped between the gel and the glass plates. A 10 ml of 10 per cent low cross linked gel solution was prepared as given in table 2 and allowed to polymerize on the first dimensional gel strip in the mold. This served as a connecting gel between the first (8%) and second dimensional (22%) polyacrylamide gels. The second dimensional 22% polyacrylamide gel solution was prepared according to Table 2. About one third of the gel solution was pipetted down the spacers and the assembly was transferred to the water tank. It was then filled with the remaining gel solution upto 2 cm from the top. The gel was allowed to polymerize for about 1 h.

Fig 3

Second dimensional electrophoresis in progress

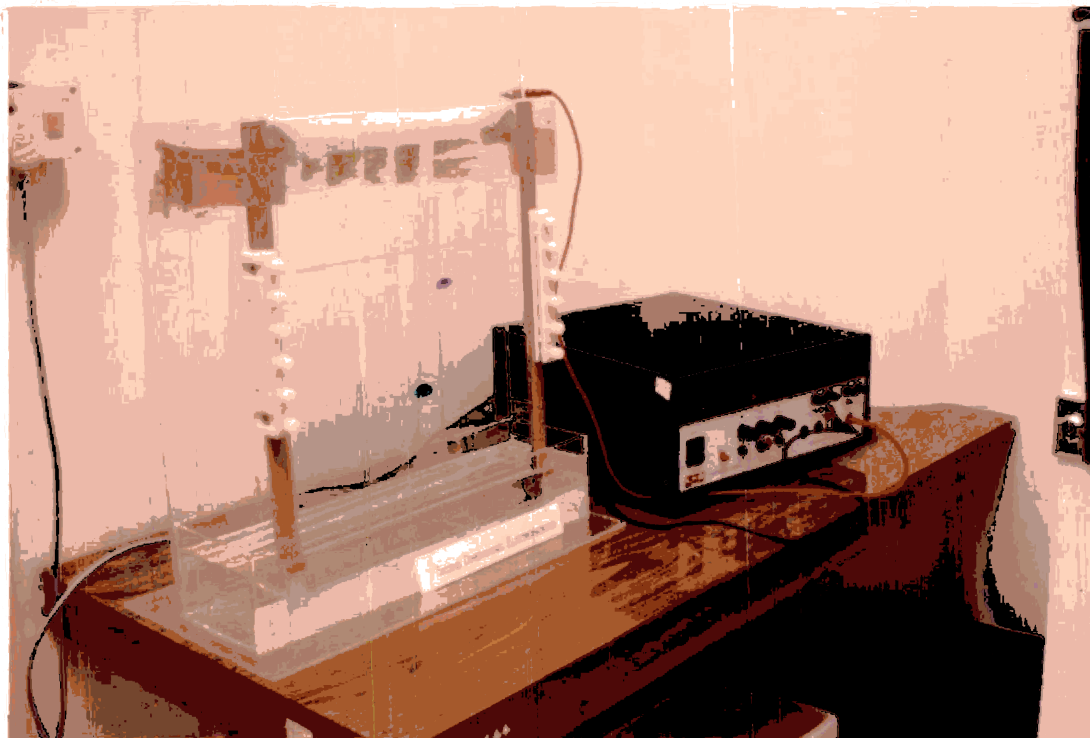


Table -2: Stock solutions for second dimensional gel system.

Component	Volume	
	Connecting gel	Separating gel
Acrylamide : Bis (23.2:1.55)	-	209.0 ml
Acrylamide : Bis (40:0.5)	2.5 ml	-
Tris-borate, 1 M, pH (8.2)	1.0 ml	11.0 ml
Deionized H ₂ O	6.4 ml	-
Degass		
TEMED	25 ul	0.1 ml
Ammonium persulfate (10%)	125 ul	0.4 ml
Electrode buffer : 50 mM Tris borate (pH 8.2)		

The bottom sealing tape was removed. The assembly was placed in the electrophoresis chamber filled with buffer, tris borate (50 mM, pH 8.2),

The gel was connected to the upper tank buffer using Whatman No. 1 filter paper Wicks. Electrophoresis was carried out at 16 mA (limiting), maximum power output of 12 W and variable voltage until the BPB dye migrated 25.5 cm in the second dimensional gel. Fig. 3 shows the second dimensional electrophoretic run.

Autoradiography

The second dimensional glass plate assembly was opened. A whatman 3 MM filter paper cut to match the size of the gel (36 x 44 cm) was gently pressed over the gel on the glass plate and pulled backwards to transfer the gel onto the filter paper. It was transferred to an X-ray film exposure cassette fitted with intensifying screens. The gel surface was covered with a cellophane sheet. Working in a photography room, a medical X-ray film (Indu) was placed over the gel. The X-ray film was exposed at -70°C for 15-24 h. The film was developed manually.

The X-ray film exposure cassette was opened in the darkroom. The X-ray film was clipped to a film holder and immersed sequentially in the following solutions.

Water bath	Dips to wet the film
X-ray developer (Agfa)	5 minutes
Water bath	1 minute
Rapid fixer (Agfa)	5 minutes
Water bath	1 minute

The film was washed under running tap water for 15 min. and then air dried.

Study of Poliovirus Proteins By Polyacrylamide Gel Electrophoresis

Preparation of radiolabelled cell lysates

Poliovirus proteins were radiolabelled and electrophoretically separated as described by Minor et al²²⁸. Monolayer cultures of HEp-2 cells (80 to 100% confluent in 25 cm² flasks) were washed twice with Hanks' BSS and infected with the poliovirus strains at multiplicity of infection of 50-60 TCID₅₀ per cell. Following virus adsorption for 30 min at room temperature, 4 ml of methionineless medium supplemented with 2 per cent FBS (dialysed) was added. The cultures were incubated at 36°C for 3.5 h. The medium was then replaced with 2 ml of methionine less medium containing 7 μ Ci [³⁵S] methionine and the cultures were labeled for 2.00 h at 36°C. The radioactive medium was removed and the cells were washed with BSS. Cultures were replenished with 4 ml of chase medium (MEM with 10 mM unlabeled methionine and 2 percent FBS). After 1.0 h incubation at 36°C, the monolayers were washed with TE buffer (20 mM Tris HCl pH 6.8, 2 mM EDTA) and lysed in 0.5 ml of lysis buffer.

SDS PAGE

Vertical slab polyacrylamide gel electrophoresis was carried out in a Hoeffer SE 600 apparatus using gels of size 14 x 16 cm and 0.75 mm thickness. 10% SDS containing polyacrylamide gel solution was prepared as given in table 3 and filled in the gel chamber to a level of about 4 cm from the top. Water saturated n- butanol was overlayered and the gel was

allowed to polymerize for more than 1 h. The stacking gel solution (table 3) was prepared and filled into the assembly. A 12 well comb was immediately inserted and the gel was allowed to polymerize for 1.0 h. The [^{35}S] methionine labeled infected cell lysates were heated for 90 sec. in a boiling water bath and 5 μl of each lysate were loaded in the individual wells. The glass plate assembly was transferred to the electrophoresis unit filled with the tank buffer (0.025 M tris pH 8.3, 0.192 M glycine, 0.1% SDS). Electrophoresis was performed at 15 mA (constant current), until the dye front migrated to the bottom of the gel. The gel was removed from the plates and fixed in 50% methanol, 12% acetic acid solution. The gel was dried in a gel drier and autoradiographed for a period of 2-3 days at room temperature. The X-ray films were developed as described earlier.

Table -3: Stock solutions for SDS- PAGE

Component	Volume	
	Separating Gel	Stacking gel
Acrylamide : Bis (14.6:0.4)	12 ml	1.33 ml
4 X running gel buffer (1.5M Tris-HCl pH8.8)	9 ml	-
4 X stacking gel buffer (0.5 M Tris-HCl pH 6.8)	-	2.5 ml
SDS [10% (w/v)]	0.36 ml	0.1 ml
Deionized H ₂ O	14.46 ml	6.1 ml
TEMED	12 μl	5 μl
Ammonium per sulfate (10%)	180 μl	50 μl

RESULTS

Poliovirus Preparation And Purification

Poliovirus type 1 strains selected for the oligonucleotide fingerprinting analysis were grown on HeLa cell cultures. Infected cell culture harvests having viral titre in the range of $10^{8.5}$ to $10^{9.375}$ TCID₅₀ per ml were routinely obtained. About 360 ml of virus suspension was used as the starting material for concentration and purification of each strain. Table 4.1 shows results of the typical experiments of concentrating polio-virus by PEG 6000 precipitation method. In sample 1, of the total virus titre of $10^{11.77}$ TCID₅₀ present in the culture harvest, $10^{11.52}$ TCID₅₀ was recovered in the PEG precipitate. The recovery at this step was estimated to be 56 percent. The PEG supernate showed $10^{11.14}$ TCID₅₀ of virus titre which corresponded to 23 per cent of the total virus titre. It indicated that 23 per cent of the virus could not be precipitated and was thus lost.

The PEG precipitated virus was resuspended in 10-12 ml of buffer and ultracentrifuged at $243000 \times g$ for 3.30 h to pellet the virus. Since the pelleted virus was dissolved in 500 μ l of buffer, the virus suspension was not titrated. Virus recovery was judged from the titration of supernatant fluid only. The supernatant fluid showed $10^{9.125}$ TCID₅₀ of virus titre equivalent to only 0.22 per cent of the virus loss at this step. Conversely, it suggested 99.7 per cent recovery of the virus in the pellet. The final virus recovery of sample 1 was estimated to be 56 per cent. The observations clearly determined that the lower final recovery was due to virus loss during the PEG precipitation step as nonprecipitated virus and also as the virus which could not be resuspended from the precipitate. The PEG precipitation method although preferred by many, was found inefficient for virus concentration^{208,228}.

Table 4.1 Poliovirus Purification by PEG Precipitation

Purification Step	Total Virus Recovered Log ₁₀ TCID ₅₀			
	S1	S2	S3	S4
Infected Culture Supernate	11.77	11.395	10.94	10.89
PEG, NaCl Precipitation. PEG Pellet in TS buffer	11.52 (56%)	11.15 (56%)	10.625 (48%)	10.50 (40%)
PEG Supernatant	11.14 (23%)	10.765 (23%)	10.32 (23%)	10.52 (42%)
Virus Pelleting at 243000 X g. Ultra Supernatant*	9.125 (0.22)	8.875 (0.3)	8.5 (0.36)	8.125 (0.17)
Final Recovery (%)	56	56	48	40

Figures in bracket represent percent recovery

* Virus in pellet was not titrated

Table 4.2 Poliovirus Purification by Differential Centrifugation

Purification Step	Total Virus Recovered Log ₁₀ TCID ₅₀					
	Isolate 512	Isolate 525	Isolate 587	Isolate 1176	Isolate 1177	Isolate 1197
Infected Culture Supernate	11.55	11.625	11.80	11.30	11.30	11.425
Ultracentrifugation 1 (132000 x g, 5h) Pellet in TS Buffer	11.395 (70.3)	11.50 (75)	11.645 (70)	11.272 (93.7)	11.25 (89)	11.325 (79.5)
Ultra-1 Supernatant	9.675 (1.33)	9.925 (2)	10.3 (3.1)	9.425 (1.3)	9.8 (3.1)	9.925 (3.1)
Ultracentrifugation 2 (243000 x g, 3.30h) Supernatant*	9.04 (0.3)	9.3 (0.4)	9.165 (0.2)	8.79 (0.3)	8.75 (0.28)	8.915 (0.3)
CsCl Gradient (virus fraction)	11.375	11.425	11.625	11.25	11.147	11.272
Final Recovery (%)	67	63	67	89	70	70

Figures in bracket represent percent recovery

* Virus in pellet was not titrated

The culture harvest was therefore ultracentrifuged at $132000 \times g$ for 5.0 h to pellet the virus. As shown in Table 4.2, of the total $10^{11.55}$ TCID₅₀ of virus in the starting fluid (sample 512), $10^{11.395}$ of virus could be recovered in the virus pellet indicating 70 per cent virus recovery. Virus titre of $10^{9.675}$ TCID₅₀ in the supernatant fluid denoted only 1.33 per cent of virus loss. The near 100% recovery indicated by virus titration of the supernatant fluid was not found in the pellet as some amount of the virus from it could not be resuspended. This amount was found to vary slightly in different preparations. Finally, Between 63 to 90 per cent of the total virus could be obtained in the concentrated form. Comparison of the results of the two procedures favoured ultracentrifugation as the method of choice for concentration of the virus. All the poliovirus strains selected for the present study were concentrated by pelleting in the ultracentrifuge at $132000 \times g$ for 5.0 h.

Density Gradient Centrifugation

Poliovirus purification by rate zonal centrifugation was performed on 15 to 30 per cent (w/v) continuous sucrose gradient centrifuged at $140000 \times g$ for 2.30 h. When the gradient was observed under scattered light, no virus band could be located. Titration results of the gradient fractions collected from a typical sucrose density gradient run are presented in table 5.1. Fractions 7 to 10 showing high viral titres were pooled for further work. The virus recovery in these fractions was about 36 per cent. The virus from the pooled fractions was pelleted at $243000 \times g$ for 3.30 h and resuspended in 400 ul buffer for the RNA extraction. Purification of poliovirus by sucrose density gradient centrifugation was found inefficient as the virus tend to spread in 2 to 3 ml fractions and needed an additional ultracentrifugation run to concentrate the virus to 400 ul.

Table 5.1

Viral titres of sucrose density gradient fractions.

Fraction No	Virus titre, log ₁₀ TCID ₅₀	
	Sample 1	Sample 2
1	< 6.0	< 6.0
2	< 6.0	< 6.0
3	< 6.0	< 6.0
4	< 6.0	< 6.0
5	< 6.0	< 6.0
6	7.75	7.5
7	9.25	9.0
8	9.75	9.5
9	9.375	9.5
10	8.75	8.75
11	8.0	8.5
12	8.5	8.75

- Fractions numbered from top to bottom of the gradient

Table 5.2

Viral titres of CsCl density gradient fractions.

Fraction No	Virus titre, log ₁₀ TCID ₅₀	
	Sample1	Sample2
1	< 6.0	< 6.0
2	< 6.0	< 6.0
3	< 6.0	< 6.0
4	< 6.0	< 6.0
5	< 6.0	< 6.0
6	< 6.0	< 6.0
Upper band	< 6.0	< 6.0
8	< 6.0	< 6.0
Lower band	11.25	11.375
10	< 6.0	< 6.0
11	< 6.0	< 6.0
12	< 6.0	< 6.0

- Fractions numbered from top to bottom of the gradient

Poliovirus purification by equilibrium density gradient centrifugation was performed on 10 to 45 per cent (w/v) CsCl density gradient centrifuged at 194000 x g for 16 h. As shown in fig. 4, two bands were observed. The upper band was dense and particulate whereas the lower band was compact. The results of virus titration of the gradient fractions recovered after a typical cesium chloride gradient run (Table 5.2) proved that the lower band contained most of the virus. It was also noted that the virus loss in other fractions was minimum. Virus recovery was estimated to be 74 per cent.

The CsCl density profile in the gradient was extrapolated from the refractive index measurements of fractions obtained from the tube used for the counterbalance (Fig.5). The density of the fraction containing maximum virus (lower band) was 1.326 g/cm³. In routine experiments it was not necessary to fractionate the complete gradient. Instead only the lower band could be collected by piercing the ultracentrifuge tube with a sterile, 22 gauge needle. As the virus was recovered in about 400 to 500 ul, the virus fraction (after dialysis) could be used directly for the RNA extraction. CsCl gradient purification was, therefore, found to be advantageous for poliovirus purification.

The final, standardized procedure employed for the purification of the poliovirus strains thus, consisted of the following steps.

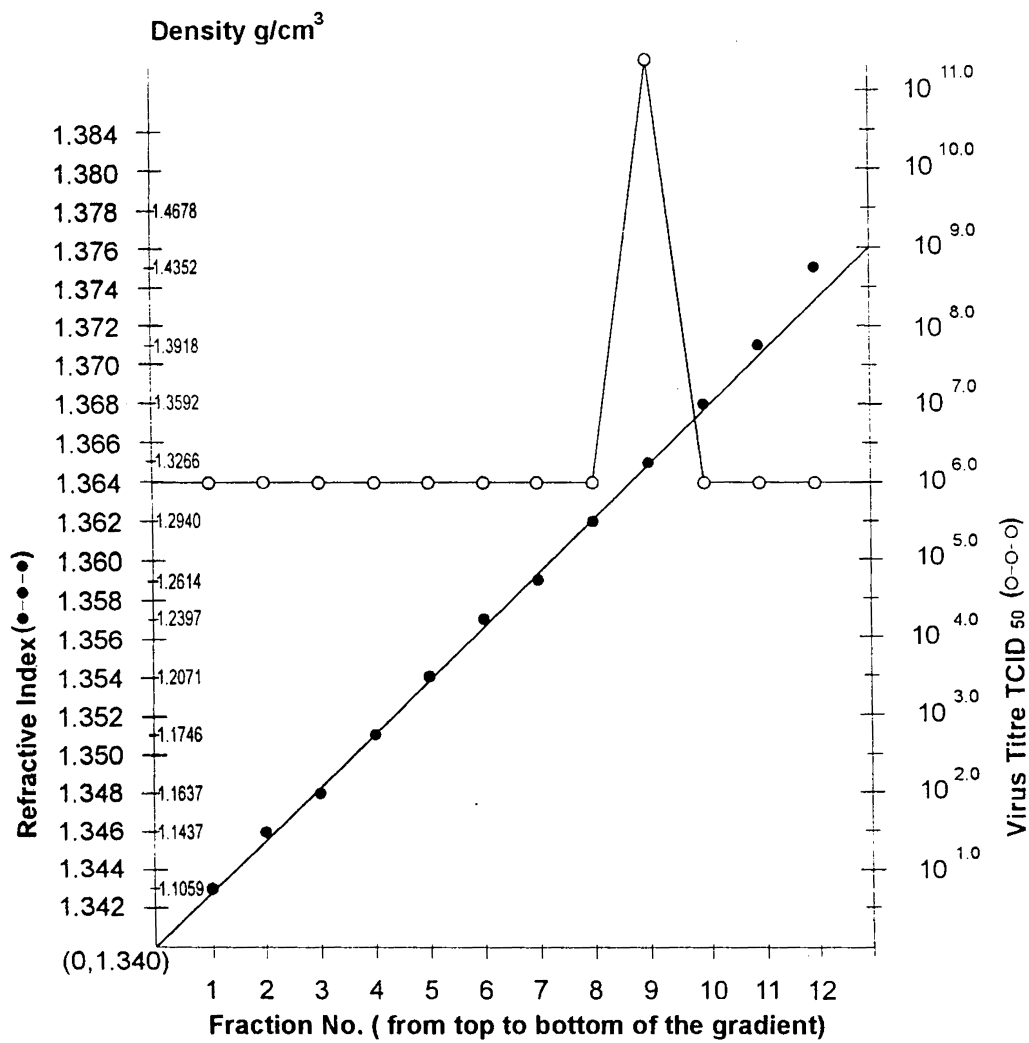
1. Centrifugation of the culture harvest at 17000 x g for 30 min.
2. Ultracentrifugation of the clarified culture supernate at 132,000 x g for 5 h. Virus pellet was resuspended in 10 ml TS buffer and clarified at 5000 x g for 10 min.
3. Pelleting of the virus suspension at 243000 x g for 3.30 h
Pellet collected in 400 ul of TSE buffer

Fig. 4
Poliovirus purification in CsCl density gradient



Fig. 5.

CsCl density profile



4. Purification of the concentrated virus on 10 to 45 per cent (w/v) CsCl density gradient at $194000 \times g$ for 16.00 h.

Virus fraction (lower band) was collected and dialysed against TSE buffer.

Quantitation Of Viral RNA

Concentration of the viral RNA extracted from purified poliovirus was determined spectrophotometrically using yeast tRNA as the reference material. Approximately, 10 ug of RNA was obtained from virus equivalent to $10^{11.25}$ TCID₅₀ (Fig.6). The ratio of the optical density readings at 260 nm and 280 nm (OD_{260}/OD_{280}) in the range of 1.8 to 2.0, provided an estimate of purity of the RNA preparations²²⁹.

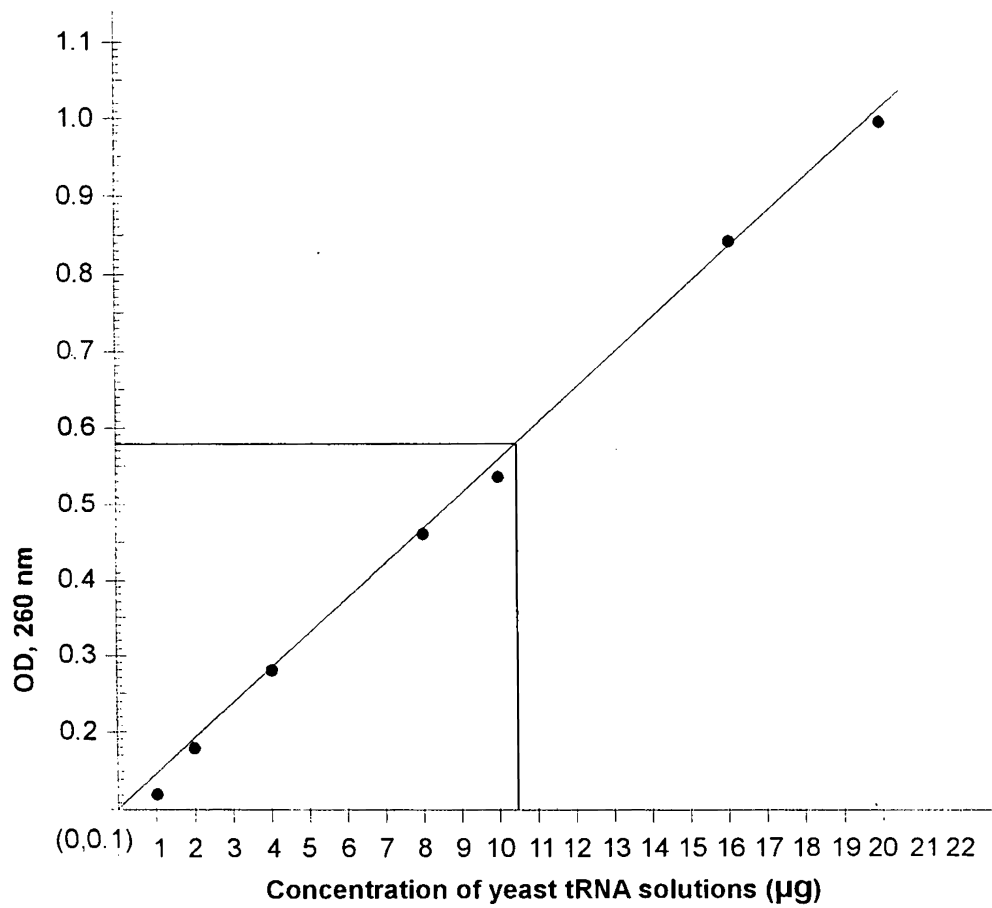
Oligonucleotide Fingerprinting

The genome of poliovirus isolates were compared by the electrophoretic profile of large oligonucleotides produced by digestion of RNA with the site specific Ribonuclease T1. A brief description of the oligonucleotide fingerprinting technique is presented (Fig.7).

Ribonuclease T1 specifically cleaves the phosphodiester bonds adjacent to the guanine (G) residues of the RNA. Extensive digestion of RNA produces oligonucleotides resistant to further cleavage with the same enzyme. The end product is a set of oligonucleotides of varying lengths, each terminating with a 5' hydroxyl group and a 3' guanosine monophosphate. The resultant oligonucleotide fragments are then resolved by a two dimensional polyacrylamide gel electrophoresis technique. For visualisation of the separation, the oligonucleotides are labeled with a radioactive isotope [³²P]. The procedures employed for

Fig. 6.

Standard curve of yeast tRNA solutions

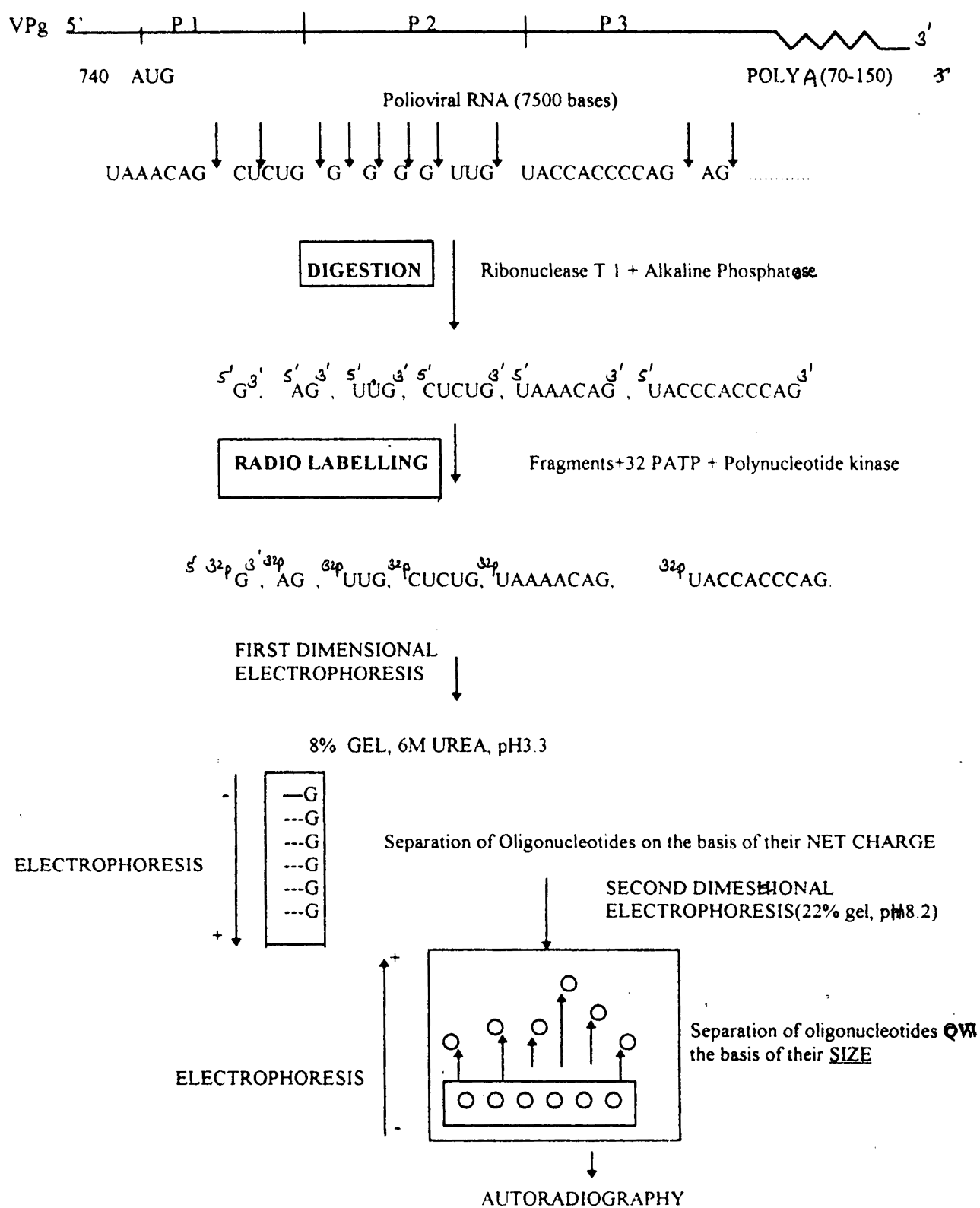


radiolabelling vary in different laboratories. Radiolabelling can be performed by biosynthetic incorporation of ortho [^{32}P] phosphate during the virus infectious cycle or by in vitro transfer of the isotope from labelled ATP to the 5' end or to the 3' end of the oligonucleotides²¹⁷. The advantage of in vitro labeling of the RNA is that the isotope is handled only in small quantities and possible contamination of the laboratory equipment is minimal. In our laboratory, the oligonucleotides were radiolabeled by the in vitro transfer of radiophosphate from [γ - ^{32}P] ATP to 5' hydroxyl groups of the oligonucleotides using T4 polynucleotide kinase. The 3' terminal phosphate groups of the oligonucleotides were removed by alkaline phosphatase to compensate for the added negative charge.

First dimensional electrophoresis was performed in 8 per cent polyacrylamide gel buffered at pH 3.3, and containing 6 M urea. At the acidic pH, the four nucleotide bases have maximum charge differences. The net negative charge and thus the electrophoretic mobilities increase in the order, Up > Gp > Ap > Cp. The oligonucleotides migrate according to the total charge contributions of their constituent base residues. A highly cross linked 22 per cent polyacrylamide gel buffered at pH 8.0 was employed for the electrophoresis in the second dimension. The four bases are largely uncharged at this pH. The charges on the oligonucleotides derive only from the phosphodiester bonds and the charge to mass ratios for all the oligonucleotides are nearly constant. The oligonucleotides, therefore, migrate in the gel on the basis of their size and shape.

After the two dimensional electrophoresis, each oligonucleotide attains a characteristic position relative to others. The large oligonucleotides are retained in the lower region of the gel, whereas the short ones migrate longer distance. The separation pattern resembles to the fingerprint of a human hand, hence the technique is referred to as fingerprinting. Nucleotide

FIG. 7 Diagrammatic representation of ribonuclease T1 resistant Oligonucleotide fingerprinting technique.



sequences become highly specific as the length of the fragment used for comparison increases. The electrophoretic mobility of the oligonucleotide fragments of size 10 bases, or larger in the gel therefore attain 'diagnostic' significance.

RNA molecules which share 85, 50 or 25 per cent of the diagnostic oligonucleotides in their fingerprints diverge at 1.0, 5.0, or 10 per cent of the total genome sequences respectively²³⁰. Comparison of the fingerprint patterns (maps) are efficient when 50 percent or more of the oligonucleotide spots comigrate. When less than 25 per cent of the oligonucleotides of the two patterns comigrate, the residual pattern similarities are difficult to detect and further comparison may not be possible unless genetic relatedness is suspected on other grounds such as epidemiological data or the existence of the intermediate strains²³¹.

The oligonucleotide fingerprinting technique was standardized using poliovirus type 1 and type 2 Sabin vaccine strains. The oligonucleotide fingerprint patterns of these two strains shown in Fig. 8a and 8b correlated well with the published fingerprints obtained by others^{207,232}. Reproducibility of the procedure was confirmed by a) repeatation of fingerprinting using the same RNA preparation and b) by using the RNA extracted from two separate preparations of the same strain.

FIG. 8

a



POLIOVIRUS TYPE 1 VACCINE STRAIN

b



POLIOVIRUS TYPE 2 VACCINE STRAIN

This thesis presents oligonucleotide fingerprints of twenty six poliovirus type 1 isolates of known epidemiologic background. The fingerprints were examined by spot to spot comparison and the characteristic patterns produced by relative electrophoretic mobilities of the spots. The data thus analysed was logically arranged to explain genomic relationship between the poliovirus strains.

The twenty six poliovirus type 1 strains selected for the present study included those isolated in the year 1986. A total of 1268 cases of paralytic poliomyelitis were reported in Bombay in 1986. The distribution of these cases in the sixteen wards of the Municipal Corporation of Greater Bombay is shown in table 6.1. A total of 28, 32, and 76 paralytic poliomyelitis cases from the three wards S,R and M were studied for viral diagnosis during the routine surveillance studies. Of these 25 cases from S ward, 29 from R ward and 58 from M ward were identified as having caused due to poliovirus type 1 infection. The monthwise distribution of the poliovirus type 1 cases in these wards is given in table 6.2²³³. For the purpose of genetic characterization, 8, 10 and 8 poliovirus type 1 isolates from wards S,R and M respectively were taken from the virus collection at the Enterovirus Research Centre.

Table - 6.1
Paralytic Poliomyelitis (1986)
Bombay
Geographical Distribution

Ward	Area (sq. km)	Population (1981) (in lacs)	No. of cases	Incidence / 100,000 Population
A	11.4	1.68	21	12.5
B	2.4	1.47	13	8.8
C	1.7	2.67	11	4.1
D	6.6	3.84	23	5.9
E	7.4	5.17	70	13.5
F	21.1	8.14	99	12.1
G	17.8	9.82	160	16.2
H	21.0	7.06	103	14.5
K	47.4	9.25	137	14.8
L	13.4	4.34	105	24.1
M	54.9	5.65	174	30.7
N	55.4	3.83	53	13.8
P	64.27	6.63	98	14.7
R	77.5	5.63	111	19.7
S	Not Know	4.94	76	15.3
T	Not known	2.22	14	6.3
	Total		1268	14.08

Table - 6.2
Monthwise distribution of poliovirus type 1
cases in the three selected wards

Month	Wards		
	S	R	M
JANUARY		3	1
FEBRUARY	2	1	2
MARCH	2		3
APRIL			2
MAY			3
JUNE			7
JULY	5	11	7
AUGUST	4	3	11
SEPTEMBER	4	3	8
OCTOBER	3	5	6
NOVEMBER		2	3
DECEMBER	5	1	5
TOTAL	25	29	58

S Ward Isolates : Details of the eight poliovirus type 1 strains selected for the oligonucleotide fingerprinting analysis from S ward are presented in table 7.

Table 7.

*	Isolate No.	Dt. of onset of disease	Age in months/ Sex	Vaccination status	Patients Residential Area
1.	424	30.6.1986	23/M	0	Railwayline zopadpatti, BhandupVillage, Bhandup (W) , Bombay -78.
2.	512	13.7.1986	15/M	0	Tembepada, Bhandup (W), Bombay - 78.
3.	525	14.7.1986	3/F	0	Pratap Nagar Road, Bhandup (W), Bombay-78.
4.	587	21.7.1986	18/M	2 /4th 5th month	Kannamwar Nagar, Vikroli (E), Bombay-83
5.	1176	30.11.1986	30/F	3	Chawl No.288, Tagore Nagar, Vikroli (E), Bombay - 83.
6.	1177	03.12.1986	30/F	0	Zopada No.3, Tagore Nagar, Vikroli (E), Bombay - 83.
7.	1197	12.12.1986	5/M	0	Anandgad Area, Parksite, Vikroli (E), Bombay - 79.
8.	1199	14.12.1986	14/F	0	Chawl No.287, Tagore Nagar, Vikroli (E), Bombay - 83.

Analysis of the fingerprint patterns of the above strains is expressed to show genetically most related virus isolates followed by those with the distant relationships. After spot to spot comparison of the fingerprints, isolate 1176 was set at the apex for comparison. The pattern and its corresponding tracing are shown in Fig. 9a and 10a. A total of 76 oligonucleotides (spots) were numbered in the fingerprint of isolate 1176 (Fig 10a). In Fig. 9b and 10b is presented the fingerprint of isolate 1199. The two fingerprints reveal that 74 (97%) spots in the 1199 pattern had equivalent electrophoretic mobility as compared to the fingerprint of isolate 1176. The differences observed were; a) presence of an extra spot indicated by a solid circle near spot no. 3 in the fingerprint of isolate 1199 (Fig. 10b) and b) the absence of spot no. 15 and 39 (marked with arrows) in the fingerprint of isolate 1199. Equivalent migration of 97 per cent of the oligonucleotides in the two fingerprints clearly indicated close genomic relationship between the two isolates. As can be seen from the Table 7, the two strains were isolated from cases of paralytic poliomyelitis which occurred in the same residential area within a period of fifteen days.

Oligonucleotide fingerprint of the isolate no. 1177 (Fig. 9c and 10c) showed all the major spots representative of the 1176 - 1199 pattern. Spot no. 22 in the 1177 map showed a lateral shift towards right (marked with dotted arrow in Fig. 10C). A lateral shift due to the altered first dimensional mobility of an oligonucleotide may result from even a single base change²¹⁷. Eight additional spots were observed in the 1177 pattern which are denoted by solid circles in Fig. 10C. The fingerprint clearly indicated that the strain 1177 was genetically related to 1176 and 1199. The Epidemiological background of the isolate 1177 was also very similar to the previous two strains.

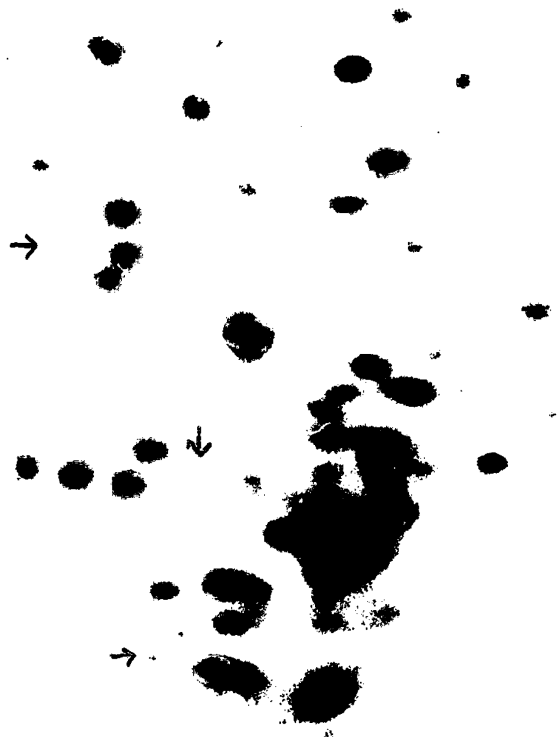
NOTE : The notations as solid circle, arrow or dotted arrow are referred to the markings done in the tracings of the fingerprints. In the photographs, the extra spots are marked with red arrows, missing spots with black arrows and shift in the oligonucleotides with green arrows.

a



ISOLATE 1176, S WARD.

b



ISOLATE 1199, S WARD.

c

1177, 67:15-18
28-1-13



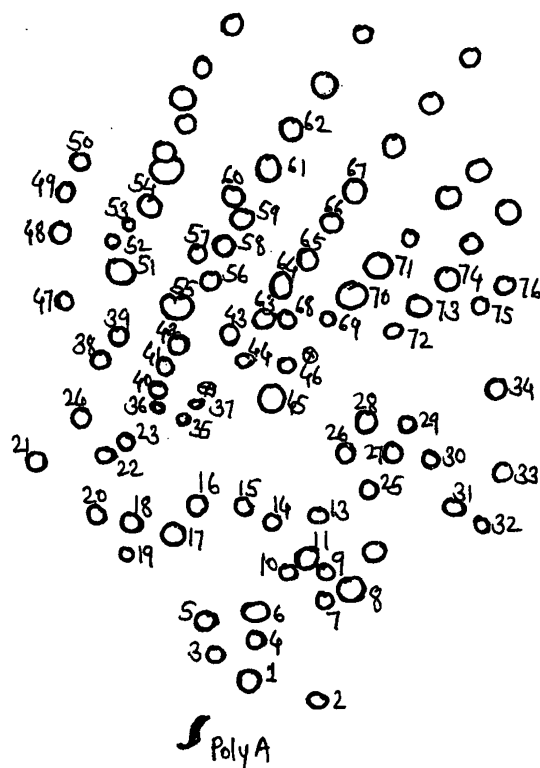
ISOLATE 1177, S WARD.

d

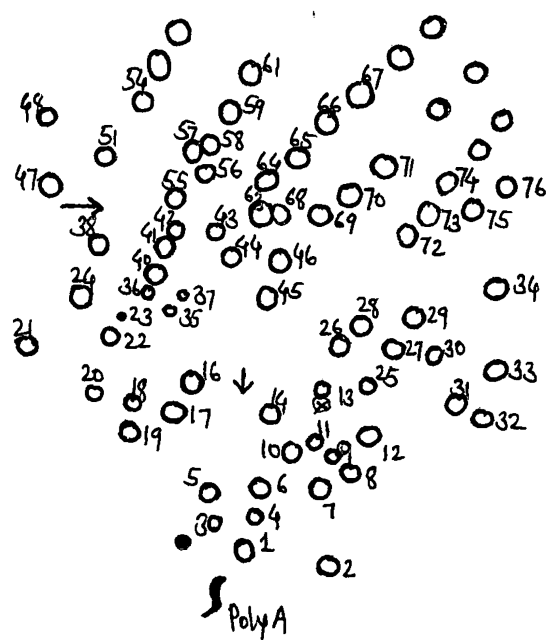


ISOLATE 587, S WARD.

FIG 10.

a

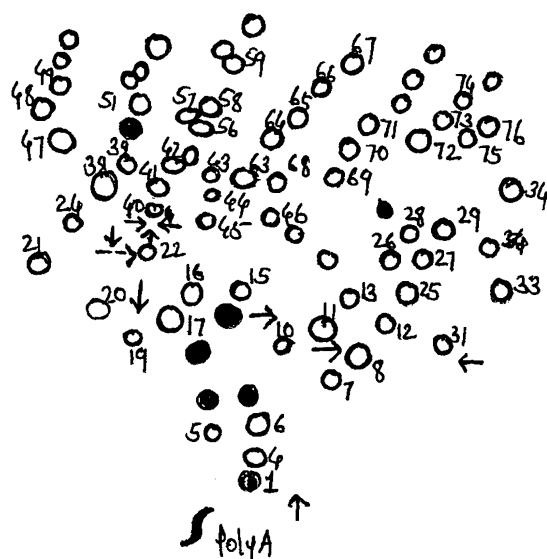
ISOLATE 1176, S WARD

b

ISOLATE 1199, S WARD

c

ISOLATE 1177, S WARD

d

ISOLATE 587, S WARD

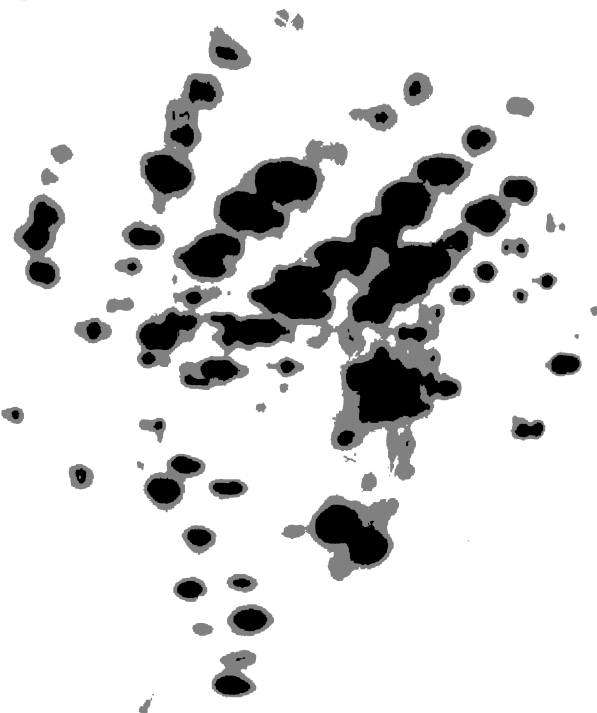
Isolate 587 also yielded a fingerprint resembling those of the above three isolates. Comparison (Fig 9a & d; and Fig 10a & d) detected that the electrophoretic separation of 58 oligonucleotide spots of the fingerprint of isolate 587 was similar to that in the pattern of isolate 1176. The differences included absence of spots 2,9,14,18,23,30,32,35 to 37 (marked with the arrows) and few additions (marked with solid circle in Fig. 10d). The lateral shift in the position of oligonucleotide 22, as observed in 1177 was also present in 587. The two fingerprints revealed 76 per cent similarity which in turn reflected about 95 per cent sequence similarity in the two strains. The strain 587 was isolated in the month of July from the same locality as that of the other three isolates in December, 1986.

The fingerprint of isolate 424 was found to be related to that of isolate 587 (Fig. 11a to d). About 49 oligonucleotides (75 per cent of total) of the fingerprint of isolate 424 had electrophoretic mobility equivalent to spots on fingerprint of isolate 587. All the oligonucleotide spots in fingerprint of 587 are numbered. However, in 424 only those which correlated with 587 fingerprint are numbered and shown.

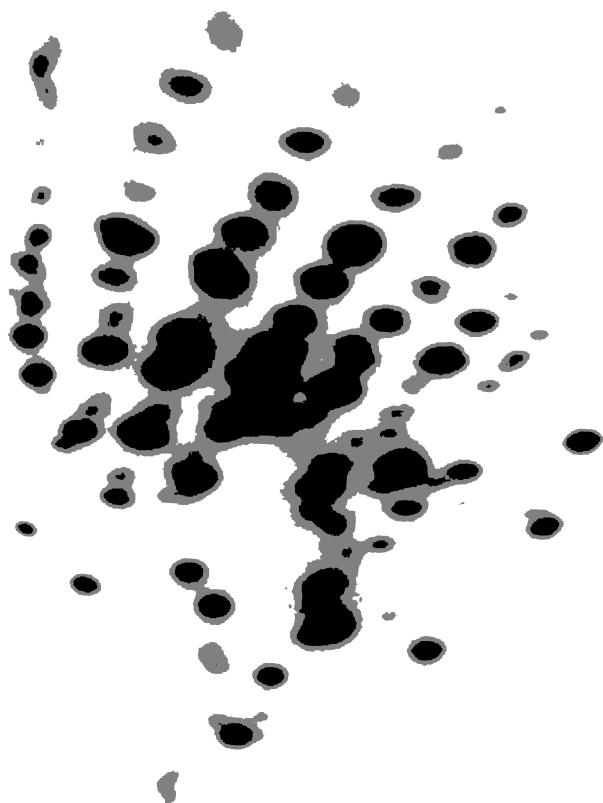
Isolates 512, 525 and 1197 displayed the fingerprint patterns which could not be correlated to any other fingerprints (Fig. 12 a,b and c).

Oligonucleotide fingerprinting analysis thus determined genomic relationship between the three strains 1176, 1199, 1177 isolated during the month of December and strain 587 isolated in July from S ward. Relationship could also be traced between the two July isolates 587 and 424. Although, the direct comparison of 424 fingerprint with the 1176 denoted less than 50 percent correlation, the two strains could be related on the basis of the existence of intermediate pattern of the strain 587. These five strains which could be related to each other on the basis of their

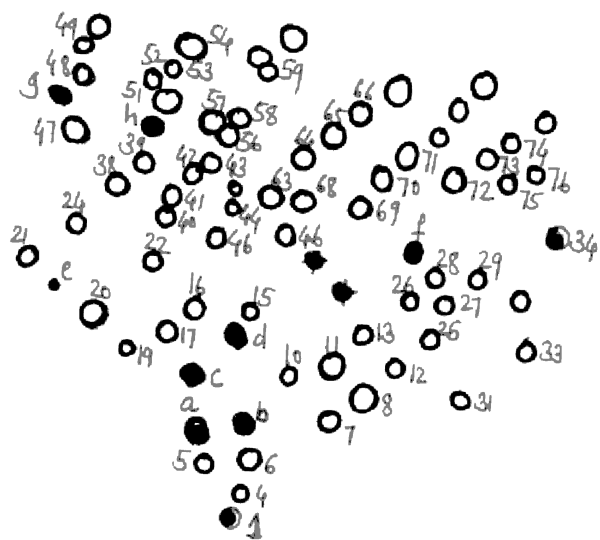
a



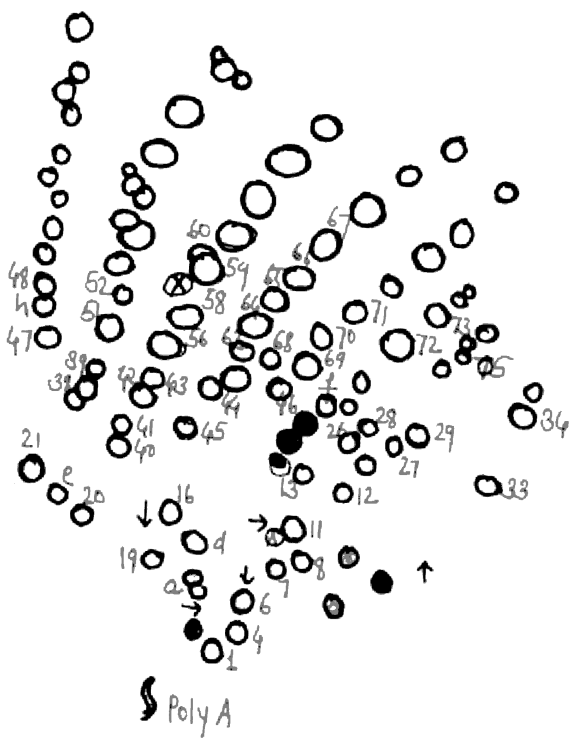
b



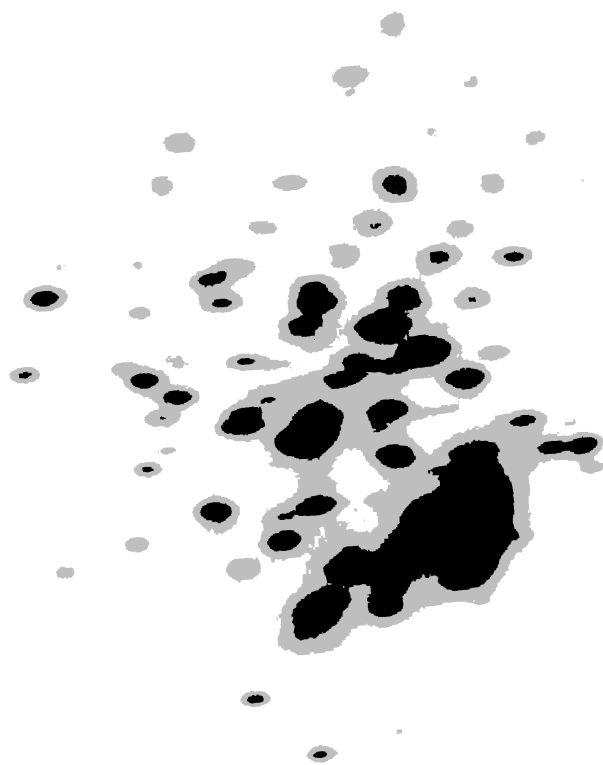
c



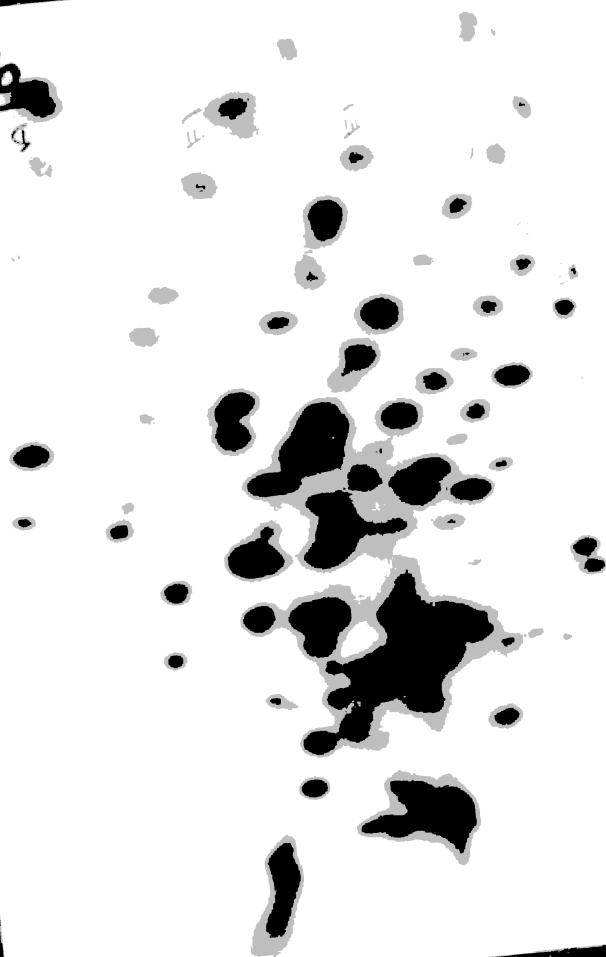
d



a



b



c



oligonucleotide fingerprint patterns constituted one of the genotype circulating in S ward. The remaining three strains 512, 525 and 1197 which yielded unrelated fingerprint patterns may be the representatives of other genotypes circulating in S ward.

R Ward Isolates : Ten poliovirus type 1 isolates obtained during the period from June to October were studied by oligonucleotide fingerprinting. Table 8 describes the epidemiological details of the isolates.

Table 8.

No.	Isolate No.	Dt. of onset of disease	Age in months/ Sex	Vaccination status	Patients Residential Area
1.	423	28.06.1986	07/F	0	Raidongari Road, Borivali (E), Bombay-66
2.	426	27.06.1986	14/M	0	Ganesh Nagar, Kandivali (E), Bombay 67.
3.	509	04.07.1986	12/M	0	Damu Nagar, Akurli Road, Kandivali (E), Bombay .
4.	558	15.07.1986	13/M	0	Dahisar (E), Bombay-68
5.	604	24.07.1986	12/F	0	Jawahar Nagar, Borivali (W), Bombay - 92
6.	605	25.07.1986	18/F	0	Nutan Nagar, Borivali (W), Bombay - 92.
7.	981	24.09.1986	18/F	3 /5th, 6th, 7th month	Garden Road, Poisar, Kandivali (E), Bombay - 67.
8.	995	02.10.1986	13/M	0	Rajendra Nagar, Borivali (E), Bombay-66.
9.	1045	19.10.1986	07/M	1 /3rd month	Gandhi Nagar, Lalji Pada, Kandivali (W), Bombay 67.
10.	1061	24.10.1986	24/M	3 /7th, 8th, 9th month	Devipada, Borivali (E), Bombay 66.

Oligonucleotide fingerprints of the two isolates 423 and 426 are shown in Fig. 13a and b. Spot to spot comparison could not determine any relationship between them. These strains were isolated from the patients who suffered from poliomyelitis on the consecutive days in two different suburbs of R ward.

The fingerprint of isolate 509 and its corresponding tracing are presented in Fig. 14a and 15a in which 88 oligonucleotide spots are numbered. The pattern denoted similarity with the fingerprint of isolate 426 (Fig. 14b). Between the 509 and 426 fingerprint, 61 spots out of the 88 were shared as having similar electrophoretic migration. As the greater degree of differences were observed in the two patterns only the corresponding spots in the 426 pattern are numbered (Fig. 15b). Where as the noncorresponding spots are marked as solid circles. It was observed that the region encircled by the dotted line in Fig. 15b correlated well in the two patterns. Migration of only five spots differed in this region. Where as greater number of differences were seen in the remaining portion. Altogether, the comparison indicated 69% similarity in the two fingerprints. Epidemiologically, the two isolates 509 and 426 were isolated from the Kandivali area within a period of 10 days.

Similar observations were made for isolates 995 and 1045, obtained in the month of October. Of the 88 spots numbered in 509 fingerprint, 48 spots had equivalent electrophoretic migration in the fingerprint of isolate 995 as well as 1045 (54 per cent similarity). Also again, only the left hand portion demarcated by dotted lines contained most of the matching oligonucleotides. A comparison with strain 426 revealed that the spots marked as solid circles (unmatched oligonucleotides with 509) differed markedly among the three strains. As will be shown later, the oligonucleotide fingerprint pattern of isolate 1045 had greater similarity with isolate 337 of M ward.

a



b



a



b



c



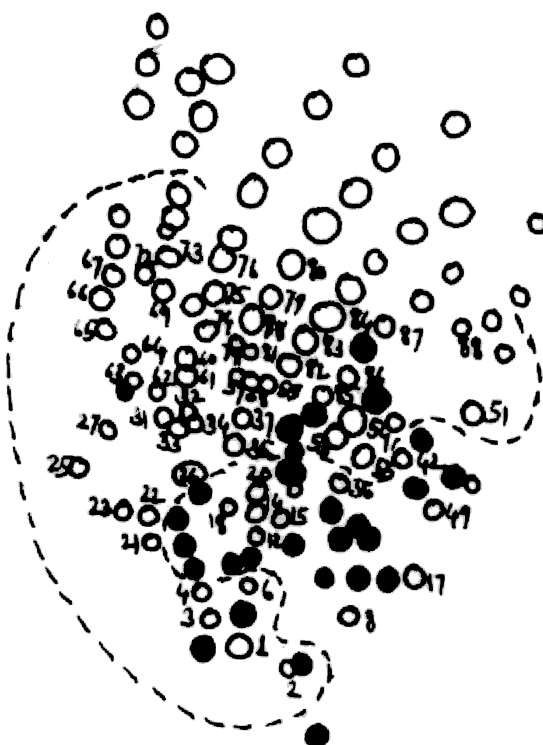
d



a



b



c



d



Oligonucleotide maps of the isolates 604 and 605 isolated from Borivali on the other hand exhibited striking similarity (Fig. 16a and b; 17a and b). Of the 74 oligonucleotide spots numbered in the 604 fingerprint pattern, electrophoretic migration of 72 spots was similar in the 605 fingerprint. Spot nos. 17 and 25 of 605 (marked with arrows in Fig 17b) showed first dimensional mobility shift towards left as compared to those of isolate 604. Extra spots (denoted by solid circles) between spots 1 and 3, 5 and 6, 7 and 8 and near 17, 24, 25 and 37 were noted in 605 fingerprint. Ninety per cent similarity observed in the two fingerprints reflect 99 per cent base sequence similarity in the two genomes. The two strains were also epidemiologically closely associated as they were isolated on the consecutive days from the nearby area (Table 8).

The 604-605 fingerprint pattern was also observed for the strain 1061 isolated three months later from the R ward (Fig. 16c; 17c). The isolate 1061 was definitely closer to the isolate 605 than to isolate 604. This was deduced from the position of spots 17 and 25. There were additions of two spots (marked with arrows in Fig. 17c) near the spots 35 and 48. The most conspicuous difference between the 604/605 profile and that of 1061 was the circular (wheel like) shape attained due to mobilities of spots numbered 16 to 25. Total of 88 per cent similarity was observed between the 605 and 1061 fingerprint patterns.

The circular appearance of spots 16 to 25 in the 1061 pattern could be traced back to the fingerprint of isolate 509. The comparison of the two patterns is given in Fig 18 a to d. The positioning of the spots 16 to 25 was very similar in the two fingerprints. However, no other similarity was observed between the two fingerprints and thus between the two viral genomes.

a



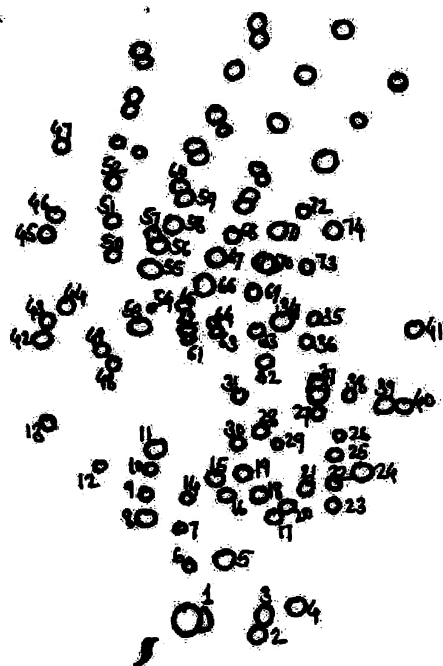
b



c



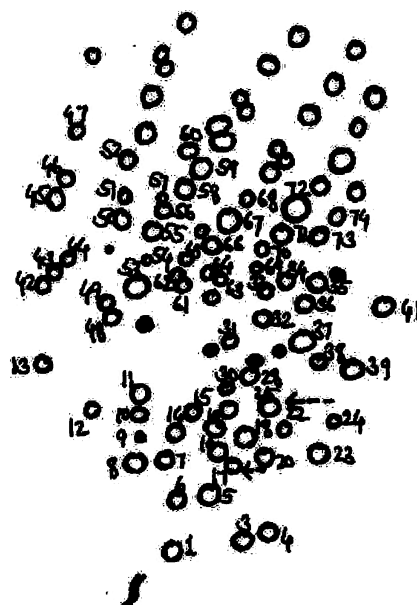
Fig 17.

a

ISOLATE 604, R WARD

b

ISOLATE 605, R WARD

c

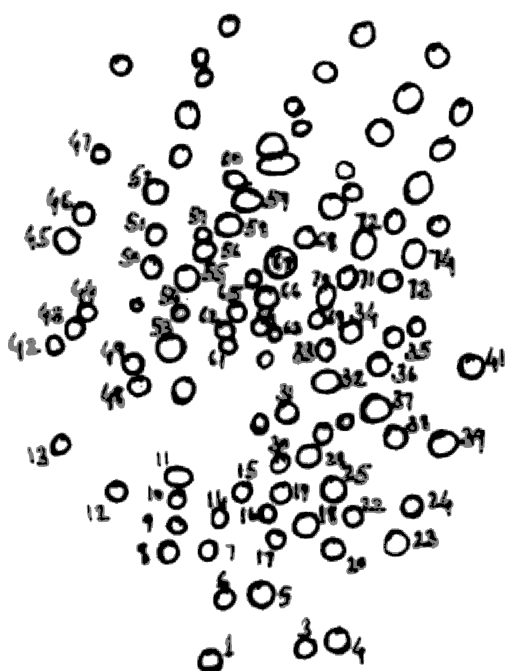
a



b

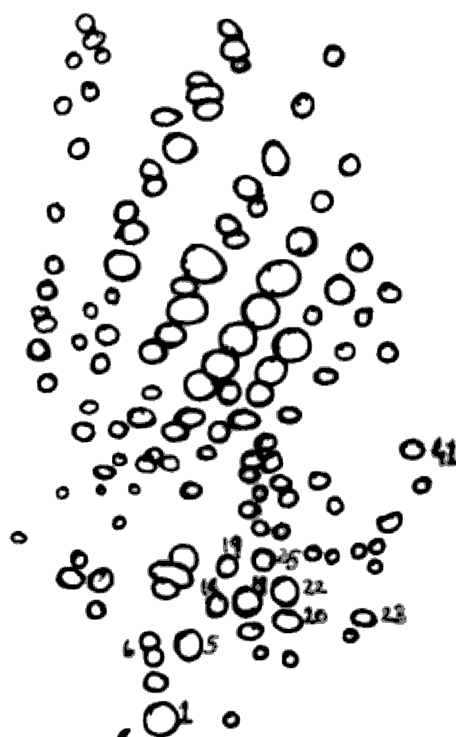


c

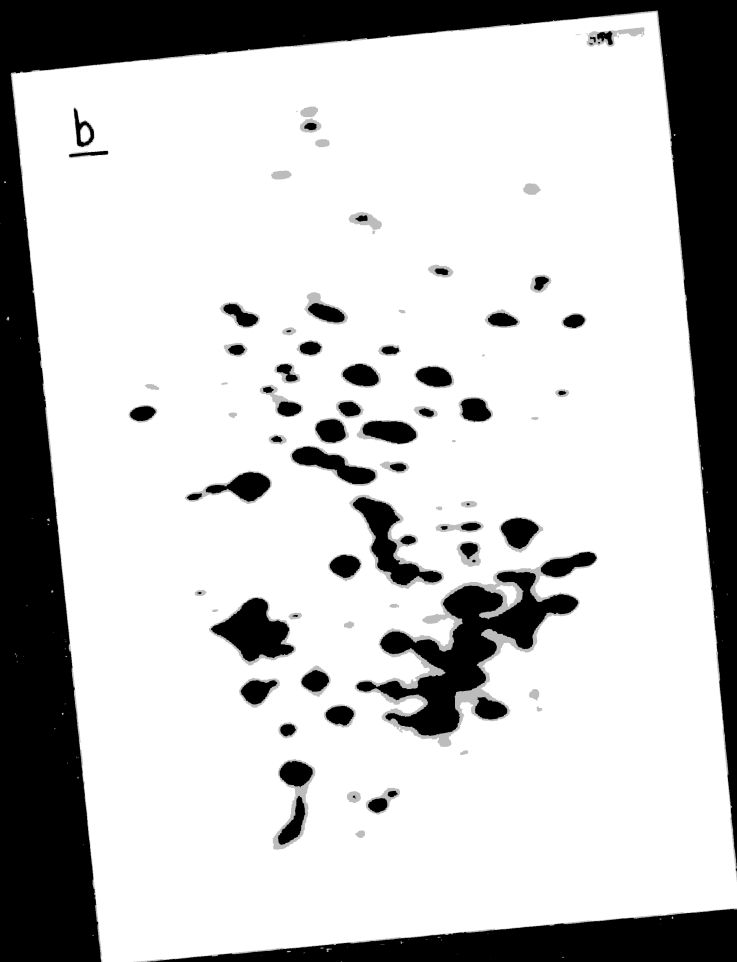


Poly A

d



Poly A



The genomes of isolates 558 and 981 (Fig. 19 a,b) of the R ward yielded oligonucleotide fingerprints which could not be matched with those of the other isolates.

M Ward Isolates : Epidemiologic background of the eight poliovirus type 1 strains selected for oligonucleotide fingerprinting from M ward was as shown in Table 9.

Table 9.

No.	Isolate No.	Dt. of onset of disease	Age in months/ Sex	Vaccination status	Patients Residential Area
1.	330	08.06.1986	18/M	1 / 3rd month	Panchsheela Nagar, Chembur, Bombay - 89.
2.	337	06.06.1986	15/F	0	St. Anthony Road, Chembur, Bombay - 71.
3.	358	16.06.1986	24/M	0	Shell Colony, Zopadpatti, Chembur, Bombay - 71.
4.	377	21.06.1986	48/F	1 / 3rd month	Gautam Nagar, Govandi, Bombay - 43.
5.	407	23.06.1986	09/M	0	Baiganwadi, Govandi, Bombay - 88
6.	652	27.07.1986	07/M	2 / 5th, 6th month	Siddharth Colony, Chembur, Bombay - 71.
7.	655	27.07.1986	08/M	1 / 5th month	Janata Nagar, Chembur, Bombay 89.
8.	663	30.07.1986	30/M	0	Gautam Nagar, Govandi, Bombay - 43.

Inspection of all the fingerprints of the M ward isolates suggested that the fingerprint of the isolate 337 could be related to several others. Hence, the oligonucleotides of the 337 fingerprint were numbered and compared for the similarities and/or differences with the others. A total of 103 oligonucleotide spots were numbered in the 337 fingerprint pattern (Fig. 20a, 21a). Of these, 99 spots showed equivalent migration in the fingerprint of isolate 330 (Fig. 20a and b; 21a and b). The differences included four deletion (spots 8,30,34 and 51) and two additions (below oligonucleotide no. 25 and the other above 27). The most prominent difference was the absence of spot no. 34 in the fingerprint of isolate 330. Total of 94 per cent similarity was detected in the two fingerprints.

Comparison of the fingerprints of isolates 358 and 337 (Fig. 20 a and c; Fig. 21 a and c) revealed that 89 spots of 358 could be matched with corresponding spots in fingerprint of isolate 337. The matched spots corresponded to 86 per cent similarity in oligonucleotides migration profile. It was also observed that the four spots which were absent in fingerprint of isolate 330 were also absent in 358 fingerprint (marked with arrows in Fig. 21 c). Moreover, absence of eight other spots (15,26,33,38,42,43,45, and 58) was observed. Two extra spots; one above spot 3 and other near no. 21 were detected. Comparison of the 358 fingerprint with 330 fingerprint revealed 87 per cent similarity. Isolates 337, 330 and 358 were epidemiologically closely associated.

The fingerprint pattern of isolate 663 obtained two months later from a different suburb of M ward could also be related to the above pattern. Its comparison with the 337 pattern revealed equivalent migration of 77 spots indicating 74 per cent of map similarity (Fig. 20 a and d; Fig. 21 a and d). Isolate 663, on the other hand showed close similarity with the 358 pattern. Spots 8,15,26,30,33,34,41,45,58 were missing as observed in the 358

a



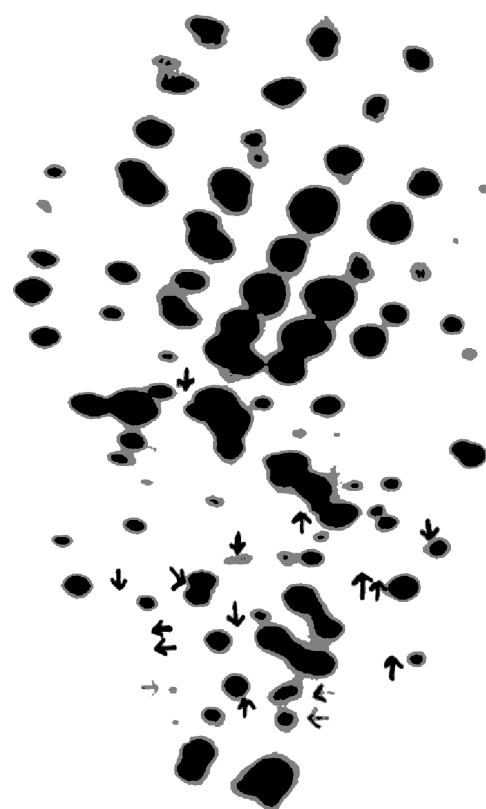
b



c



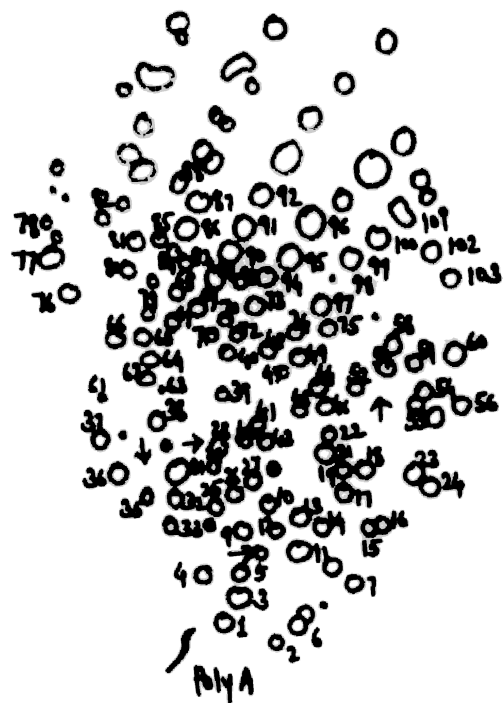
d



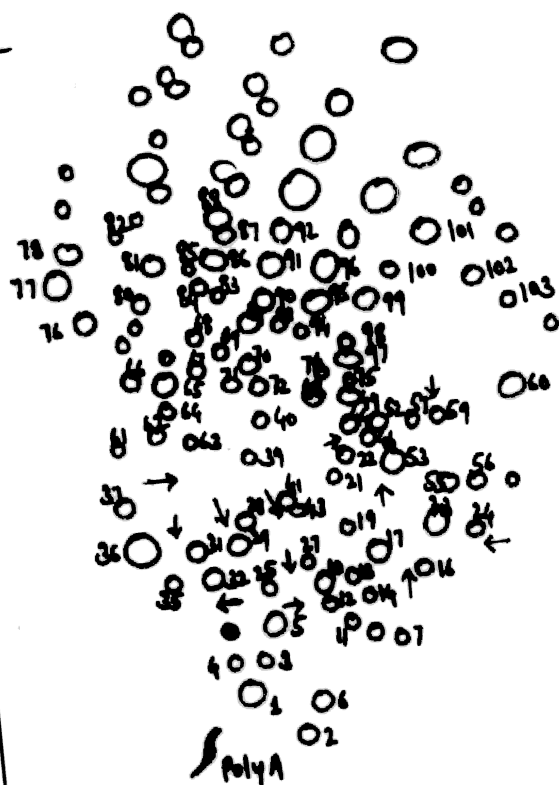
a



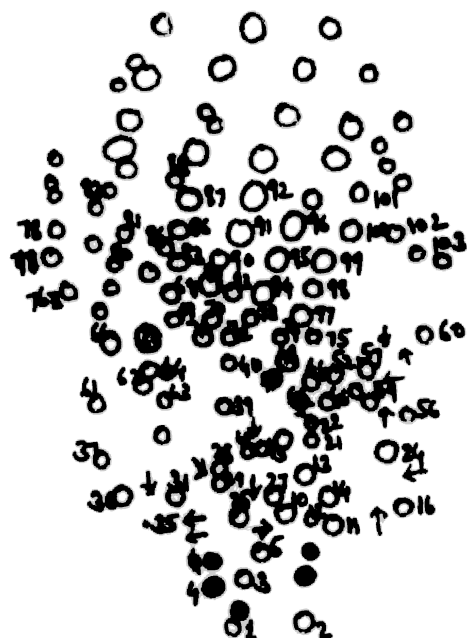
b



c



d

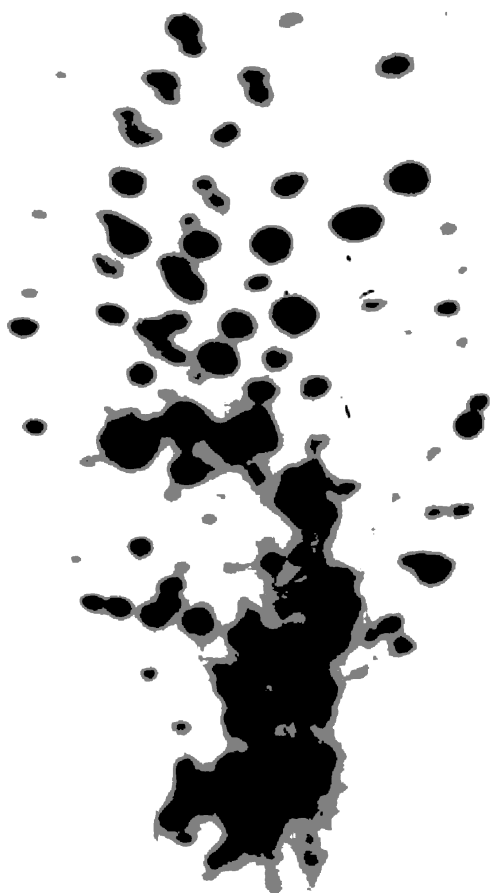


fingerprint. Absence of two more spots 32 and 61 was also observed. The spot 38 that was missing in 358 reappeared in 663. Total 88 per cent of correlation was detected in the two patterns.

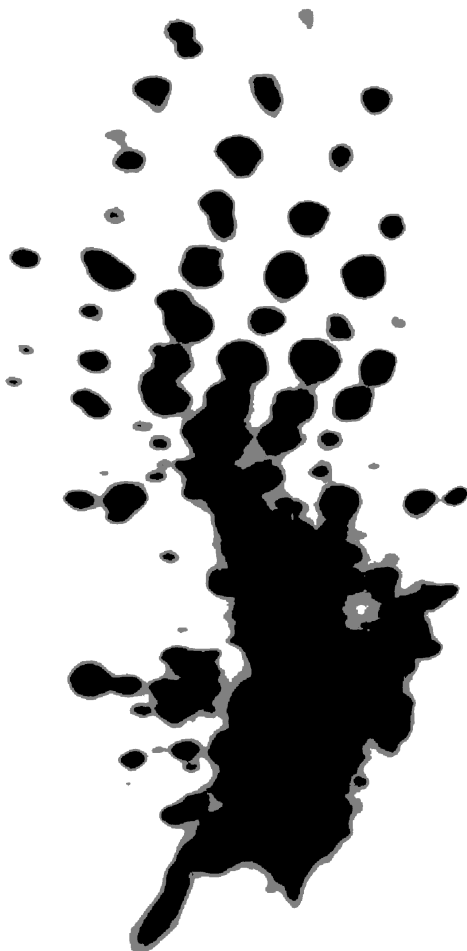
Fingerprints of isolates 377 and 407 could also be related to that of isolate 337 (Fig. 22 a to d). Out of the 103 spots of the 337 pattern, 59 spots in the 377 pattern showed similarity suggesting 57 per cent correlation. Only those spots corresponding to the 337 pattern are numbered in Fig. 22 C. In the 407 pattern, only 45 spots showed correlation implying 45 per cent similarity (Fig. 22b and d). These two isolates thus appeared to be distantly related to the previous group of isolates of M ward.

Fingerprint patterns of the isolates 652 and 655 (Fig. 23 a and b) could not be matched with any other.

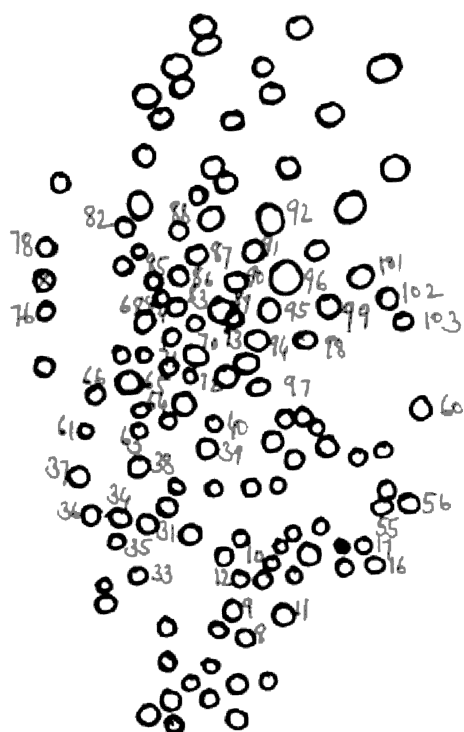
a



b



c



d



a



b



To determine the genetic relationship between isolates obtained from different wards, the 26 fingerprints were examined as one large group. Isolate 337 of M ward showed relationship with isolate 1177 of S ward (Fig. 24 a-d). While comparing the 337 and 1177 fingerprints, only the matching spots of the isolate 1177 pattern are numbered (in Fig. 24 d) for clarity of expression. Spots 1-5, 10-14, 17, 21-24, 27, 44, 45, 47, 53-56 could be identified. Spots 15 and 16 were however not seen in the 1177 pattern. Towards the left hand side, the positioning of spots 31, 34 and 36 was similar. The spot no. 36 showed a slightly altered position. Spots 32, 33, 37, 28, 29 and 30 were absent. Instead a spot above spot no. 31 was observed in the 1177 pattern. The upper portion of the two fingerprints were almost similar. However, Spot no. 77 was missing, 80 shifted to the right and the intensity of spot 79 altered in the 1177 pattern. An extra spot appeared below 61 and 62. The separation pattern of all remaining spots was very similar in the two maps. Together, out of the 103 oligonucleotide spots, 66 oligonucleotides were shared suggesting 65 per cent similarity in the two fingerprints.

Isolate 337 also showed correlation with isolate 1045 of R ward (Fig. 25 a to d). Out of the 103 spots, 76 spots (73 per cent) showed equivalent migration. The differences included absence of spots 6, 9, 12, 13, 16, 25, 54, 55, 58, 30, 32, 34, 37 and 63 in the 1045 pattern. Two extra spots were observed near spot no. 38.

Finally, the genetic relationship of the 26 poliovirus isolates could be presented in the form of a dendrogram shown in Fig. 26. As has been indicated in the dendrogram the subgroups related by the 337, 1045 and 1176 fingerprint relationships together form one major genotype circulating

A black and white photograph showing a large number of small, dark, irregularly shaped objects, possibly seeds or small stones, scattered across a light background. The objects are concentrated in the upper half of the frame, with a few scattered in the lower half. They vary in size and shape, some being round, some elongated, and some with irregular edges. The lighting is even, highlighting the dark color of the objects against the light background.

S

Poly A

a



b



c



d

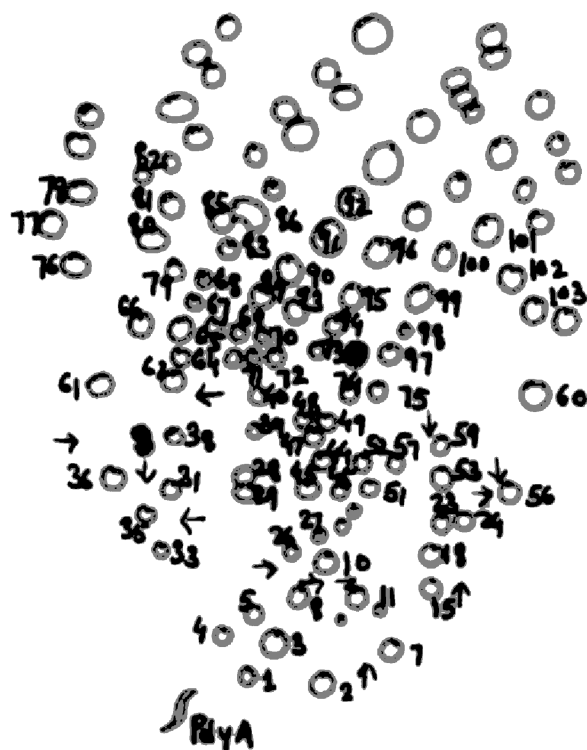
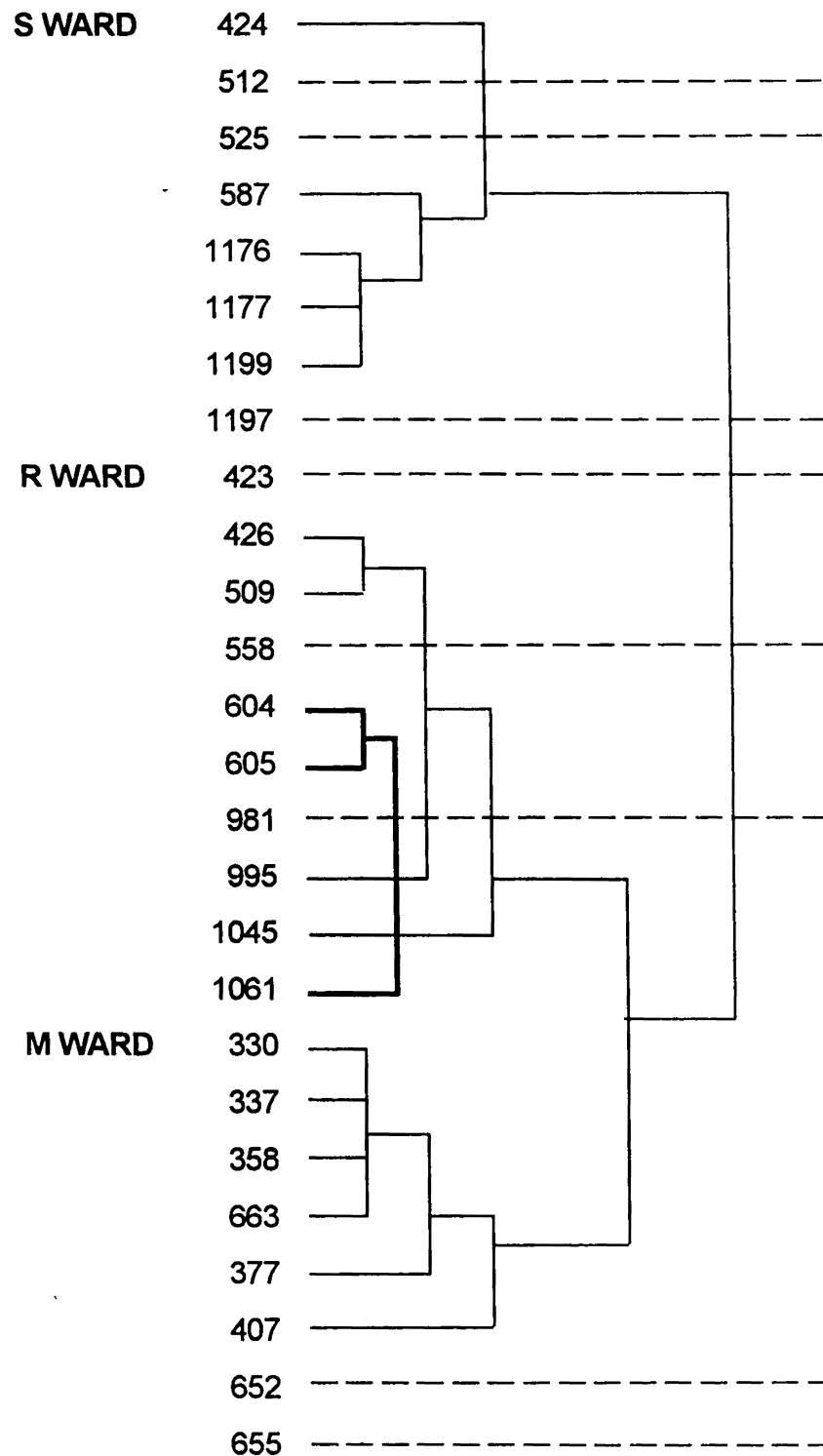


Fig 26. Dendrogram, constructed according to oligonucleotide fingerprint relatedness among poliovirus type 1 strains isolated from wards S, R and M of Bombay in 1986.



in Bombay. Another subgroup represented by isolates 604, 605 and 1061 constitute a second distinct genotype. It is noteworthy that a total of 8 strains including representatives of all the three wards could not be placed in the form of genotypes. However, the data is clear to show that in each of these areas more than one genotype circulated during the period under study. One genotype among them predominated.

Comparative Study Of Poliovirus Proteins By Polyacrylamide Gel Electrophoresis

Poliovirus type 1 strains analysed by oligonucleotide fingerprinting were also compared on the basis of the electrophoretic separation patterns of their viral proteins. Poliovirus proteins were radiolabeled by biosynthetic incorporation of [35 S] methionine during the virus infectious cycle. The infected cell lysates were then resolved by electrophoresis on 10% SDS polyacrylamide gels. The poliovirus type 1 vaccine strain (Sabin) and the locally isolated poliovirus type 1 wild strain (M₂b) were included as the reference strains. The polypeptide separation profile of the vaccine strain (lane 6, Fig. 27 a) was similar to the profile published by Nottay et al²⁰⁸. The profile was reproducible in the repeat experiments. Total of 16 protein bands were observed in the polypeptide separation profile presented by poliovirus type 1 vaccine strain. The bands (proteins) were numbered serially. as the exact functional identity of the labelled proteins was not determined. However, by comparison with the published reports, the proteins could have been identified as shown in Table 10²⁰⁸.

Table 10.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
-	-	NC VP 2	-	NC VP -3a	NC VP 3b	NC VP 5b	-	VP O	NC VP 6a	NC VP X	VP 1.	VP2/ 6b	VP 3	-	7a

The polypeptide separation patterns of the reference strain M₂b and the selected poliovirus type 1 field isolates differed from the poliovirus type 1 vaccine strain. Comparison of the vaccine strain and the M₂b strain (Fig 27a, lanes 6 and 7) showed presence of two extra bands, one below the band 6 and other above the band 12 in the M₂b profile. Mobility differences were also observed in band 7,9,13 and 14. The two extra bands of M₂b were, however, not seen in the pattern displayed by the S ward isolates (Fig. 27 a) Isolates 424, 587, 1176, 1177, 1197, and 1199 (lanes 1,4,5,8,9 and 10) displayed very similar protein profiles. An R ward isolate 423 (lane 11) also yielded a similar profile. Mobility differences were detected in the profiles of isolates 512 and 525 (lanes 2,3). Increased mobility of bands 10 and 13 was observed. An extra band as observed in M₂b was seen in 512, where as it was absent in 525.

Fig. 27b shows the electrophoretic separation patterns of the R ward isolates. The two isolates 604 and 605 displayed identical patterns (lanes 3,4). Mobility differences in bands 9 and 10, decreased mobility of band 12 and increased mobility of band 13 were the prominent features of these strains. As expected, similar pattern was observed for isolate 1061 (lane 10). Slight difference in the mobility of band 10 was however noted. Isolates 995 and 1045 (lanes 8,9) also gave identical patterns. Absence of band 10 and presence of an extra band above 12 was observed in these isolates. The profile of isolate 509 was similar to 995 except the band 10 was retained (lane 2). The profiles displayed by isolates 426, 981 and 558 of R ward (lanes 1,5,11) differed from those of the other isolates. The protein profile of isolate 423 was very similar to the S ward isolates (Fig. 27a, lane 11).

Five isolates 330, 337, 358, 652 and 807 of M ward were analysed. Isolates 330, 337, 358 and 807 (Fig. 27 c, lanes 3, 4, 5 and Fig. 28a, lanes

FIG. 27

Q

b



C

E				P1	E	E	G	E	E
10	330	337	358	AHm	2	10	17	18	26
2	3	4	5	6	7	8	9	10	12

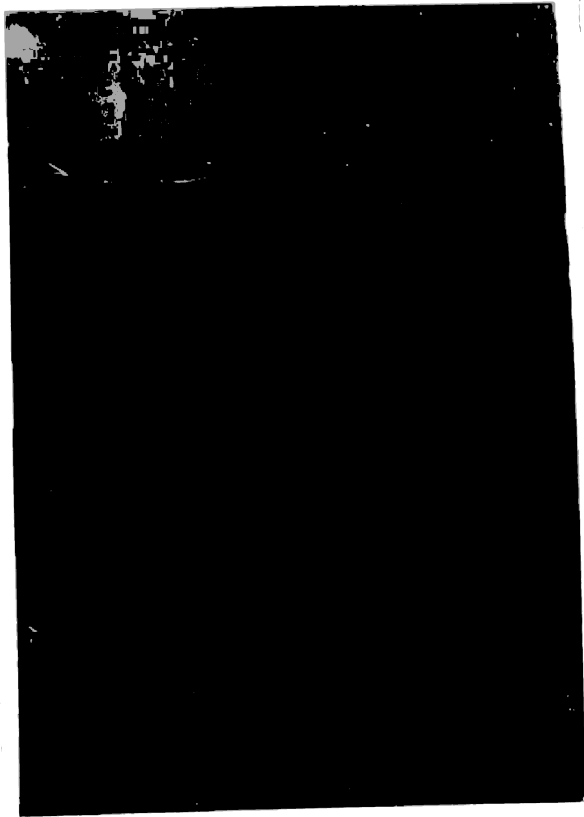
Five isolates 330, 337, 358, 652 and 807 of M ward were analysed. Isolates 330, 337, 358 and 807 (Fig. 27 c, lanes 3, 4, 5 and Fig. 28a, lanes 6, 7 and 9) displayed very similar protein profiles. The pattern was characterized by the absence of band 10 and presence of an extra band above band 12 and thus could be related to the pattern shown by isolates 995 and 1045 of R ward. The pattern of the isolate 652 (Fig. 28a, lane 7) differed from the other isolates.

In the year 1986, two poliomyelitis outbreaks caused due to poliovirus type 1 were reported in India. One of the epidemic occurred in the Marathwada region in Maharashtra and the other in Emmeneshwaram in Ramnad district of the State of Tamil Nadu^{234, 235}. Five isolates from the Marathwada and six from the Emmeneshwaram epidemic were also included in this study. The three Marathwada isolates 846, 965 and 1073 (Fig. 28 b, lanes 3,4 and 5) showed identical pattern. The two other strains 651 and 838 were however different (Fig. 28 b, lanes 1,2). In contrast, all the isolates of Emmeneshwaram epidemic (Fig. 28 b, lanes 8,9,10 and Fig. 28 a, lanes 1,2 and 3) displayed very similar protein separation patterns.

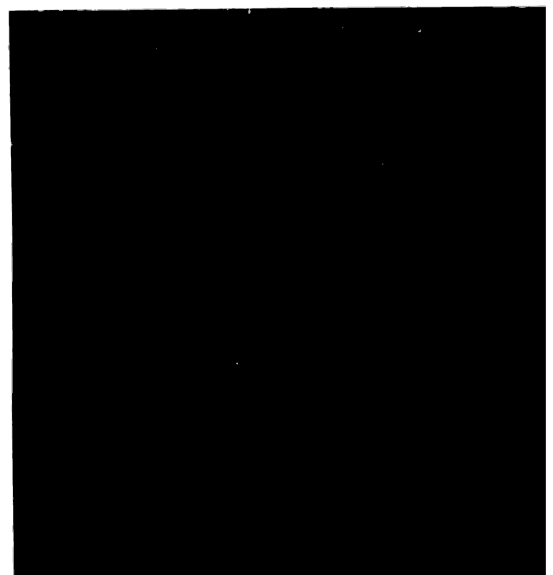
The poliovirus type 1 isolates could be classified into four groups on the basis of electrophoretic separation of [³⁵ S] methionine labeled viral polypeptides in the 10 per cent SDS PAGE.

Fig. 28

a



b



Group - 1

No.	Isolate No.	Source
1.	424	S Ward, Bombay
2.	587	S Ward, Bombay
3.	1176	S Ward, Bombay
4.	1177	S Ward, Bombay
5.	1197	S Ward, Bombay
6.	1199	S Ward, Bombay
7.	423	R Ward, Bombay

Group - 2

No.	Isolate No.	Source
1.	604	R Ward, Bombay
2.	605	R Ward, Bombay
3.	1061	R Ward, Bombay
1.	995	R Ward, Bombay
2.	1045	R Ward, Bombay
3.	330	M Ward, Bombay
4.	337	M Ward, Bombay
5.	358	M Ward, Bombay
6.	807	M Ward, Bombay

Group - 3

No.	Isolate No.	Source
1.	M 846	Marathwada
2.	M 965	Marathwada
3.	M 1073	Marathwada
1.	E-2	Emmeneshwaram
2.	E-10	Emmeneshwaram
3.	E-11	Emmeneshwaram
4.	E-17	Emmeneshwaram
5.	E-18	Emmeneshwaram
6.	E-26	Emmeneshwaram

Non Grouped Strains

No.	Isolate No.	Source
1.	512	S Ward, Bombay
2.	525	S Ward, Bombay
3.	426	R Ward, Bombay
4.	558	R Ward, Bombay
5.	981	R Ward, Bombay
6.	652	M Ward, Bombay
7.	M 838	Marathwada
8.	M 651	Marathwada

The grouping achieved through polypeptide analysis was in agreement with the previous analysis of the strains by oligonucleotide fingerprinting.

DISCUSSION

Polioviruses have the potential to undergo genomic variation during replication in humans. Analysis of poliovirus strains at the genomic level provided the possibility to discriminate epidemiologically distinct wild polioviruses from each other and from vaccine derived strains. Determination of the patterns of genomic variation among the poliovirus strains has been identified as a powerful approach for establishing their epidemiologic interrelationships²⁰⁹. In the present study, genomic relationship among the poliovirus strains circulating in Bombay was analysed using oligonucleotide fingerprinting technique of the polioviral RNA.

Surveillance studies have shown that the incidence rate of paralytic poliomyelitis in Bombay ranged from 11.3 per 100000 population to 14.3 per 100000 population (occurrence of over 1000 cases annually) during the years 1972 to 1987. Immunization coverage levels improved with the launching of UIP and the declining trend in the incidence was observed since 1988. Reduction in the incidence rate from 14 per 100000 population in 1987 to 6.8 in 1988 and to 2.3 in 1992 has been noted²³⁶. As the genomic characteristics and possible variations among the circulating poliovirus strains were not known, selection of strains that would be able to determine genomic relationship was a prerequisite for this first attempt of genomic study. It was envisaged that the poliovirus strains obtained during high endemicity of the disease would be genetically related. Poliovirus type 1 strains isolated during the year 1986 were selected for the present study. Twenty six poliovirus type 1 strains of the three epidemiologically important wards S,R and M of the year 1986 were analysed by ribonuclease T1 resistant oligonucleotide fingerprinting.

As presented in Table 6.1, a total of 1268 cases of paralytic poliomyelitis were observed in Bombay in the year 1986. From the surveillance records it was noted that the incidence of poliomyelitis had been relatively steady for many years in S ward which included Vikroli, Bhandup and Mulund suburbs of the Bombay city. A total of 76 cases were reported in S ward in the year 1986. Samples from 28 cases were virologically investigated through the routine surveillance system of which twenty five (89.28%) were identified as caused due to poliovirus type 1. The eight strains from S ward analyzed by oligonucleotide mapping were divided into two temporal groups. The first group consisted of four consecutive isolates (424, 512, 525 and 587) obtained during the month of July. The remaining four strains (1176, 1177, 1197, 1199) were isolated in the month of December. The two groups of strains were, thus, separated by a period of five months.

Comparison of the oligonucleotide fingerprints presented by the July isolates (Fig. 11 and 12) denoted that none of the large oligonucleotides could be shared in the maps of three isolates 424, 512 and 525. The three isolates were obtained from poliomyelitis cases reported in the same locality (Bhandup (W), Bombay-78) within a period of fifteen days. On the epidemiologic background, genetic relationship among these isolates could be expected. Clearly distinct oligonucleotide maps of the three strains, however, provided evidence for no relationship among them.

The fourth isolate 587, obtained from a slightly distant area, of Vikroli (E), Bombay-83, showed 75 per cent map similarity with the Bhandup isolate 424 (Fig. 11). The differences observed in the two fingerprints indicated about 5 per cent of sequence divergence (approximately, 350 base substitutions) between the two viral genomes. Accumulation of so many mutations in the genomes of the strains separated by a period of fifteen

days appears inconceivable. The observation, therefore, suggests that the two strains may have derived from separate lineages of a common ancestral strain. Genetic heterogeneity was thus observed among geographically closely associated poliovirus strains isolated from S ward during the month of July. Conditions permissive for such an observation probably arise due to suboptimum immunization coverage. Separate evolution of poliovirus genotypes may occur when the population of susceptibles is sufficiently large to maintain local circulation of specific genotypes for the extended period²¹⁸. Furthermore, morbidity peak of poliovirus infections is observed during the monsoon months from July to September. Virus transmission is very rapid during this period and maximum intermixing of the strains is expected which may result in the presence of genetically diverged isolates.

In contrast to the isolates of July, the December isolates showed high degree of map similarity. Only three spot differences were observed in the fingerprints of isolates 1176 and 1199 and 9 spots differed in the pattern of isolate 1177 (Fig. 9 and 10). Epidemiologically, these strains were isolated from poliomyelitis cases reported in the same residential area in Tagore Nagar, Vikroli (E), (Bombay-83). More than 90 per cent similarity observed in the three fingerprints indicated 99 per cent sequence similarities in the genomes of the three strains. Close similarities in the oligonucleotide maps have been demonstrated among the isolates taken during the course of the same epidemic^{209,228,237}. Nottay et al (1981) defined the correlation among the successive strains isolated from paralytic poliomyelitis cases and their immediate contacts during the thirteen months transmission of an epidemic from Netherlands to USA via Canada (1978-79). In the fingerprints of successive strains majority of the oligonucleotides migrated to equivalent position but no two maps were identical or differed from the reference map (index case) in the same way. Small map changes were consistently observed in the serial isolates which revealed the continual mutation of the

poliovirus genome during replication in humans. The oligonucleotide maps of the successive isolates provided a measure of rate at which selected changes occurred in the viral genome. It was estimated that about 1 to 2 base substitutions get fixed over the entire viral genome in a period of one week. Accordingly relatively few map changes were observed in the temporally consecutive isolates, while the maps of the isolates taken over long intervals showed greater differences²⁰⁸.

Close similarity in the fingerprints of the three December isolates 1176, 1177 and 1199 thus provided evidence for the same genetic lineage of the three strains. It revealed that the virus strain responsible for the poliocases in this area have originated from a common focus. The fingerprint comparison also detected that isolate 1176 had a greater map correlation with isolate 1199 than to the 1177 indicating its genomic proximity to isolate 1199. The strain 1177 was isolated from the case which occurred only three days after the 1176 case, where as 1199 was isolated 15 days later. The epidemiological data and the fingerprint analysis together could be used to provide evidence of virus transmission route. It is probable that the patients 1176 and 1177 were infected by virus originated from a common ancestral strain but were separated by unknown number of multiplications in susceptible children. The almost identical maps of strain 1176 and 1199 suggests that the virus excreted by patient 1176 was closely responsible to cause paralytic disease in patient 1199. The results provided a clear molecular evidence of transmission of a poliovirus strain within the community. The fourth strain 1197 isolated in the month of December from a slightly distant area (Anandgad, Vikroli (E), Bombay - 79), presented a different oligonucleotide separation pattern which proved its nonrelationship with the three December strains of Tagore Nagar.

Genomic relationship between the strain 587 isolated from Vikroli (E) in the month of July and the strain 1176 isolated from the same area in the month of December was also deduced from fingerprint comparison (Fig. 9). The two strains were separated by a period of six months. In consequence, greater degree of map differences were observed in these two isolates than those observed for the temporally consecutive isolates of December. Fingerprint similarity provided evidence for the common genetic lineage of the two strains. Consequently, it also provided evidence for the common genetic lineage of the two July 424 and 587 and the three December 1176, 1177 and 1199 strains.

The data collected through fingerprint analysis of the S ward isolates showed that when poliovirus transmission was at its peak (July), strains revealing genetic diversity were detected. This indicated circulation of poliovirus strains of different lineages in the community. Isolation of genetically related strains in the month of December (low polio season) indicated that one genotype may predominate when small number of poliocases are observed. Genomic relationship between the July isolates and those of the December strains proved continual circulation of a poliovirus genotype in the same geographical area for long periods of time. The molecular method thus provided evidence to show the epidemiological link between paralytic poliomyelitis cases.

The R ward which included the suburbs Kandivali, Borivali and Dahisar of the Bombay city covered a relatively larger area (77.5 Km²) than the other two wards. A total of 111 cases of paralytic poliomyelitis (incidence rate of 19.7 per 100000 population) were recorded in R ward in the year 1986. Samples from 32 poliomyelitis cases were virologically investigated, out of which 29 (90.62%) were identified to have been caused due to poliovirus type 1. Ten poliovirus type 1 isolates selected for the

fingerprinting analysis from the months June to October included five isolates from Borivali (423, 604, 605, 995, 1061), four from Kandivali (426, 509, 981, 1045) and one (558) from the Dahisar region.

Very close genomic relationship was observed between the isolates 604 and 605 obtained from poliomyelitis cases reported on two consecutive days in Borivali (W). The fingerprint similarity provided a clear evidence of a single focus of infection. The July isolates were genetically closely related with the October isolate 1061 from Borivali (E) (Fig. 16). Circulation of a Borivali (W) genotype of July in Borivali (E) after a period of three months could thus be demonstrated. Isolates 423 (July) and 995 (October) showed no genetic similarity among them even though they were isolated from Borivali (E). More than one genotype of poliovirus type 1, thus, circulated in this area.

Of the four isolates studied from Kandivali, the two isolates 426 and 509 obtained in the month of July had 69 per cent map similarity (Fig. 14), indicating the same genetic lineage of the two strains. The Kandivali (E) genotype represented by 509 could be genetically traced to Kandivali (W) isolate 1045 of the month of October and Borivali (E) isolate 995 of the same month (Fig. 14). The three strains, however, represented as the three corners of a triangle on the basis of fingerprint comparison. It is therefore suggested that the 509 genotype continued circulation in Borivali (E) and Kandivali (W), each giving rise to separate lineages diverging from each other. The Kandivali isolate 981 and the Dahisar isolate 558 were from the independent lineages.

An unusual feature was observed during fingerprint comparison of isolates 509 and 1061. While no similarity was noted in the oligonucleotide separation patterns of the two isolates, a mobility of group of

oligonucleotides (spot no. 16 to 25) forming a wheel like constellation in the right corner of the two fingerprints was very similar (Fig. 18). Since the two strains were grouped into two separate lineages, three possibilities could be envisaged to produce this relationship; i) Natural divergence in poliovirus type 1 strains to common points; ii) Recombination of parenteral strains of 509 and 604/605 with a third independent strain possessing a wheel like distribution of the said (particular) oligonucleotides; iii) The third genotype recombined with a parent of 509 during July, also continued its own circulation and had a chance of recombination with the progeny of 604/605 to yield a strain 1061. The evidence for the recombination among the poliovirus strains has been demonstrated in the virus strains excreted by healthy vaccinees, and by vaccine associated poliomyelitis cases^{238,239}. Recombinant genotypes among the wild poliovirus strains have also been demonstrated²¹⁸. Although, the actual frequency of the recombination phenomena among poliovirus strains has not yet been established, it has been recognized as an important mechanism for broadening the number of poliovirus genotypes in nature²⁴⁰.

In conclusion, multiple genotypes of poliovirus were observed in R ward. Each locality presented with a predominant lineage. As was observed in S ward isolates, circulation of a genotype for long periods was also seen in this ward.

Highest number of paralytic poliomyelitis cases among the residents of Bombay were reported from M ward which included Chembur, Govandi and the Mankhurd areas of the Bombay City. The ward covering an area of 54.9 Km², recorded 174 poliomyelitis cases and an incidence rate of 30.7 per 100000 population in the year 1986. Of the 76 cases of acute flaccid paralysis studied for virus isolation, 58 (76.31%) were caused due to poliovirus type 1 infection. Five isolates obtained from Chembur (330, 337,

358, 652, 665) and three isolates (377, 407, 663) obtained from Govandi during the three month period, June to August were analysed by oligonucleotide fingerprinting.

The oligonucleotide position similarity between isolates 330 and 337 and isolates 330 and 358 were 94 and 87 per cent, respectively (Fig. 20). Epidemiological details of the three cases revealed that 330 and 337 were reported within a two day period in the month of June, whereas 358 case was reported ten days later. As was suggested, while discussing the maps of S ward isolates, that very closely occurring cases such as 330 and 337 may not serve as source of infection for either instead could be infected by virus circulating in the environment. Thus, the isolates 330 and 337 may have been infected by the virus excreted by the previous case. The close similarity of 330 and 358 maps was suggestive of the 358 isolate being in direct lineage of the isolate 330.

The two contemporary strains of Govandi 377 and 407 showed only 57 per cent and 45 per cent map similarity with the Chembur isolate 337 which reflected their distant relationship. On the contrary, the strain 663 isolated from Govandi in the month of August showed 94 per cent map similarity with the Chembur isolate 358. Close similarity in the fingerprint patterns clearly suggest migration of the Chembur strain to Govandi. The four closely related isolates 330, 337, 358, 663 and the two distantly related isolates 377 and 407 thus formed one of the genotype circulating in M ward. The two strains 652 and 655 isolated in the month of August in Chembur on the other hand, gave oligonucleotide maps showing no relationship with each other or with other isolates. Evidence for the existence of different lineages circulating simultaneously in M ward was thus obtained.

The isolate 337 of M ward also showed fingerprint correlation with isolate 1177 of S ward (Fig. 24). Sixty six per cent map similarity observed in the two patterns suggested that the two strains belong to the same genotype. Isolate 337 also showed 73 per cent map correlation with isolate 1045 of R ward (Fig. 25). The interward genomic relationship revealed by map similarity in the isolates 337, 1177 and 1045 provided a scope to presume that the isolates related by 337 in M ward, 1045 in R ward and 1177 in S ward may all in turn be related to each other. The inter-ward fingerprint comparison was therefore critically reexamined. It was observed that while majority of these strains shared less than 50 per cent map similarity, the electrophoretic migration of some of the cardinal spots such as i) 'Y' shaped constellation formed by oligonucleotide no. 1 to 6 at the bottom of the fingerprint; ii) characteristic separation of the group of 4 to 6 oligonucleotides on the left; iii) separation pattern of majority of the spots comprising of the upper portion of the fingerprint (especially 'U' and the inverted 'V' shaped constellations at the upper central and upper right side respectively) remained unchanged in all these fingerprints. It was earlier shown by Frisby et al (1977) that some of the oligonucleotides are specific for the fingerprints of poliovirus strains and are conserved among otherwise fairly dissimilar ones²⁰⁵. Kew et al (1981) observed that some of the oligonucleotide spots in maps from successive isolates often disappeared and reappeared in the later isolates suggesting the existence of highly mutable region(s) of the viral genome. Certain other large oligonucleotides on the other hand did not change their position which indicated the presence of conserved regions²⁴¹. If poliovirus can accumulate only a finite number of mutations in its genome, the various lineages of one ancestral strain (genotype) would diverge upto a limiting point only. Oligonucleotide fingerprints of isolates 337, 1177 and 1045 of wards M, S and R respectively, probably revealed inter-relationship between the three apparently separated lineages seen in the 3 wards. The presence of the

cardinal spots and the intermediate genomic relationship demonstrated by isolates 337, 1176 and 1045, thus provide a strong evidence to assume that fourteen strains are of the same genetic lineage and constitute one of the major genotype circulating in the year 1986 in Bombay.

The isolates 604, 605 and 1061 of R ward formed another genotype circulating in Bombay. The oligonucleotide maps of this genotype did not show relationship with the above mentioned genotype. The strains which displayed the unrelated oligonucleotide fingerprint pattern were probably the representatives of other genotypes circulating in the population. It was however not possible to determine whether these strains represented the diverged strains of the same genotype or they are the members of the separate genotype. Such long range relationships may be elucidated by partial genomic sequencing.

For polioviruses, alteration in the oligonucleotide maps were accompanied by shifts in the electrophoretic mobilities of the virus proteins^{208,241}. Several mobility differences were detected in the protein profiles of the parent Mahoney and the Sabin vaccine strain LSc 2ab. Increased mobility of the capsid protein VP1 was identified to be a feature which distinguished LSc 2ab from all other poliovirus type 1 strains. Mobility differences were also detected in VP0, VP2, VP3 and in non-capsid proteins 2 and 4. Viral proteins of the epidemic strains showed differences in the mobility of each of these proteins. Poliovirus type 3 virulent strains could be distinguished from the vaccine strains by the presence of two additional polypeptides. Migration of these polypeptides varied slightly from strain to strain²²⁸. PAGE analysis was increasingly employed to analyze the relatedness of poliovirus isolates²⁴².

In the present study, the polypeptide analysis was applied to determine relationship among the poliovirus strains. The polypeptide analysis data and the results of the fingerprint analysis were compared. The genomic relationship detected by fingerprint similarity among the strains 424, 587, 1176, 1177, 1199 of S ward; 604, 605, 1061 and 1045, 995, 509 of R ward and 330, 337 and 358 of M ward were reflected exactly in the electrophoretic separation pattern of the viral proteins. The differences observed through the oligonucleotide fingerprints for strains 512, 525, 558, 981 and 652 of the three wards were also detected in the polypeptide analysis. The protein profiles observed for the isolates 330, 337 and 358 of M ward was very similar to the profile presented by the strains 995 and 1045 of R ward. Thus, the interward relationship demonstrated by isolates 337 of M ward and 1045 of R ward could also be detected by electrophoretic separation pattern of the viral proteins.

The polypeptide analysis data however did not correlate for strains 1197 and 423. 1197 was related to the genotype represented by 1176 of S ward by polypeptide analysis but not by oligonucleotide fingerprinting. The protein profile of R ward isolate 423 grouped it with the S ward genotype which is also in disagreement with the oligonucleotide fingerprinting results. Similarly, the fingerprint correlation observed among the isolates 426 and 509 of R ward; isolate 337 of M ward and 1177 of S ward could not be detected on the basis of their polypeptide separation pattern.

The isolates associated with the Emmeneshwaram epidemic produced very similar polypeptide separation pattern. From the results of the strains studied by both the methods (Bombay isolates), it can be suggested that Emmeneshwaram isolates would turn out to be of a single genotype. Similar results were however not observed for the strains associated with the Marathwada epidemic. Of the five strains, three strains

produced identical polypeptide separation pattern. Whereas the two strains showed unrelated patterns. The surveillance data showed that the Marathwada epidemic was on much larger scale than the Emmeneshwaram outbreak and involved Aurangabad, Beed, Jalna and Parbhani districts of the Maharashtra State²³⁴. It is therefore probable that the outbreak strains varied genetically.

The results of the SDS-PAGE analysis of the poliovirus strains were thus, in general, in consistence with the fingerprinting analysis. The technique was however, found to be less sensitive to detect the genomic changes. It may be of value as a preliminary screening method to select strains for oligonucleotide fingerprinting (genetic analysis) when large number of isolates are available.

The data presented in this thesis showed the usefulness of oligonucleotide fingerprinting of the poliovirus isolates to determine relationship between them. Similarity in the oligonucleotide fingerprint patterns clearly reflected the genomic relationship among the poliovirus strains. Isolates obtained even after extensive multiplication in the community for as long as six months could be traced back to their progenitor strains. Relationships between the strains could be pointed out even if they were isolated from different geographic locations. It was also possible to deduce the order of infection and migration of a strain from one place to other from the fingerprint correlation. The data revealed that more than one genotype of poliovirus type 1 were in simultaneous circulation in each ward during the period of study. One genotype among them however predominated in each ward. Fingerprint correlation was also observed among the predominating genotypes. A genotype represented by fourteen poliovirus type 1 isolates constituted one of the major genotype circulating in Bombay during the year 1986. At least two genotypes circulating during

the period of study could be defined. Analysis of the viral polypeptide patterns corroborated with the fingerprinting results.

The fingerprints are of significant practical value to show survival of certain genotypes in the environment. Fingerprinting analysis of the representative strains of the successive years will be able to determine the genotypes which have survived, diverged and have migrated from the adjacent area. Survival of a genotype in a given population also reflects its selective preference over the other genotypes and would be of interest for further study. As the number of poliomyelitis cases declines to a significant level, it will be possible to characterize all the isolates and determine relatively few number of genotypes circulating in the population.

The efforts for eradication of poliomyelitis from our country will depend heavily on the application of vaccines and the immunization strategies. Oral polio vaccine although has a very good safety record and has unequivocally been opted as a standard EPI antigen for the control and eradication of poliomyelitis, its three limitations have been identified. It is highly thermolabile and rapidly loses its potency when exposed to higher temperature. Loss of vaccine potency has been the most common reason cited for TOPV failure in the tropical areas. For the reasons that are not clearly known, lower antibody response to 3 doses of OPV has been observed in the developing countries⁹⁹. Lower efficacy of TOPV has also been observed in India^{243,98}. The epidemiological data from Bombay and New Delhi have shown that significant proportion (20-22 per cent) of the total paralytic poliomyelitis cases occur among the vaccinated children^{236,244}. Recent outbreaks in Andhra Pradesh (1992, 400 cases) and Marathwada (1990, 355 cases) reported involvement of over 25 per cent of the vaccinated children²⁴⁵. In Marathwada, as high as 60 per cent of the patients reported a vaccination history providing evidence for the less

effectiveness of vaccine in response to high challenging virus dose²⁴⁶. Lacunae in vaccination coverage and limitations to vaccine efficacy can play a substantial role for the survival of the wild poliovirus within the population.

The epidemic events reported in the poliocontrolled regions during the last decade have demonstrated that even a highly satisfactory vaccination coverage and an excellent immunity status following vaccination may not always guarantee a complete protection. In the Netherlands, the religious community refusing vaccination resulted in the accumulation of the population susceptible for the emergence of outbreaks (1978, 1993), despite 97 per cent of the vaccination coverage (IPV) in the general population⁸¹. In Finland, the epidemic strain which had migrated from the Mediterranean countries differed in its immunological and molecular properties from the previously characterized type 3 poliovirus strains. The unusual antigenicity accounted for the spread of the epidemic strain in well immunized population of Finland after twenty years of freedom from poliomyelitis¹⁰⁷. Reports from Gambia, Taiwan, Oman, South Africa and Namibia provided evidence for the break-throughs in OPV vaccinated communities. Even with the 87 per cent of OPV immunization coverage, the number of susceptible people was large enough to sustain transmission of wild virus. In Oman, as many as 94 per cent, 79 per cent and 51 per cent of case patients had received at least 1, 2 or 3 doses of OPV respectively. These outbreaks which occurred in distant regions, under vastly different environmental and social circumstances and caused by unrelated types of poliovirus, in general, raised a warning for unexpected drawbacks in vaccine induced immunity. They emphasized the need to sustain high immunization coverage and develop the sensitive systems of epidemiologic and laboratory surveillance to identify areas of potential susceptibility for outbreaks.

As the eradication programme in our country continues, extensive laboratory support will be needed for the detection of reservoirs of poliovirus transmission, identification of the origin of outbreak strain, population at risk and transmission of wild poliovirus from one place to another for implementing the prompt outbreak control and supplemental immunization activities. In the places, where high levels of immunization coverage have greatly reduced the number of poliomyelitis cases, virus isolated from all the suspected cases will require virological investigations including intratypic differentiation to clarify their origin. Recognition of the most likely portals of entry for extrinsic genotypes would direct the intensive surveillance and immunization activities to those critical areas. Poliovirus also finds its way into the environment through the excretion in faeces and survives in waste water for several months, where it may be the source of new infections. Environmental surveillance will therefore be crucial to the success of the eradication programme. After successful elimination of poliomyelitis caused due to wild poliovirus for a period of time, full implementation of AFP surveillance is still considered essential for providing the evidence needed for the eventual certification. Genomic characterisation of wild poliovirus strains will thus formulate as essential aspect of the eradication programme.

The availability of detailed information on the circulation of specific poliovirus genotypes, coupled with improved control measures, should allow us to witness eradication of poliomyelitis caused by wild poliovirus from our country.

SUMMARY AND CONCLUSIONS

Poliovirus strains isolated from the patients of paralytic poliomyelitis were characterized genomically using the technique of ribonuclease T1 resistant oligonucleotide fingerprinting. Isolates were propagated on cell cultures and the poliovirus from the infected culture suspension was concentrated by differential centrifugation. Poliovirus preparation was further purified by equilibrium density gradient centrifugation using CsCl. The purified polioviral particles were extracted with phenol chloroform to isolate viral RNA. The RNA genomes of poliovirus isolates were compared by the electrophoretic profile of the oligonucleotides produced by cleavage of RNA molecule with the site specific ribonuclease T1. Complete digestion of viral RNA with ribonuclease T1 produced oligonucleotides resistant to further digestion with the same enzyme. The digest (oligonucleotides) were end labeled with a radio-isotope [^{32}P] and resolved by two dimensional polyacrylamide gel electrophoresis which employed 8 per cent polyacrylamide gel buffered at pH 3.3 in the first dimension and 22 per cent polyacrylamide gel buffered at pH 8.0 during the second dimension. The oligonucleotides separated into a pattern (fingerprint/map) highly specific for a given RNA sequence. The large, unique oligonucleotides attained a characteristic position relative to others within the fingerprint which represented many independent points of reference for comparison.

Oligonucleotide fingerprint patterns of the twenty six poliovirus type 1 strains isolated during the year 1986 from three wards S, R and M of the Bombay city were analysed. Equivalent migration of the diagnostic oligonucleotides in the fingerprint patterns revealed genomic relationship among the poliovirus strains. Poliovirus strains related on the basis of the fingerprint similarity were grouped together to represent the genotypes. Poliovirus type 1 isolates of more than one genotype were observed. A

predominating genotype and the other minor genotypes existed in each ward. Fingerprint relationship was also observed among the predominating genotypes of the three wards. These strains constituted one of the major genotype circulating in Bombay. At least two genotypes circulating during the period of study were defined. Poliovirus strains of the same genotype were identified even if they were isolated from different geographic locations. Circulation of a genotype within the community for at least a period of six months was demonstrated. From the fingerprint correlation, it was also possible to determine the order of infection and migration of a genotype from one place to another. Oligonucleotide fingerprinting analysis thus provided valuable information on the relationship among the poliovirus strains circulating in the population during the period of study. The catalogue of oligonucleotide fingerprints presented in this thesis will serve for the comparison of the genetic characteristics of the strains isolated during the successive years and form the baseline data for the future studies.

The selected poliovirus strains were also analysed by electrophoretic separation pattern of the [^{35}S] methionine labeled viral proteins. The SDS-PAGE patterns of the strains were in good agreement with the oligonucleotide maps in establishing poliovirus strain relatedness. However, strain relatedness was more accurately recognized with the oligonucleotide fingerprinting analysis. It is suggested that the SDS-PAGE may be of value as a preliminary screening method to select isolates for the genomic studies when large number of isolates are available.

APPENDIX.

I. Composition of Media and Other Reagents Used in Cell and Virus Culture.

- i. Minimum Essential Medium (HI MEDIA, AT 047)
(With Earle's Salts, Non essential amino acids, L- Glutamine).

The contents of 'one unit' (10 g) of powdered medium were dissolved in 10 l. of Milli Q purified water. The solution was sterilized by filtration through 0.22 μ membrane Millipore filter and distributed into 1 litre volumes in tightly capped bottles. Stored at 4°C.

- ii. L Glutamine (3%)

L Glutamine	3g
H ₂ O	100 ml.

Sterilized by filtration through 0.22 μ membrane filter; dispensed in 5 ml volumes and stored under sterile conditions at -20°C.

- iii. Sodium bicarbonate (7.5%)

Sodium bicarbonate	7.5 g
H ₂ O	100 ml.

Sterilized by filtration; distributed in 30 ml volumes and stored at 4°C.

- iv. Penicillin and Streptomycin

1 x 10⁶ units of crystalline Penicillin G and 1.0 g Streptomycin sulphate were dissolved in 100 ml PBS; distributed into 1 ml volumes and stored at -20°C.

- v. Kanamycin

0.5 g Kanamycin was dissolved in 25 ml of sterile PBS; distributed into ml volumes and stored at - 20°C.

- vi. Phosphate buffered saline (PBS)
(without Calcium and Magnesium ions)

Sodium chloride	8.0 g
Potassium chloride	0.20 g
Disodium hydrogen phosphate 12H ₂ O	2.89 g
Potassium dihydrogen orthophosphate	0.20 g
H ₂ O	1.0 litre

Sterilized by autoclaving at 15 lbs pressure for 15 min. stored at 4°C.

NOTE : Milli Q purified (> 18 M Ω) water was used throughout this work.

vii. Cell culture Growth and Maintenance medium

Ingredient	Medium	
	Growth	Maintenance
1. Eagle's Minimum Essential Medium	95 ml	95 ml
2. L Glutamine (3%)	1 ml	1 ml
3. Sodium bicarbonate (7.5%)	3 ml	3 ml
4. Penicillin-Streptomycin solution	0.1 ml	0.1 ml
5. Kanamycin	0.1 ml	0.1 ml
6. Serum*	5 ml	2 ml

- * 5 ml of foetal bovine serum (GIBCO) for HEp-2 cell culture and;
10 ml of sheep serum for HeLa cell culture growth medium.

viii. Trypsin solution (TPVG)

1. Trypsin solution

1 g of Trypsin (Difco 1:250) was dissolved in 400 ml of PBS by agitation with a magnetic stirrer for 30 min at 36°C; sterilized by filtration through 0.22 µ membrane; dispensed into 25 ml volumes and stored at -20°C.

2. D Glucose (10%)

D Glucose	5 g
H ₂ O	50 ml

Sterilized by filtration and stored at 4°C.

3. Working solution (TPVG)

Trypsin solution	25 ml
PBS	420 ml
EDTA (0.2 M)*	50 ml
Glucose (10%)	2.5 ml
Penicillin/Streptomycin	1.0 ml

pH adjusted to 7.5 using sterile 1 N NaOH; dispensed in 30 ml volumes and stored at 4°C.

- * Composition of 0.2 M EDTA is given in Section IV.

II. Preparation of methionine less medium for radiolabelling the viral proteins.

Hanks' Basic Salt Solution (Hanks' BSS)
Solution A (Stock)

NaCl	40.00 g
KCl	2.00 g
MgSO ₄ 7H ₂ O	0.50 g
MgCl ₂ 6H ₂ O	0.50 g
H ₂ O	to 200 ml

0.70g of CaCl₂ was dissolved separately in 30 ml H₂O; mixed with solution A and volume made to 250 ml.

Solution B (Stock)	
Na ₂ HPO ₄ 12H ₂ O	0.76 g
KH ₂ PO ₄	0.30 g
Dextrose	5.60 g
H ₂ O	to 200 ml

Working solution	
Solution A	50 ml
Solution B	50 ml
H ₂ O	870 ml
0.4% Phenol red	2 ml

Sterilized by autoclaving and stored at 4°C.

Stock solution 1 (x100) : Following L-aminoacids were dissolved in 100 ml of Hanks' BSS by heating to 80°C.

Arginine	1.05 g
Histidine	0.24 g
Lysine	0.58 g
Leucine	0.52 g
soleucine	0.52 g
Phenylalanine	0.32 g
Threonine	0.48 g
Tryptophan	0.1 g
Valine	0.46 g

Sterilized by filtration and stored at 4°C.

Stock solution 2 (x100) : Following L-amino acids were dissolved in 100 ml of 0.1 N HCl

Tyrosine	0.36 g
Cystine	0.24 g

Sterilized by filtration and stored at 4°C.

Stock solution 3 (x1000) : Following vitamins were dissolved in 100 ml of Hanks' BSS.

Choline	0.1 g
Nicotinic acid	0.1 g
Pantothenic acid	0.1 g
Pyridoxal phosphate	0.1 g
Riboflavin	0.01 g
Thiamin	0.1 g
Inositol	0.2 g

Sterilized by filtration and stored at 4°C.

Stock solution 4 (x100) : 0.01 g of folic acid was dissolved in 100 ml of Hanks' BSS by addition of few drops of 0.5 N NaOH. Sterilized by filtration and stored at 4°C.

Methionine less Growth Medium	
Hanks' BSS	92 ml
Stock solution 1	1 ml
Stock solution 2	1 ml
Stock solution 3	0.1 ml
Stock solution 4	1 ml
D Glucose (10%)	1 ml
Sodium bicarbonate (7.5%)	3 ml
Glutamine (3%)	1 ml
Penicillin and Streptomycin	0.1 ml
Kanamycin	0.1 ml
dialysed foetal bovine serum	2 ml

III. Reagents Used In Poliovirus Purification And Oligonucleotide Fingerprinting.

- i. Polyethylene glycol (40% w/v)

Polyethylene glycol 6000	40.0 g
H ₂ O	to 100 ml

 Sterilized by autoclaving; stored at room temperature.
- ii. Sodium chloride (23% w/v)

Sodium chloride	23.0 g
H ₂ O	to 100 ml

 Sterilized by autoclaving; stored at 4°C

- iii. Sucrose solutions (15% w/v and 30% w/v)
 Sucrose powder was dissolved in TSE buffer (pH 8.0) to obtain solutions of Refractive Index of 1.355 g/cm^3 (15% W/V) and 1.381 g/cm^3 (30%) w/v. Solutions sterilized by filtration and stored at 4°C .
 * Composition of TSE buffer (pH 8.0) is given in Section IV.
- iv. CsCl solutions (10% w/v and 45% w/v)
 CsCl powder was dissolved in TSE buffer (pH 8.0) to obtain solutions of Refractive index 1.3405 g/cm^3 (10% w/v) and 1.3807 g/cm^3 (45% w/v). Solutions sterilized by filtration and stored at 4°C .
- v. Phenol
 Phenol was allowed to warm to room temperature and then melted at 60°C . Liquified phenol was distilled and the fraction that eluted at 170°C was collected. 8-hydroxyquinolin was added (final conc. 0.1%) and phenol was stored in brown bottles in dark at -20°C .
- vi. Lithium chloride (8M)
 Lithium chloride (anhydrous) 19.96 g
 H₂O to 50 ml
 Sterilized by filtration; distributed into 50 ul aliquots; stored at -40°C .
- vii. Ammonium acetate (0.6 M)
 Ammonium acetate 4.624 g
 Diethylpyrocarbonate (DEPC)
 treated sterile water 100ml
 Sterilized by filtration, distributed into 100 ul aliquots and stored at -40°C .

For Diethylpyrocarbonate treatment, 1 ml of DEPC (Sigma) was added to 1.0 l of Milli Q purified water. Solution was stirred overnight at room temperature. On the following day, DEPC treated water was autoclaved at 15 lbs pressure for 15 min.

IV. Buffers

Stock solutions

- i. 1 M Tris HCl pH 7.8 (5°C)
 Tris base 1.34 g
 Tris HCl 14.04 g
 H₂O to 100 ml
 Sterilized by autoclaving and stored at 4°C .

- ii. 1 M Tris HCl pH 8.0 (5°C)
Tris base 12.70 g
Tris HCl 2.36 g
H₂O to 100 ml
Sterilized by autoclaving and stored at 4°C.
- iii. 1 M Tris HCl pH 8.9 (5°C)
Tris base 10.64 g
Tris HCl 1.92 g
H₂O to 100 ml
Sterilized by autoclaving and stored at 4°C.
- iv. 40 mM Tris HCl pH 8.0
Tris base 0.20 g
Tris HCl 0.35 g
DEPC treated H₂O 100 ml
Sterilized by autoclaving. Distributed into 100 ul aliquots and stored at - 40°C.
- v. 1 M NaCl
NaCl 5.844 g
H₂O to 100 ml
Sterilized by autoclaving and stored at 4°C.
- vi. 0.2 M EDTA,
EDTA 7.444 g
Dissolved in 80 ml of water while stirring continuously on a magnetic stirrer and pH adjusted to 8.0 using NaOH. Volume was made to 100 ml. Sterilized by autoclaving at 15 lbs pressure for 15 min.
- vii. 100 mM K₂HPO₄
K₂HPO₄ 1.741 g
DEPC treated H₂O 100 ml
Sterilized by autoclaving and stored at 4°C.
- viii. 100 mM K₃PO₄ (Potassium phosphate, Tribasic)
K₃PO₄ 2.123 g
DEPC treated H₂O 100 ml
Sterilized by autoclaving and stored at 4°C.

- ix. 100 mM Magnesium acetate, tetrahydrate.
 Magnesium acetate $4\text{H}_2\text{O}$ 2.144 g
 DEPC treated water 100 ml
 Sterilized by filtration and distributed into 100 μl aliquots, stored at -40°C .

- x. 100 mM Dithiothreitol (DTT)
 Dithiothreitol 0.309 g
 0.01 M Sodium acetate (pH 5.2) 20 ml
 Sterilized by filtration, dispensed in 50 μl aliquots and stored at -40°C .

For 0.01 M sodium acetate, pH 5.2

Sodium acetate 0.136 g
 dissolved in 80 ml of DEPC treated water, pH adjusted to 5.2 with glacial acetic acid and volume made to 100 ml. Sterilized by autoclaving.

Working solutions -

- i. TS buffer pH 7.8 (50 mM Tris HCl pH 7.8, 50 mM NaCl)
 1 M Tris HCl pH 7.8 5 ml
 1 M NaCl 5 ml
 Autoclaved water to 100 ml
 Stored at 4°C .
- ii. TSE buffer pH 8.0 (10 mM tris HCl pH 8.0, 50 mM NaCl, 1 mM EDTA)
 1 M Tris HCl pH 8.0 1 ml
 1 M NaCl 5 ml
 0.2 M EDTA 0.5 ml
 Autoclaved H_2O to 100 ml
 Stored at 4°C .
- iii. TE buffer pH 8.9 (100 mM tris HCl pH 8.9, 10 mM EDTA)
 1 M Tris HCl pH 8.9 10 ml
 0.2 M EDTA 5 ml
 Autoclaved H_2O to 100 ml
 Stored at 4°C .

- iv. PNK buffer (10 mM K_2HPO_4 - K_3PO_4 (pH 9.5),
 10 mM $\text{Mg}(\text{oAc})_2$, 5 mM DTT)
- | | |
|--|--------|
| 100 mM K_2HPO_4 - K_3PO_4 pH 9.5 | 100 ul |
| 100 mM Magnesium acetate | 100 ul |
| 100 mM Dithiothreitol | 50 ul |
| DEPC treated H_2O | 750 ul |
- Prepared freshly.

V. Electrophoresis Reagents

Two dimensional polyacrylamide gel electrophoresis.

Chemical purities of the electrophoresis reagents were especially critical to the production of high quality fingerprints. All the stock solutions were prepared using molecular biology, ultrapure grade reagents obtained from Sigma Chemical Co., USA.

1. First dimensional electrophoresis

i. Acrylamide monomer solution (40:0.5)

Acrylamide	40 g
N,N' Methylene - bis - acrylamide	0.5 g
H_2O	to 100 ml

Purified by stirring with Amberlite MB1 monobed resin for 12 h at room temperature followed by filtration through 0.22 um millipore filter
 Stored in the dark at 4°C.

ii. 9 M Urea

Urea	270.27 g
H_2O	to 500 ml

Stirred continuously with Amberlite MB1 monobed resin(20 g/l) for 12.0 h at room temperature and filtered through a bed of silicic acid. Bed of silicic acid was prepared by suspending 20 g of powdered silicic acid in 200 ml of deionized H_2O , and filtering the suspension under suction through a Whatman no. 1 paper .
 Urea solution was stored at 4°C.

iii. $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.16% w/v)

$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	0.16 g
H_2O	100 ml

Stored in 1 ml aliquots at -20°C.

- iv. Ascorbic acid (1.0% w/v)
 Ascorbic acid 0.25 g
 H₂O 25 ml
 Stored in 3 ml aliquots at -20°C.
- v. H₂O₂ (30%). Distributed into 1 ml aliquots and stored at 4°C.
- vi. 25 mM Citric acid pH 3.3 (Tank buffer)
 Citric acid 15.76 g
 H₂O 3 litre.
 pH adjusted to 3.3 using 10 N NaOH
- vii. 10 N NaOH -
 NaOH pellets 20 g
 H₂O to 50 ml
- viii. Sample buffer (9M Urea, 50% glycerol, 0.25% bromophenol blue, 0.25% Xylene Cyanol FF).
 Urea 5.4 g
 H₂O 2.5 ml
 Glycerol 5 ml
 Bromophenol blue 25 mg
 Xylene cyanol FF 25 mg
 Heat the solution to dissolve the urea.
 Store at 4°C Warm before use.

2. Second dimensional electrophoresis

- i. Acrylamide monomer solution (23.2 : 1.55)
 Acrylamide 232 g
 N,N' Methylene-bis-acrylamide 15.5 g
 H₂O to 1.0 litre
 Purified by stirring with Amberlite MB1 monobed resin (20 g/l) for 12 h at room temperature followed by filtration through 0.22 um millipore filter. Stored in dark at 4°C.
- ii. 1 M Tris borate (pH 8.2)
 Tris base 121 g
 Boric acid 63 g
 H₂O to 1 litre
 Filtered and stored at room temperature.

- iii. 50 mM Tris borate (pH 8.2)
 Reconstituted from 1 M Tris borate stock solution.
 1 M Tris borate 250 ml
 H₂O to 5 litre

- iv. Ammonium persulfate (10% w/v) (prepared fresh)
 Ammonium persulfate 0.1 g
 H₂O 1 ml

3. SDS PAGE

- i. Acrylamide monomer solution
 Acrylamide 14.6 g
 N,N' Methylene-bis-acrylamide 0.4 g
 H₂O to 50 ml
 Filtered and stored at 4°C in dark.
- ii. 4 x running gel buffer. (1.5 M Tris-HCl pH 8.8)
 Tris base 36.3 g
 pH adjusted to 8.8 with Conc. HCl
 H₂O to 200 ml
 Stored at 4°C .
- iii. 4 x stacking gel buffer. (0.5 M Tris-HCl pH 6.8)
 Tris base 3.0 g
 pH adjusted to 6.8 with HCl
 H₂O 50 ml
 Stored at 4°C .
- iv. SDS (10% w/v)
 SDS 1 g
 H₂O 10 ml
 Stored at 4°C .
- v. 2 x Treatment (lysis) buffer (0.125 M Tris-Cl pH 6.8, 4% SDS,
 20% glycerol, 10% 2 mercaptoethanol)
 4 X stacking gel buffer 2.5 ml
 SDS (10% w/v) 4.0 ml
 Glycerol 2.0 ml
 2 mercaptoethanol 1.0 ml
 H₂O to 10 ml

- vi. Tank buffer(0.025 M Tris, 0.192 M glycine, 0.1% SDS)

Tris	12 g
Glycine	57.6 g
SDS	4.0 g
H ₂ O	to 4.0 litre

- vii. Fixing solution (50% methanol, 12% acetic acid, 0.1% formalin)

Methanol	50 ml
Acetic acid	12 ml
Formalin	0.1 ml
H ₂ O	to 100 ml

VI Developer (Agfa - Gevaert, T 230)

Contents of the packets (940 g) were dissolved in 9.0 l of H₂O by stirring continuously. Stored in dark at room temperature.

VII. Hardening Fixer (Agfa - Gevaert T 201 P)

The powdered contents of the packet were dissolved in 9.0 l of H₂O. Allowed to stir for 30 min. Stored in dark at room temperature.

VIII. Enzymes

- i. Ribonuclease T1 (Pharmacia, Sequence grade, no : 27-0991)
(200 U/Vial)

The contents of the vial was dissolved in 20 ul of sterile water and stored at - 20°C.

Working solution : Stock solution diluted four fold in 40 mM Tris HCl pH 8.0. Used 1 ul per reaction.

- ii. Phosphatase, Alkaline (E coli) (Pharmacia, no. 27-0598) (210 U/ml)
10 ul of E coli Alkaline phosphatase suspension was centrifuged in a microfuge for 5 min. at 4°C. Pellet was resuspended in 10 ul of 40 mM Tris HCl (pH 8.0).

For working solution, the stock solution was diluted 200 fold in 40 mM Tris HCl (pH 8.0) and stored at 4°C, 1 ul of working solution (2×10^{-3} U) was used per reaction.

- iii. Polynucleotide kinase (from phage T4 infected E coli) (Boehringer Mannheim, no. 174645).
(200 U/vial)
Dispensed into 2 ul aliquots. Used 0.5 ul per reaction.
- iv. Pronase E (Protease; Type XXV; SIGMA; no P6911)
Powdered pronase was dissolved in 10 mM Tris HCl (pH 7.5), 10 mM NaCl to a final concentration of 20 mg/ml and self digested for 1.0 h at 37°C.
Dispensed in 25 ul aliquots and stored at -20°C.
Used at 1mg/ml concentration in the reaction.
- v. Yeast t RNA (SIGMA)

100 A₂₆₀ Units of t RNA was dissolved in 1 ml of sterile water;
dispensed in 25 ul volumes and stored at -40°C.
(2.5 U = 100 ug t RNA).

REFERENCES

1. Melnick JL. The Picornaviruses. Portraits of viruses. A history of virology. Fenner F, Gibbs A eds. Basel, Karger. 1988 : 147-88.
2. Heine J. Observations on paralytic conditions of the lower extremities and their treatment. Stuttgart, Kohler, 1840.
3. Charcot JM, Jaffroy A. Case of infantile spinal paralysis with lesions of the anterior horns of the gray substance of spinal cord. Arch de Physiol Norm et Path 1870; 3 : 134.
4. Medin O. On an epidemic of spinal infantile paralysis. Verhandl Proc d X Internat med Kongr 1891; 2 : 37.
5. Caverly CS. Preliminary report of an epidemic of paralytic disease, occurring in Vermont, in the summer of 1894. Yale MJ 1894; 1 : 1.
6. Taylor JM. An epidemic of poliomyelitis. Philadelphia MJ 1898; 1 : 208.
7. Chantemesse A. Ascending paralysis; investigation of an epidemic in the insane asylum of Sainte Gemmes. d. trav Comite consult d. hyg Pub de France 1899; 28 : 195.
8. Bulow - Hansen, Harbitz F. Contribution to the study of acute poliomyelitis. Norsk Mag f Laegevidensk 1898; 59 : 1170.
9. Flexner S. The mode of infection in epidemic poliomyelitis. JAMA 1912; 59 : 1371.
10. Landsteiner K, Popper E. Transmission of acute poliomyelitis to monkeys. Und Exper Therap (Orig.) 1909; 2 : 377-90.
11. Flexner S, Lewis PA. Experimental epidemic poliomyelitis in monkeys. Ascending nasal infection; characteristic alterations of the cerebrospinal fluid and its early infectivity; infection from human mesenteric lymph node. JAMA 1910; 54 : 1140-57.
12. Lavinder CH, Freeman AW, Frost WH. Epidemiologic studies of poliomyelitis in New York City and northeastern United States during the year 1916. Public Health Bull 1918; 91 : 1-309.
13. Sabin AB, Ward R. The natural history of human poliomyelitis. I. Distribution of virus in nervous and non nervous tissues. J Exp Med 1941; 73 : 771-93.
14. Bodian D, Horstmann DM. Polioviruses. Viral and Rickettsial Infections of Man. 4th ed. Lippincott, Philadelphia : FL Horsfall, I Tamm eds 1965; 430-73.

15. Hashimoto I, Hagiwara A, Komatsu T. Possible sites for poliovirus replication. Acta Neuropathol 1984; 64 : 53-60.
16. Horstmann DM. Clinical aspects of acute poliomyelitis. Am J Med 1949; 5 : 592-605.
17. Gilliam AG, Hemphill FM, Greende JH. Poliomyelitis incidence and epidemic recurrence. Pub Health Rep 1949; 64 : 1575-95.
18. Sabin AB. Transmission of poliomyelitis virus. Analysis of differing interpretations and concepts. Practical implications. Chicago M Soc Bull 1951; 54 : 163-71.
19. Melnick JL. Studies on the serological epidemiology of poliomyelitis as an index of immunity in certain Carribbean islands, British Guiana and Equador. WI Med J 1959; 8 : 275-98.
20. Sabin AB. Epidemiologic patterns of poliomyelitis in different parts of the world. Int Polio Conf 1949; 1 : 3 - 28.
21. Flexner S, Amoss HL. An immunizing strain of virus of poliomyelitis. J Exp Med 1924; 3 : 625-32.
22. Aycock WL, Kagan JR. Experimental immunization in poliomyelitis. J Immunol 1927; 14 : 85-9.
23. Brodie M, Park WH. Active immunization against poliomyelitis. Am J Public Health 1936; 26 : 119-25.
24. Leake JP. Poliomyelitis following vaccination against this disease. JAMA 1935; 105 : 2152-60.
25. Kolmer JA. Vaccination against acute anterior poliomyelitis. Am J Public Health 1936; 26 : 126-30.
26. Lennette EH, Gorden FB. Immunological differences between the MV and the Philadelphia - 1932 strains of poliomyelitis virus. J Bact 1940; 39 : 64-5.
27. Kessel JF, Moore FJ, Pait CF. Differences among strains of poliomyelitis virus in *Macaca mulatta*. Am J Hyg 1946; 42 : 82-9.
28. Committee on Typing of the National Foundation for Infantile paralysis : Immunologic classification of poliomyelitis viruses. Discussion of results and general summary. Am J Hyg 1951; 54 : 191-274.
29. Enders JF, Weller TH, Robbins FC. Cultivation of the Lansing Strain of poliomyelitis in cultures of various human embryonic tissues. Science 1949; 109 : 85-7.

30. Jenner E. Further observations on the variole vaccine or cow pox, variole London. Reprinted in : Camac CNB ed Classics of medicine and surgery, New York, Dover 1959; 241-76.
31. Theiler M, Smith HH. The effect of prolonged cultivation in vitro upon the pathogenicity of yellow fever virus. J Exp Med 1939; 65 : 767-87.
32. Theiler M. Studies on poliomyelitis. Medicine 1941; 20 : 443-62.
33. Bodian D, Morgan IM, Schwerdt CE. Virus and host factors influencing the titer of Lansing poliomyelitis virus in monkeys, cotton rats and mice. Am J Hyg 1950; 51 : 126-33.
34. Melnick JL. Differences in the degree of infectiousness of two related strains of poliomyelitis virus following their oral administration in monkeys. J Immunol 1951; 67 : 219-24.
35. Koprowski H, Jervis GA, Norton TW. Immune responses in human volunteers upon oral administration of rodent adapted strain of poliomyelitis virus. Am J Hyg 1952; 55 : 108.
36. Cabasso VJ. Poliomyelitis : Propagation of MEF 1 strain of poliomyelitis virus in developing chick embryo by allantoic cavity inoculation. Proc Soc Exp Biol Med 1952; 81 : 525-31.
37. Enders JF, Weller TH, Robbins FC. Alteration in pathogenicity for monkeys of Brunhilde strain of poliomyelitis virus following cultivation in human tissue. Fed Proc 1952; 11 : 467.
38. Sabin AB. Present status and future possibilities of a vaccine for the control of poliomyelitis. Am J Dis Child 1953; 86 : 301-10.
39. Sabin AB, Winser J, Hennessen WA. Differences in pathogenic spectrum of poliomyelitis viruses propagated in different hosts and tissues. Int Cong Micro 1953; 3 : 156-9.
40. Sabin AB, Hennessen WA, Winser J. Studies on variants of poliomyelitis virus : I Experimental segregation and properties of avirulent variants of three immunologic types. J Exp Med 1954; 99 : 551-76.
41. Sabin AB. Avirulent viruses for immunization against poliomyelitis. In : Poliomyelitis - papers and discussion presented at the Third International Poliomyelitis Conference Rome 1954. Philadelphia : Lippincott 1955 : 186-94.

42. Jungherr EL, Cabasso VJ, Moyer AW, Cox HR. Proposed method and evaluation of the monkey neurovirulence test for attenuated poliovirus vaccine. J INFECT DIS 1961; 108 : 247-61.
43. Sabin AB, Boulger LR. History of Sabin attenuated poliovirus oral live vaccine strains. J Biol Stand 1973; 1 : 115-8.
44. Dick GWA, Schwerdt CE, Huber W, Sharpless GR, Howe HA. Immunization of cotton rats with inactivated Lansing poliomyelitis virus - II. Inactivation by physical methods. Am J Hyg 1951; 53 : 131-8.
45. Milzer A, Oppenheimer F, Levinson SO. A new method for the production of potent inactivated vaccines with ultraviolet irradiation. III. A completely inactivated poliomyelitis vaccine with Lansing strain in mice. J Immunol 1945; 50 : 331-40.
46. Schwerdt CE, Dick GWA, Herriott RM, Howe HA. Immunization of cotton rats with inactivated Lansing poliomyelitis virus I. Inactivation by chemical agents. Am J Hyg 1951; 58 : 121-30.
47. Salk JE, Krech U, Youngner JS, Bennet BL, Lewis LJ, Bezeley PL. Formaldehyde treatment and safety testing of experimental poliomyelitis vaccines. Am J Public Health 1954; 44 : 563-70.
48. Francis T Jr, Korns RF, Voight RB, Boisen M, Hemphill FM, Napier JA, Tolchinsky E. An evaluation of 1954 poliomyelitis vaccine trials : Summary report. Am J Public Health 1955; 45 (Suppl) : 1-66.
49. Salk JE. Poliomyelitis vaccine in the fall of 1955. Am J Public Health 1956; 46 : 1-14.
50. Salk JE. Vaccination against poliomyelitis. An ounce of prevention. Roy Soc Health J 1959; 79 : 310-23.
51. Nathanson N, Langmuir AD. The cutter incident : Poliomyelitis following formaldehyde - inactivated poliovirus vaccination in the United States during the spring of 1955. II. Relationship of poliomyelitis to Cutter vaccine. Am J Hyg 1963; 78 : 29-60.
52. Devis DC, Lipson MJ, Carver DH, Melnick JL, Robbins FC. The degree and duration of poliomyelitis virus excretion among vaccinated contacts of clinical cases of poliomyelitis. Pediatrics 1958; 22 : 33-9.
53. Fox JP, Gelfand HM, LeBlanc DR, Rowan DF. The influence of natural and artificially induced immunity on alimentary infections with poliovirus. Am J Public Health 1958; 48 : 1181-92.

54. Lepow M, Robbins FC, Woods WA. Influence of vaccination with formalin inactivated vaccine upon gastrointestinal infection with polioviruses. Am J Public Health 1960; 50 : 531-42.
55. Merrill RE, Batson R, Kinsmon D. Vaccination status of patients in an outbreak of acute poliomyelitis. Am J Dis Child 1960; 100 : 857-60.
56. Sabin AB. Present status of attenuated live virus poliomyelitis vaccine. J Am Med Assn 1956; 162 : 1589-96.
57. Smorodintsev AA, Drobyshevskaya AI, Ilyenko VI, Klyuchareva TE, Chalkina OM. Experimental and epidemiological data on the effectiveness of live poliomyelitis vaccine. Part I. Experience in the production, biological control, and use of Live Poliomyelitis Vaccine made from Sabin strains. First International Conference on Live Poliovirus Vaccines. Scientific publication. Washington D.C. Pan American Sanitary Bureau 1959; 44 : 305-12.
58. Chumakov MP, Voroshilova MK, Drozdov SG, Dzagurov SG, Lashkevich VA, Mironova LL, et al. On the course of mass immunization of the population in the Soviet Union with live poliovirus vaccine from Albert B. Sabin's strains. Poliomyelitis papers and discussion presented at the Fifth International Poliomyelitis Conference. Copenhagen July 26-28, 1960. Philadelphia Lippincott 1961 : 228-39.
59. Sabin AB. Oral poliovirus vaccine recent results and recommendations for optimum use. Roy Soc Health J 1962; 82 : 51-8.
60. Robertson HE, Acker MS, Dillenberg HO, Woodrow R, Wilson RJ, Ing WK, MacLeod DRE. Community wide use of a 'balanced' trivalent oral poliovirus vaccine (Sabin) : a report of the 1961 trial at Prince Albert, Saskatchewan. Can J Public Health 1962; 53 : 179-91.
61. Koprowski H, Norton TW, Jervis GA, Nelson TL. Clinical investigations of attenuated strains of poliomyelitis virus. Use as a method of immunization of children with living virus. JAMA 1956; 160 : 954-66.
62. Cox HR, Cabasso VJ, Markham FS, Moses MJ, Moyer AW, Roca-Garcia M, Ruegsegger JM. Immunological response to trivalent oral poliomyelitis vaccine. Br Med J 1959; 2 : 591-7.
63. Expert Committee on poliomyelitis second report (Geneva 1957). WHO Tech Rep Ser 1958; 145 : 1-83.
64. Steigman AJ, Lipton MM. Poliovirus neutralizing antibody in faeces of poliomyelitic children. Lancet 1959; 2 : 272-3.

65. Ogra PL, Karzon DT, Righthand F, MacGillivray M. Immunoglobulin response in serum and secretions after immunization with live and inactivated poliovaccine and natural infections. N Engl J Med 1968; 279 : 893-900.
66. Ogra PL, Karzon DT. Formation and function of poliovirus antibody in different tissues. Prog Med Virol 1971; 13 : 156-93.
67. Ogra PL. Mucosal immune response to poliovirus vaccines in childhood. Rev Infect Dis 1984; 6 (Suppl) S361-8.
68. Salk J, Salk D. Control of influenza and poliomyelitis with killed virus vaccines. Science 1977; 195 : 834-47.
69. Salk D, van Wezel AL, Salk J. Induction of long term immunity to paralytic poliomyelitis by use of non-infectious vaccine. Lancet 1984; ii : 1317-21.
70. van Wezel AL, van Steenis G, Hannik CA, Cohen H. New approach to the production of concentrated and purified inactivated polio and rabies tissue culture vaccines. Dev Biol Stand 1978; 41 : 159-168.
71. Faden H, Modlin JF, Thomas ML, McBean AM, Ferdon MB, Ogra PL. Comparative evaluation of immunization with live attenuated and enhanced - potency inactivated trivalent poliovirus vaccines in childhood : Systemic and local immune responses. J Infect Dis 1990; 162 : 1291-7.
72. Sabin AB, Michaels RH, Ziring P, Krugman S, Warren J. Effect of oral poliovirus vaccine in newborn children. II. Intestinal resistance and antibody response at 6 months in children fed type 1 vaccine at birth. Pediatrics 1963; 31 : 641-50.
73. Fox JP. Modes of action of poliovirus vaccines and relation to resulting immunity. Rev Infect Dis 1984; 6 (Suppl) : S352-5.
74. Cockburn WC, Drozdov SG. Poliomyelitis in the world. Bull WHO 1970; 42 : 405-7.
75. Schonberger LB, Kaplan J, Kim-Farley R, Moore M, Eddins DL, Hatch M. Control of paralytic poliomyelitis in United States. Rev Infect Dis 1984; 6 (Suppl) : S424-6.
76. Nkowane BM, Wassilak GF, Orenstein WA, Bart KJ, Schonberger LB, Hinman AR, and Kew OM. Vaccine associated paralytic poliomyelitis in United States : 1973 through 1984. JAMA 1987; 257 : 1335-40.
77. Kim Farley RJ, Bart KJ, Schonberger LB, Orenstein WA, Nkowane BN, Hinman AR, et al. Poliomyelitis in the USA : Virtual elimination of the disease caused by wild virus. Lancet 1984 : 1315-16.

78. Varughese PV, Carter AO, Acres SE, Furesz J. Eradication of indigenous poliomyelitis in Canada : impact of immunization strategies. Can J Public Health 1989; 80 : 363-8. 9
79. Robertson SE, Chan C, Kim-Farley R, Ward N. Worldwide status of poliomyelitis in 1986, 1987 and 1988 and plans for its global eradication by the year 2000. Wld Hlth Statist Quart 1990; 43 : 80-90. 3
80. Lepinlemu K, Stenvik M. Experiences with polio vaccination and herd immunity in Finland. Dev Biol Std 1981; 47 : 241-6.
81. Oostvogel PM, van Wijngaarden JK, vander Avoort HGAM, Mulders MN, Conynvan Spaendonck MAE, Rumke HC, et al. Poliomyelitis outbreak in an unvaccinated community in the Netherlands 1992-93. Lancet 1994; 344 : 665-70.
82. Nicholas DD, Kratzer JH, Ofosu-Amaah S, Belcher DW. Is poliomyelitis a serious problem in developing countries - The Danfa experience. Br Med J 1977; 1 : 1009-12.
83. Ofosu-Amaah S, Kratzer JH, Nicholas DD. Is poliomyelitis a serious problem in developing countries - lameness in Ghanaian schools. Br Med J 1977; 1 : 1012-4.
84. Bernier RH. Some observations on Poliomyelitis Lameness Surveys. Rev Infect Dis 1984; 6 (Suppl) : S371-75.
85. WHO : Expanded Programme on Immunisation. Resolution WHA 27.57, 1974.
86. Control of poliomyelitis : A continuing paradox [Presidents pages]. J Infect Dis 1982; 146 : 540-51.
87. Sabin AB, Silva E. Residual paralytic poliomyelitis in a tropical region of Brazil, 1969-1977. Prevalence surveys in different age groups as indicators of changing incidence. Am J Epidemiol 1983; 117 : 193-200.
88. World Health Organization. Expanded Programme on Immunization. Survey for residual poliomyelitis paralysis. Wkly Epidemiol Rec 1977; 52 : 269-71.
89. Poliomyelitis in 1984. Wkly Epidemiol Rec 1986; 61 : 229-36.
90. Sabin AB, Ramos - Alvarez M, Alvarez-Amezquita J, Pelon W, Michales RH, Spigland I, et al. Live orally given poliovirus vaccine : effects of rapid mass immunization on population under conditions of massive enteric infection with other viruses. JAMA 1960; 173 : 1521-6.

91. Cruz RR. Cuba : mass polio vaccination program, 1962-1982. Rev Infect Dis 1984; 6 (Suppl) : S408-12.
92. Risi JB Jr. The control of poliomyelitis in Brazil. Rev Infect Dis 1984; 6 (Suppl) : S400-3.
93. Goal for 1990 : eradication of poliomyelitis in the Americas. Wkly Epidemiol Rec 1985; 60 : 394-5.
94. Patriarca PA, Palmeira G, Lima Filho J, Cordeiro MT, Laender F, Couto Oliveira MJ, et al. Randomized trial of alternative formulation of OPV in Brazil. Lancet 1988; 1 : 429-33.
95. Hanlon P, Hanlon L, Marsh V, Bypass P, Sillah H, Hayes R, et al. Serological comparisons of approaches to poliovaccination in The Gambia. Lancet 1987; 1 : 800-1.
96. Schoub BD, Johnson S, McAnerney JM, Kustner HGV, vander Merwe LA. A comprehensive investigation of immunity to poliomyelitis in a developing country. Am J Epidemiol 1986; 123 : 316-24.
97. Oduntan S, Lucas AO, Wenner EM. The immunological response of Nigerian infants to attenuated and inactivated poliovaccines. Ann Trop Med Parasitol 1978; 2 : 111-5.
98. Deshpande JM, Kamat JR, Rao VK, Nadkarni SS, Kher AS, Salgaokar SD, Rodrigues JJ. Prevalence of antibodies to polioviruses and enteroviruses excreted by healthy children in Bombay. Indian J Med Res 1995; 101 : 50-4. 20 / 5
99. Patriarca PA, Weight PF, John TJ. Factors affecting the immunogenicity of oral poliovirus vaccine in developing countries : Review. Rev Infect Dis 1991; 13 : 926-39.
100. Sabin AB. Oral poliovirus vaccine : History of its development and use and current challenge to eliminate poliomyelitis from the world. J Infect Dis 1985; 151 : 420-36.
101. Pandit CG, Ramlingaswami V. Memorandum on poliomyelitis. ICMR publication, 1955.
102. Gharpure PV, Dave KH. Poliovirus vaccine, live, oral (Sabin); first field trial in India. Ind J Med Sci 1962; 16 : 1-27.
103. Basu RN. Magnitude of problem of poliomyelitis in India. Indian Pediatr 1981; 18 : 507-11. 21 / 6
104. Sokhey J. Poliomyelitis surveillance in India. Indian Pediatr 1992; 29 : 677-82.

105. Sokhey J, Mathor YN, Beillik R, Country Overview - A report of the international evaluation of the immunization programme in India. Indian Pediatr 1993; 30 : 153-74. 22/8
106. Bijkerk H. Poliomyelitis epidemic in The Netherlands. Dev Biol Stand 1978; 43 : 195-206.
107. Hovi T. Outbreak of poliomyelitis in Finland in 1984-85. Significance of antigenic variation of type 3 poliovirus and site specificity of antibody response in antipolio immunization. Adv Virus Res 1989; 37 : 243-75.
108. Slater PE, Orenstein WF, Morag A, Avni A, Handscher R, Green MS. et al. Poliomyelitis outbreak in Israel in 1988 : a report with two commentaries. Lancet 1990; 335 : 1192-8.
109. Kim-Farley RJ, Rutherford G, Lichfield P, Orenstein WA, et al. Outbreak of paralytic poliomyelitis, Taiwan. Lancet 1984; 2 : 1322-4.
110. Deming MS, Jaiteh KO, Otten MW Jr, Flagg EW, Jallow M, Cham M, et al. Epidemic poliomyelitis in the Gambia following the control of poliomyelitis as an endemic disease. I. Descriptive findings. II. Clinical efficacy of trivalent oral polio vaccine. AM J Epidemiol 1992; 135 : 381 - 408. 22/9
111. Sutter RW, Patriarca PA, Brogan S, Malankar PG, Pallansch MA, Kew OM, et al. Outbreak of paralytic poliomyelitis in Oman : evidence for widespread transmission among fully vaccinated children. Lancet 1991; 338 : 715-20. 22/12
112. Pan American Health Organization. Polio incidence rises in Brazil's northeast. EPI Newsletter 1986; 8 : 4-5.
113. EPI : Poliomyelitis outbreak. Bulgaria. Wkly Epidemiol Rec 1992; 95 : 336.
114. Van Niekerk ABW, Vries JB, Baard J, Schoub BD, Chezzic, Black burn NK. Outbreak of paralytic poliomyelitis in Namibia. Lancet 1994; 344 : 661-7.
115. Forty-first World Health Assembly. The global eradication of poliomyelitis by the year 2000. Agenda item 12, 13, May, 1988. (WHA 41.28).
116. Committee on the Enteroviruses : Classification of human enteroviruses. Virology 1962; 16 : 501-4.
117. Cooper PD, Agol VI, Bachrach HL, Brown F, Ghendon Y, Gibbs AJ, et al. Picornaviridae : Second report. Intervirology 1978; 10 : 165-80.

118. Melnick JL. Classification of hepatitis A virus as enterovirus type 72 and hepatitis B virus as hepadnavirus type 1. Intervirology 1982; 18 : 105-6.
119. Schwerdt CE, Schaffer FL. Some physical and chemical properties of purified poliomyelitis virus preparations. Ann N Y Acad Sci 1955; 61 : 740-53.
120. Schaffer FL, Frommhagen LH. Similarities of biophysical properties of several human enteroviruses as shown by density gradient ultracentrifugation of mixtures of viruses. Virology 1965; 25 : 662-64.
121. Rueckert RR. On the structure and morphogenesis of picornaviruses. Comprehensive Virology (Frankel - Conrat H., Wagner R.R. eds), New York : Plenum Press 1976; 6 : 131-213.
122. Mapoles JE, Anderegg JW, Rueckert RR. Properties of poliovirus propagated in medium containing cesium chloride : Implications for picornaviral structure. Virology 1978; 90 : 103-11.
123. Paul AV, Schultz SE, Pincus S, Oroszlan S, Wimmer E. Capsid protein VP4 is N-myristoylated. Proc Natl Acad Sci USA 1987; 84 : 7827-31.
124. Schaffer FL, Schwardt CE. Crystallization of purified MEF-1 poliomyelitis virus particles. Proc Natl Acad 1955; 41 : 1020-3.
125. Finch JT, Klug A. Structure of poliomyelitis virus. Nature 1959; 183 : 1709-14.
126. Horne RW, Nagigton J. Electron microscope studies of the development and structure of poliomyelitis virus. J Mol Biol 1959; 1 : 333-8.
127. Maizel JV. Evidence for multiple components in the structural protein of type 1 poliovirus. Biochem Biophys Res Commun 1963; 13 : 483-9.
128. Summers DF, Maizel JV, Darnell JE. Evidence for virus specific noncapsid proteins in poliovirus infected HeLa cells. Biochemistry 1965; 54 : 505-13.
129. Rueckert RR. Picornaviral architecture. Comprehensive virology. Maramorosch K, Kurstak E eds. New York Academic Press 1971 : 255-306.
130. Jacobson MF, Asso J, Baltimore D. Further evidence on the formation of poliovirus proteins. J Mol Biol 1970; 49 : 657-69.
131. Hamman A, Wiegers KJ, Drzenick R. Isoelectric focussing and 2D-analysis of poliovirus proteins. Virology 1977; 78 : 359-62.

132. Wetz K, Habermehl KO. Topographical studies on poliovirus capsid proteins by chemical modification and cross-linking with bifunctional reagents. J Gen Virol 1979; 44 : 525-34.
133. Hordern JS, Leonard JD, Scraba DG. Structure of the mengovirion. VI. Spatial relationship of the capsid polypeptides as determined by chemical crosslinking analysis. Virology 1979; 97 : 131-40.
134. Wetz K, Habermehl KO. Specific crosslinking of capsid proteins to virus RNA by ultraviolet irradiation of poliovirus. J Gen Virol 1982; 59 : 397-401.
135. Kaper JM. The chemical basis of virus structure, dissociation and reassembly. *Frontiers of Biology* 39 Neuberger, Tatum eds. Amsterdam : North Holland, 1975.
136. Hogle JM, Chow M, Filman DJ. Three dimensional structure of poliovirus at 2.9 A resolution. Science 1985; 229 : 1353-65.
137. Wychowski C, vander Werf S, Siffert O, Crainic R, Bruneau P, Girard M. A poliovirus type 1 neutralization epitope is located within amino acid residues 93 to 104 of viral capsid polypeptide VP1. EMBO 1983; 2 : 2019-24.
138. Minor PD, Ferguson M, Evans DMA, Almond JW, Icenogle JP. Antigenic structure of poliovirus of serotypes 1,2 and 3. J Gen Virol 1986; 67 : 1283-91.
139. Minor PD, Antigenic structure of picornaviruses. Current Topics in Microbiol Immunol 1990; 161 : 121-54.
140. Colter JS, Bird HH, Moyer AW, Brown RA. Infectivity of ribonucleic acid isolated from virus infected tissues. J Virol 1957; 4 : 522-7.
141. Granboulan N, Girard M. Molecular weight of poliovirus RNA. J Virol 1969; 4 : 475-9.
142. Kitamura N, Semler BL, Rothberg PG, Larsen GR, Adler CJ, Dorner AJ, et al. Primary structure, gene organization, polypeptide expression of poliovirus RNA. Nature 1981; 291 : 547-53.
143. Racaniello VR, Baltimore D, Molecular cloning of poliovirus cDNA and determination of the complete nucleotide sequence of the viral genome. Proc Natl Acad Sci USA 1981; 78 : 4887-91.

144. Flanagan JB, Patterson RF, Ambros V, Hewlette MJ, Baltimore D. Covalent linkage of a protein to a defined nucleotide sequence at the 5' terminus of virion and replicative intermediate RNAs of poliovirus. Proc Natl Acad Sci USA 1977; 74 : 961-5.
145. Yogo Y, Wimmer E. Polyadenylic acid at the 3' terminus of poliovirus RNA. Proc Natl Acad Sci USA 1972; 69 : 1877-82.
146. Jacobson MF, Baltimore D. Polypeptide cleavages in the formation of poliovirus proteins. Proc Natl Acad Sci USA 1968; 61 : 77-84.
147. Kitamura N, Adler C, Martinko J, Nathenson SG, Wimmer E. The genome-linked protein of picornaviruses. VIII. Genetic mapping of poliovirus VPg by protein and RNA sequence studies. Cell 1980; 21 : 295-302.
148. Reuer Q, Kuhn RJ, Wimmer E. Characterization of poliovirus clones containing lethal and nonlethal mutations in the genome linked protein VPg. J Virol 1990; 64 : 2967-75.
149. Dorner AJ, Dörner LF, Larson GR, Wimmer E, Anderson CW. Identification of the initiation site of poliovirus polyprotein synthesis. J Virol 1982; 42 : 1017-28.
150. Pilipenko EV, Blinov VM, Romanova LI, Sinyakov AN, Maslova SV, Agol VI. Conserved structural domains in the 5' untranslated region of picornaviral genomes. An analysis of the segment controlling translation and neurovirulence. Virology 1989; 168 : 201-9.
151. Skinner MA, Racaniello VR, Dunn G, Cooper J, Minor PD, Almond JW. New model for the secondary structure of the 5' non coding RNA of poliovirus is supported by biochemical and genetic data that also show that RNA secondary structure is important in neurovirulence. J Mol Biol 1989; 207 : 379-92.
152. Nicholson R, Pelletier RJ, Le SY, Sonenberg N. Structural and functional analysis of the ribosome landing pad of poliovirus type 2 : in vivo translation studies. J Virol 1991; 65 : 5886-94.
153. Stone DM, Almond JW, Brangwyn JK, Belsham GJ. Trans complementation of cap independent translation directed by poliovirus 5' non coding region deletion mutants. Evidence for RNA-RNA interactions. J Virol 1993; 67 : 6215-23.
154. Emini EA, Elzinga M, Wimmer E. Carboxy - terminal analysis of poliovirus proteins : termination of poliovirus RNA translation and location of unique poliovirus polyprotein cleavage sites. J Virol 1982; 42 : 194-9.

15

39 | 18

155. Sarnow P. Role of 3' end sequences in infectivity of poliovirus transcripts made in vitro. J Virol 1989; 63 : 467-70.
156. Ahlquist P, Kacsberg P. Determination of the length distribution of poly (A) at the 3' termini of the virion RNAs of EMC virus, poliovirus, rhinovirus, RAV 61 and CPMV, and mouse globin mRNA. Nucl Acids Res 1979; 7 : 1195-1204.
157. Cooper PD, Bennet DJ. Genetic and structural implications of tryptic peptide analysis of poliovirus structural proteins. J Gen Virol 1973; 20 : 151-60.
158. Abraham G, Cooper PD. Relations between poliovirus polypeptides as shown by tryptic peptide analysis. J Gen Virol 1975; 29 : 215-21.
159. Pallansch MA, Kew OM, Palmenberg AC, Golini F, Wimmer E, Rueckert RR. Picornaviral VPg sequences are contained within the replicase gene. J Virol 1980; 35 : 414-19.
160. Summers DF, Maizel JV Jr. Determination of the gene sequence of poliovirus with pactamycin. Proc Natl Acad Sci USA 1971; 68 : 2852-56.
161. Taber R, Rekosh D, Baltimore D. Effect of pactamycin on synthesis of poliovirus proteins, a method for genetic mapping. J Virol 1971; 8 : 395-401.
162. Cooper PD. Genetics of picornaviruses. Comprehensive virology. (Fraenket-Contrat, Wagner eds) New York. Plenum Press 1977; 9 : 133-207.
163. Korant BD. Role of cellular and viral proteases in the processing of picornavirus proteins. The Molecular Biology of Picornaviruses. (Perez-Bercott R ed) New York - London : Plenum Press 1979 : 149-73.
164. Hanecak R, Semler BL, Anderson CW, Wimmer E. Proteolytic processing of poliovirus polypeptides : antibodies to polypeptide P3-7C inhibit cleavage at glutamine - glycine pairs. Proc Natl Acad Sci USA 1982; 79 : 3973-77.
165. Nomoto A, Kitamura N, Lee JJ, Rothberg PG, Imura N, Wimmer E. Identification of point mutations in the genome of the poliovirus Sabin vaccine LSc 2ab, and catalogue of RNase T1 - and RNase A - resistant oligonucleotides of poliovirus type 1 (Mahoney) RNA. Virology 1981; 112 : 217-27.

166. Nomoto A, Omata T, Toyoda H, Kuge S, Horie H, Kataoka Y, et al. Complete nucleotide sequence of the attenuated poliovirus Sabin 1 strain genome. Proc Natl Acad Sci USA 1982; 79 : 5793-97.
167. Stanway G, Hughes PJ, Mountford RC, Reeves P, Minor PD, Schild GC, Almond JW. Comparison of the complete nucleotide sequence of the genomes of the neurovirulent poliovirus P3/Leon/37 and its attenuated Sabin vaccine derivative P3/Leon/12a1b. Proc Natl Acad Sci USA 1984; 81 : 1539-43.
168. Pollard SR, Dunn G, Cammack N, Minor PD, Almond JW. Nucleotide sequence of a neurovirulent variant of the type 2 oral poliovirus vaccine. J Virol 1989; 63 : 4949-51.
169. Hirst GK. Genetic recombination with New castle disease virus, polioviruses and influenza. Cold spring Harbar Symp. Quant Biol 1962; 27 : 303-8.
170. Agol VI. Neurovirulence of the intertypic poliovirus recombinant V³/al-25 : Characterization of strains isolated from the spinal cord of diseased monkeys and evaluation of the contribution of the 3' half of the genome. J Gen Virol 1985; 66 : 309-16.
171. Kohara M, Omata T, Kameda A, Semler BL, Itoh H, Wimmer E, Nomoto A. In vitro phenotypic markers of a poliovirus recombinant constructed from infectious C DNA clones of the neurovirulent Mahoney strain and the attenuated Sabin 1 strain. J Virol 1985; 53 : 786-92.
172. Christodoulou C, Colberg - Garapin F, Macadam A, Tafes LF, Marden S, Minor PD, Horaud F. Mapping of mutations associated with monkey neurovirulence of Sabin 1 poliovirus revertants selected at high temperature. J Virol 1990; 64 : 4922-29.
173. Westrop GD, Evans DMA, Dunn G, Minor PD, Magarath DI, Taffs F, et al. Genetic basis of attenuation of the Sabin type 3 oral poliovaccine. J Virol 1989; 63 : 1338-44.
174. Weeks-Levy C, Tatem JM, DiMichele SJ, Waterfield W, Georgiu AF, Mento SJ. Identification and characterization of a new base substitution in the vaccine strain of Sabin 3 poliovirus. Virology 1991; 185 : 934-7.
175. Macadem AJ, Pollard SR, Ferguson G, Dunn G, Skuce R, Almond JW, Minor PD. The 5' non coding region of the type 2 poliovirus vaccine strain contains determinants of attenuation and temperature sensitivity. Virology 1991; 181 : 451-8.
176. Ren R, Moss EG, Racaniello VR. Identification of two determinants that attenuate vaccine related type 2 poliovirus J Virol 1991; 65 : 1377-82.

177. Minor PD. The molecular biology of poliovaccines. J Gen Virol 1992 ; 73 : 3065-77.
178. Minor PD, Dunn G. The effect of sequences in the 5' non coding region on the replication of polioviruses in the human gut. J Gen Virol 1988; 69 : 1091-6.
179. Svitkin YV, Pestova TV, Maslova SV, Agol VI. Point mutations modify the response of poliovirus RNA to a translation of initiation factor : a comparison of neurovirulence and attenuated strains. Virology 1988; 166 : 394-404.
180. Svitkin Y, Cammack N, Minor PD, Almond JW. Translation deficiency of the Sabin type 3 poliovirus genome association with an attenuating mutation C 472 - V. Virology 1990; 175 : 103-9.
181. Minor PD, Dunn G, Evans DNA, Magrath DI, John A, Howlett J, et al. The temperature sensitivity of the Sabin type 3 vaccine strain of poliovirus : molecular and structural effects of a mutation in the capsid protein VP3. J Gen Virol 1989; 70 : 1117-23.
182. Macadam AJ, Ferguson G, Arnold C, Minor PD. An assembly defect as a result of an attenuating mutation in the capsid protein of the poliovirus type 3 vaccine strain. J Virol 1991; 72 : 2472-81.
183. Sabin AB. Reproductive capacity of poliovirus of diverse origins at various temperatures. Perspect Virology 1961; 2 : 90-108.
184. Vogt M, Dulbecco R, Wenner HA. Mutants of poliomyelitis viruses with reduced efficiency of plating in acid medium and reduced neuropathogenicity. Virology 1957; 4 : 141-55.
185. Kanda Y, Melnick JL. In vitro differentiation of virulent and attenuated polioviruses by their growth characteristics on MS cells. J Exp Med 1959; 109 : 9-24.
186. Hodes HL, Zepp HD, Ainbender E. A physical property as a virus marker. Difference in avidity of cellulose resin for virulent (Mahoney) and attenuated (LSc, 2ab) strain of type 1 poliovirus. Virology 1960; 11 : 306-8.
187. Wallis C, Melnick JL, Ferry GD, Wimberly IL. An aluminium marker for the differentiation and separation of virulent and attenuated polioviruses. J Exp Med 1962; 115 : 763-75.

188. Benyesh - Melnick M, Melnick JL. The use of in vitro markers and monkey neurovirulence tests to follow genetic changes in attenuated poliovirus multiplying in the human alimentary tract. Live poliovirus vaccines, 1st Int. Conf. on Live Poliovirus Vaccines, Washington D.C. Pan American Sanitary Bureau Publication 1959; 44 : 179-202.
189. Dane DS, Dick GWA, Briggs M, Nelson R, McAlister J, Connolly JH, et al. Vaccination against poliomyelitis with live viruses vaccines 6. Changes in Sabin type II oral vaccine virus after human passage. Br Med J 1961; ii : 256-65.
190. Cossart YE. Genetic marker studies of polioviruses. Natural Variation. J Hyg Camb 1967; 65 : 67-76.
191. Wenner HA, Kamitsuka P, Lenahan M, Melnick JL. A comparative study of type 2 poliomyelitis viruses. II antigenic differences relating to 18 type 2 strains. J Immunol 1956; 77 : 220-31.
192. Wenner HA, Archetti I, Dubes GR. Antigenic variations among type 1 polioviruses. A study of 16 wild type strains and 5 variants. Am J Hyg 1959; 70 : 66-90.
193. Dubes GR, Archetti I, Wenner HA. Antigenic variations among type 3 polioviruses. Am J Hyg 1959; 70 : 91-105.
194. Mc Bride WD. Antigenic analysis of polioviruses by kinetic studies of serum neutralization. Virology 1959; 7 : 45-58.
195. Wecker E. A simple test for serodifferentiation of poliovirus strains within the same type. Virology 1960; 10 : 376-79.
196. Nakano JH, Gelfand HM. The use of a modified Wecker technique for the serodifferentiation of type 1 polioviruses related and unrelated to Sabin's vaccine strain. Am J Hyg 1962; 75 : 363-76.
197. Nakano JH, Hatch MH, Thieme ML, Nottay BK. Parameters for differentiating vaccine derived and wild poliovirus strains. Prog Med Virol 1978; 24 : 178-206.
198. van Wezel AI, Hazendonk AG. Intratypic serodifferentiation of poliomyelitis virus by strain specific antisera. Intervirology 1979; 11 : 2-8.
199. Osterhaus ADME, van Wezel AL, van Steenis B, Drost GA, Hazendonk AG. Monoclonal antibodies to polioviruses : Production of specific monoclonal antibodies to the Sabin vaccine strains. Intervirology 1981; 16 : 218-24.

200. Crainic R, Couillin P, Blondel B, Cabau N, Boue A, Horodniceanu F. Natural variation of poliovirus epitopes. Infect Immune 1983; 41 : 1217-25..
201. Ferguson M, Yi - Hua Q, Minor PD, Magrath DI, Spitz M, Schild GC. Monoclonal antibodies specific for the Sabin vaccine strain of poliovirus 3. Lancet 1982; 2 : 122-24 .
202. Kew OM, Pallansch MA, Omilianowski DR, Rueckert RR, Changes in three of the four coat proteins of oral poliovaccine strain derived from type 1 poliovirus. J Virol 1980; 33 : 256-63.
203. Minor PD. Comparative biochemical studies of type 3 poliovirus. J Virol 1980; 34 : 73-84.
204. De Wachter R, Fiers W. Preparative two dimensional polyacrylamide gel electrophoresis of [32P] labelled RNA. Anal Biochem 1972; 49 : 184-97.
205. Frisby DP, Newton C, Carey NH, Fellner P. Oligonucleotide mapping of picornavirus RNAs by two dimensional electrophoresis. Virology 1976; 71 : 379-88.
206. Lee YF, Wimmer E. "Fingerprinting" high molecular weight RNA by two dimensional gel electrophoresis : Application to poliovirus RNA. Nucl Acid Res 1976; 3 : 1647-58.
207. Lee YF, Kitamura N, Nomoto A, Wimmer E. Sequence studies of poliovirus RNA. Nucleotide sequence complexities of poliovirus type 1, type 2 and fingerprint of poliovirus type 3 genome. J Gen Virol 1979; 44 : 311-22.
208. Nottay BK, Kew OM, Hatch MH, Hayword JT, Obijeski JF. Molecular variation of Type 1 vaccine related and wild polioviruses during replication in humans. Virology 1981; 108 : 405-23.
209. Kew OM, Nottay BK. Molecular epidemiology of polioviruses. Rev Infect Dis 1984; 3 (suppl-2) : S499-S504.
210. Young JF, Desselberger U, Palese P. Evolution of human influenza A viruses in nature : sequential mutations in the genomes of new H1N1 isolates. Cell 1979; 18 : 73-82.
211. Harris TJR, Robson KJH, Brown F. A study of the level of nucleotide sequence conservation between the RNAs of two serotypes of foot and mouth disease virus. J Gen Virol 1980; 50 : 403-18.

212. Clewley JP, Bishop DHL, Kang CY, Coffin J, Schnitzlein WM, Reichmann ME, Shope RE. Oligonucleotide fingerprints of RNA species obtained from rhabdoviruses belonging to the vesicular stomatitis virus subgroup. J Virol 1977; 23 : 152-66.
213. Repik PM, Dalrymple JM, Brandt WE, McCown JM, Russell PK. RNA fingerprinting as a method for distinguishing dengue -1 virus strains. Am J Trop Med Hyg 1983; 32 : 517.
214. Monath TP, Kinney RM, Schlesinger JJ, Brandriss MW, Btes P. Ontogeny of yellow fever 17 D vaccine : RNA Oligonucleotide fingerprint and monoclonal antibody analysis of vaccines produced worldwide. J Gen Virol 1983; 64 : 627.
215. Trent DW, Grant JA, Vorndam AV, Monath TP. Genetic heterogeneity among St. Louis encephalitis virus isolates of different geographic origin Virology 1981; 114 : 319.
216. Banerjee K, Ranadive SN. Oligonucleotide fingerprint analysis of Japanese encephalitis virus strains of different geographical origin. Indian J Med Res. 1989; 89 : 201-16.
217. Kew OM, Nottay BK, Obijeski JF. Applications of oligonucleotide fingerprinting to the identification of viruses. *Methods in Virology.* (K Maramorosch and H. Koprowski, Eds.) 1984; 8 : 41-84.
218. Rico-Hesse R., Pallansch M.A., Nottay B.K. and Kew O.M. Geographic distribution of wild poliovirus type 1 genotypes. Virology 1987; 160 : 311-22.
219. Poyry T, Kinnunen L, Hovi T. Restricted variability of a 17 nucleotide stretch within the 5' noncoding region of poliovirus genome. Epidemiol Infect 1989; 103 : 671-83.
220. Poyry T, Kinnunen L, Kapsenberg K, Kew O, Hovi T. Type 3 poliovirus Finland 1984 is genetically related to common mediterranean strains. J Gen Virol 1990; 71 : 2535-41.
221. Balanant J, Guillot S, Candrea A, Delpeyroux F, Crainic R. The natural genomic variability of poliovirus analyzed by a RFLP assay. Virology 1991; 184 : 645-54.
222. Vonsover A, Handsher R, Neuman M, Guillot S, Balanant J, Rudich H, et al. Molecular epidemiology of type 1 polioviruses isolated in Israel and defined by RFLP assay. J Infect Dis 1993; 167 : 199-204.
223. Handerson RH, The World Health Organization's Plan of Action for global eradication of poliomyelitis by the year 2000. Ann NY Acad Sci 1991; 369 : 69-85.

224. Payment P, Larose Y, Trudel M. Polioviruses and other enteroviruses in urban sewage from Laval (Canada) : Presence of nonvaccinal strains of poliovirus, Can J Microbiol 1979; 25 : 1305-9.
225. EPI. Poliomyelitis in 1991. Wkly Epidemiol Rec 1993; 68 : 17-24.
226. Progress towards global eradication of poliomyelitis 1988-1993. MMWR 1994; 43 : 499-503.
227. Lennet EH, Schmidt NJ. Diagnostic procedures for viral and rickettsial disease. American Public Health Association, Inc 1790 Broadway, NewYork. 1964 : 45-51.
228. Minor PD. Characterization of strains of type 3 poliovirus by oligonucleotide mapping. J Gen Virol 1982; 59 : 307-17.
229. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning. A laboratory manual 3. Coldspring Harbor, Laboratory Press 1989.
230. Aaronson RP, Young JP, Palese P. Oligonucleotide mapping : evaluation of its sensitivity by computer simulation. Nucl Acid Res 1982; 10 : 237-46.
231. Robson KJH, Crowther JR, King AMQ, Brown F. J Gen Virol 1979; 45 : 579-90.
232. Contreras G, Dimock K, Furesz J, Gardell C, Hazielt D, Karpinski K, et al. Genetic characterization of Sabin types 1 and 3 poliovaccine virus following serial passages in the human intestinal tract. Biologicals 1992; 20 : 15-26.
233. Annual Report. Enterovirus Research Centre 1986.
234. Deshpande JM, Dave KH. Intratypic serodifferentiation of poliovirus type1 strains isolated during outbreaks of paralytic poliomyelitis. Indian J Med Res 1988; 87 : 103-7.
235. Deshpande JM, Dave KH. Intratypic characterization of poliovirus type 1 isolates. Indian J Med Res 1991; 93 : 202-7.
236. Annual Report. Enterovirus Research Centre 1993.
237. Magarath DI, Evans DMA, Ferguson M, Schild GC, Minor PD, Horaud F, et al. Antigenic and molecular properties of type 3 poliovirus responsible for an outbreak of poliomyelitis in a vaccinated population. J Gen Virol 1986; 67 : 899-905.

70/

31

238. Cammack N, Phillips A, Dunn G, Patel V. Intertypic genomic rearrangements of poliovirus strains in vaccinees. Virology 1988; 167 : 507-14.
239. Kirkegaard K, Baltimore D. RNA recombination in poliovirus. Cell 1986; 47 : 433-43.
240. Furione L, Guillot S, Otelea D, Balanant J, Candrea A, Crainic R. Polioviruses with natural recombinant genomes isolated from vaccine associated paralytic poliomyelitis. Virology 1993; 196 : 197-208.
241. Kew OM, Nottay BK, Hatch MH, Nakano JH, Obijeski JF. Multiple genetic changes can occur in the oral poliovaccines upon replication in humans. J Gen Virol 1981; 56 : 337-47.
242. Furesz J, Armstrong RE, Contreras G, Wachmann B. Genetic characterization of poliovirus isolates in Canada 1962-1981. Rev Infect Dis 1984; 6 (Suppl) : S528-S534.
243. John TJ. Antibody response of infants in tropics to five doses of oral poliovaccine. Br Med J 1976; 1 : 812.
244. Sharma M, Sen S, Ahuja B, Dhamija K. Paralytic poliomyelitis from a Sentinel Centre. Indian Pediatr 1990 ; 27 : 143-50.
245. Murthy N, Bhaskaran P, Murali MV, Sukanya M, Shrinivasa Rao CR. An outbreak of poliomyelitis in Andhra Pradesh (South India) . Indian Pediatr 1994; 31 : 533-41.
246. Mandke VB, Pawar RM, Naik DD, Salgaokar SD. An outbreak of poliomyelitis in the Marathwada Region of Maharashtra State 1990. Indian Pediatr 1994 ; 31 : 1091-4.

**SYNOPSIS
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TO THE
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FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY IN MICROBIOLOGY**

1. TITLE OF THE THESIS : Study of Poliovirus Isolates by
Oligonucleotide Fingerprinting.
2. NAME OF THE CANDIDATE : Alpana P. Khirwadkar
3. NAME & DESIGNATION OF THE GUIDE : Dr. S.R. Sengupta, M.B.B.S., M.D.
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SIGNATURE OF CANDIDATE

INTRODUCTION :

Great strides have been made towards the control of poliomyelitis since the introduction of the two poliovaccines. This has culminated into almost total eradication of poliomyelitis from most of the developed countries of the world. However, it still remains as a disease of major public health importance in the tropical and subtropical countries. Epidemiological data from India show that poliomyelitis is endemic in most parts of the country¹.

Poliovirus, the aetiological agent of this disease exists in three serologically distinct types, type 1, type 2 and type 3. Poliovirus has a single stranded RNA genome of positive polarity, about 7500 nucleotides long. Poliovirus replication like other RNA viruses results in frequent errors that provide the virus with great potential for evolution. This is reflected by existence of several distinct genetic subgroups within each serotype^{2,3}. The standard methods to identify poliovirus isolates have been serologic using type specific antisera. Various methods were developed for intratypic analysis of each serotype. These included complement fixation⁴, measurement of neutralization kinetics⁵, modified Wecker analysis², neutralization with cross absorbed antisera⁶ and using panels of monoclonal antibodies⁷. These approaches based on antigenic analysis however could not provide evidence for intratypic genetic relatedness and for the distribution of poliovirus subpopulations among human communities. These failed to unequivocally identify the paralytic cases caused by vaccine derived strains. Hence other approaches based on the genomic relationship were introduced to characterize the poliovirus isolates.

Two dimensional gel analysis of ribonuclease T1 resistant oligonucleotides was originally described by De Wachter and Fiers (1972) to separate complex mixtures of RNA molecules⁸. The enzyme cleaves RNA specifically at guanosine residues. The large oligonucleotides were shown to electrophoretically separate in patterns characteristic of their chain-length and base composition. Comparison of the oligonucleotides separation pattern (fingerprints/maps) allowed investigators to define genomic variations between virus isolates. Evolution of RNA viruses such as foot and mouth disease virus, Influenza virus, Japanese encephalitis virus over a period of time was traced by comparison of oligonucleotide maps of their serial isolates^{9,10,11}. Lee et. al.; (1979) were the first to demonstrate that although extensive sequence homology has been observed between the RNA's of the three poliovirus serotypes, the fingerprints differed strikingly in the separation patterns of RNase T1 resistant large oligonucleotides¹².

Nottay et.al.; (1981) demonstrated that oligonucleotide fingerprinting could unequivocally identify the origin of poliovirus isolates from vaccine associated cases. It was also shown that oligonucleotide maps of epidemiologically distinct isolates were very different. Comparison of the oligonucleotide fingerprints could define the relationship between poliovirus type 1 isolates from paralytic poliomyelitis cases in the Netherlands (1978), Canada (1978) and United States (1979)¹³. Epidemiological data confirmed the possibility of migration of the Netherlands strain to United States via Canada by certain religious groups refusing vaccination.

The genomic analysis of poliovirus type 1 isolates has been extended to partial sequencing of RNA and PCR product analysis of viral genome. Rico-Hesse et.al.; (1987), by partial sequencing, have compared poliovirus isolates obtained over a 37 year period from five continents. The data identified distinct genotypes circulating in different geographic regions. Genomic relationship could establish epidemiologic links among the poliomyelitis cases and outbreaks in distant communities. It provided an overview of the complexity of the patterns of wild type poliovirus transmission^{14,15}.

The epidemiologic significance of the genomic variation among circulating poliovirus strains was first documented during Finland epidemic in 1984-85. The epidemic occurred in the nearly 100% vaccinated population. Widespread circulation of poliovirus type 3 was demonstrated. Serologic analysing using serum samples collected before the outbreak showed very low antibody titres against the epidemic strains as compared to the vaccine strain. The epidemic strains were also characterized at the molecular level. The oligonucleotide fingerprints of these strains were unrelated to the type 3 strains used in either oral poliovaccine (OPV) or inactivated poliovaccine (IPV). Further analysis by monoclonal antibodies and genomic sequencing revealed mutations responsible for establishing the strain in the immunized population¹⁶.

The recent outbreaks caused by poliovirus type 1 in Gambia (1986)¹⁷, Israel (1988)¹⁸, Oman (1990)¹⁹, and due to poliovirus type 3 in Netherlands (1992)²⁰ have illustrated the ability of wild type poliovirus to infect susceptible individuals even in extensively immunized populations. This has drawn attention towards the potential importance of genomic variation among the circulating poliovirus strains.

The above findings highlight that genomic analysis of poliovirus isolates have vital importance in studying following aspects of poliovirus epidemiology.

1. To define the genotypes of wild polioviruses.
2. To determine the spread of wild viruses from one focus to another.
3. To determine the origin of outbreak strains.
4. To identify the vaccine associated poliomyelitis cases.
5. For rapid laboratory diagnosis of poliovirus infection using genotype specific oligonucleotide probe in hybridization assay.

The World Health Assembly in 1988, declared the commitment of the WHO to the global eradication of poliomyelitis by the year 2000²¹. To achieve this goal, vaccination programme has been strengthened throughout India. As a consequence, a decline in the incidence of the disease has been observed. Epidemiological data from Bombay reports more than 1200 cases of poliomyelitis per annum till 1987. The first decline in the incidence was observed in 1988 when 549 cases were reported. The number of cases of poliomyelitis for the year 1991 were only 299²².

It is however, likely that the problems encountered presently by other countries in eradication of poliomyelitis will be encountered by India in the near future. As the country approaches this stage, the polio control programme will need extensive laboratory support in areas such as antigenic and genomic characterization of virus isolates and transmission (spread) of wild poliovirus strains. The following objectives were, thus, selected for the present thesis work.

1. To standardize oligonucleotide fingerprinting technique for the study of polioviruses.
2. To prepare a catalogue of oligonucleotide fingerprints of indigenous poliovirus type 1 strains.
3. To define the genotype(s) of poliovirus type 1 isolates.
4. To study variations in the protein profiles of these strains.

Materials and Methods

Poliovirus type 1 isolates used in the present study were obtained from the virus bank at the Enterovirus Research Centre. The virus isolation and serotyping was performed as follows :

Stool samples of patients suffering from acute flaccid paralysis were collected for virus isolation. After processing the samples to destroy contaminating microorganisms, these were inoculated on HEp-2 cell cultures. Tissue culture fluid harvests were then analysed for virus identification using type specific equine antisera.

Cell Cultures

HEp-2 : HEp-2 cells were maintained using Eagle's minimum essential medium (MEM) supplemented with non-essential amino acids and 5% foetal bovine serum.

HEp-2 cell cultures were used for preparing high titre working stock of each virus isolate, virus titration, virus neutralization tests, and for analysis of viral proteins.

HeLa Cells : HeLa S3 cells were maintained using Eagle's MEM supplemented with non-essential amino acids and 10% sheep serum.

HeLa cell cultures were used for propagation of each virus isolate for oligonucleotide fingerprinting experiments.

Virus Isolates :

Poliovirus type 1 isolates of the year 1986 were selected for the present study. Twenty three isolates from three Bombay Municipal Wards were chosen. These included eight isolates from S ward, eight isolates from R ward and seven from M ward. Poliovirus type 1 and type 2 Sabin vaccine strains obtained at (SO+2) level were used for the reference.

Virus Purification

Each virus isolate was propagated on confluent monolayers of HeLa cells grown in twelve 175 cm² tissue culture flasks. Virus was concentrated by a two step procedure involving precipitation by polyethylene glycol (PEG) and pelleting by ultracentrifugation (37,000 rpm, 3.30h, 4°C) through a 0.5 ml cushion of 30% sucrose (w/v).

Virus was further purified by equilibrium density gradient centrifugation in cesium chloride. (33,000 rpm, 16 hrs, 4°C). Poliovirus was collected by piercing a sterile needle just below the virus band. The virus fraction was dialyzed against TSE buffer (10 mM Tris HCl pH 8.0, 50 mM NaCl, 1mM EDTA).

RNA Extraction

The dialyzed virus preparation was treated with pronase, SDS, B-mercaptoethanol to disrupt the virions. RNA was isolated by phenol chloroform extraction and ethanol precipitation. Purity of RNA preparations was confirmed by agarose gel electrophoresis.

Ribonuclease T1 digestion and 5' end labelling

The procedure described by Kew et.al.²³, was used for RNA digestion and labelling of oligonucleotides. 1-2 ug of viral RNA was digested with 2.5 U of ribonuclease T1 in the presence of 2×10^{-3} U of E.coli alkaline phosphatase, (37°C, 30 min). 5' end labelling was done by addition of T4 polynucleotide kinase and 15-25 uCi [γ -³²P] ATP (5.0hr, 37°C). The reaction was terminated by addition of 0.6 M NH₄OAC. 100 ug of carrier tRNA (yeast) was added and oligonucleotides were precipitated by 2.5 vol of ethanol at -70°C. The precipitate was washed once with cold 95% ethanol, dried in vacuo, and resuspended in first dimensional electrophoresis buffer.

Electrophoresis

Ribonuclease T1 generated oligonucleotides were separated by two dimensional polyacrylamide gel electrophoresis. The technique of De Wachter and Fiers was followed⁸.

First dimensional electrophoresis : First dimensional electrophoresis was performed in 8% polyacrylamide gel containing 6M Urea at pH 3.3. The RNA digest was loaded in the sample well. Electrophoresis was carried out in cold at a maximum power output of 3.2 W (450 V, 7mA). The run was terminated when the bromophenol blue dye migrated 21.5 cm from the bottom of the sample well.

Second dimensional electrophoresis : After first dimensional electrophoresis, the gel strip containing the sample lane was cut and aligned along the lower edge of the second dimensional glass plate (35.5 x 43 cm). The second dimensional gel (22% polyacrylamide pH 8.2) was then casted. Electrophoresis was Performed at a maximum power output of 12 W (16 mA, variable voltage) at room temperature. Run was terminated when bromophenol blue dye migrated to 25.5 cm in the separating gel.

Autoradiography

The gel was transferred to X-ray cassette for autoradiography. X-ray film was exposed for 24h at -70°C using intensifying screens. The X-ray film was developed manually.

Study of protein profiles by polyacrylamide gel electrophoresis.

HEp-2 cell cultures grown in 25 cm² flasks were infected with poliovirus type 1 isolates, at the multiplicity of 50 TCID₅₀ per cell. Cells were incubated at 35°C in medium lacking methionine. At 3.5h post infection the MEM containing 10 uCi of [³⁵S] methionine was added. Labelling was continued for 2.0 hrs at 35°C, followed by a chase period of 2.0 h. Cells were then washed with cold PBS (2X) and lysed in electrophoresis sample buffer. The protein lysates were separated by electrophoresis in 10% SDS polyacrylamide gels. Electrophoresis was carried out at 40 mA. The gels were fixed in acetic acid methanol water (12:50:38), silver stained and dried. For autoradiography the X-ray films were exposed to the dried gels for 3 days at room temperature.

RESULTS :

Virus Purification :

Highly purified preparations of poliovirus are required for oligonucleotide fingerprinting. The yield of virus obtained at following steps of virus purification was monitored. Virus was estimated by virus titration.

- a. Original virus harvest.
- b. PEG Supernatant
- c. PEG Pellet resuspended, and
- d. Supernatant of centrifugation at 37,000 rpm.

Yield was calculated from the titres of virus recovered in the pellet, and/or lost in the supernatant.

Cesium Chloride density gradient centrifugation :

An opaque virus band was observed in the centrifuged gradient. In the initial experiments of standardization, the gradient was collected into 0.4 ml fractions. Each fraction was titrated to estimate the virus. It was found that virus was most concentrated in the visible band, and the band was then collected (titre 10^{11.25}TCID₅₀/ml).

RNA Extraction :

Purity and concentrations of RNA was determined by O.D. readings at 260 nm and 280 nm. It was found that around 15 to 20 ug of RNA was obtained from 10^{11} viral particles.

Results of agarose gel electrophoresis revealed a high molecular weight RNA. Only minimal degradation was observed.

Oligonucleotide fingerprinting :

Oligonucleotide fingerprinting was standardized using poliovirus type 1, and poliovirus type 2, Sabin vaccine strains. The fingerprints matched with those published by Lee et al. (1979)¹². It should be mentioned here that the earliest published work involved in vivo incorporation of radioisotope in the viral RNA. This has recently been replaced by in vitro 5' end labelling of the ribonuclease T1 generated oligonucleotides using polynucleotide Kinase (PNK). The reproducibility of oligonucleotide fingerprinting procedure was confirmed by a) repeatation of fingerprints using the same RNA preparation and, b) using two separate preparations of virus stocks of the same strain after two passages in cell culture.

Oligonucleotide fingerprinting was carried out for twenty five poliovirus isolates. These were, Poliovirus type 1 and type 2, Sabin vaccine strains, and twenty three isolates from three wards of the Bombay Municipality Corporation.

M Ward Isolates : Seven isolates from this ward were mapped. These were isolated from paralytic poliomyelitis cases reported during June to August 1986. The fingerprint pattern of five strains were similar to each other. The fingerprints of the remaining two strains appeared unrelated.

S Ward Isolates : Eight isolates from S ward were studied. Four of these isolates were obtained from polio patients during July 1986, and the remaining were from December. The four isolates of July were represented by distinct fingerprint patterns which could not be grouped. On the otherhand, three out of the four strains isolated during December had almost identical fingerprints. Oligonucleotide map of one of the July isolates was similar to the December pattern.

R Ward Isolates : Eight isolates of R ward included five isolates of July and three from October. Oligonucleotide maps of most of the isolates were unrelated to each other. Two of the R ward October strains were found to be genetically related to M ward strains isolated during July 1986.

SDS PAGE :

Genomic variation among the poliovirus strains would result in changes in viral polypeptides. A comparison of total protein profiles of the selected poliovirus type 1 isolates was studied. Slight but consistent variation in the migration rates of proteins was observed. Majority of the S ward strains showed similar protein profiles. Similarly M ward strains could be grouped together whereas R ward strains showed number of differences in them.

Discussion

The molecular epidemiology of poliovirus has assumed great importance as the country is approaching towards the control of poliomyelitis. Such investigations will enable characterization of sporadic cases, define relationship between strains and determine transmission of different wild strains. The present study is aimed at defining the genomic characteristics of poliovirus strains circulating in our region.

The incidence of poliomyelitis in Bombay has declined from 14.08 per 10^5 population in 1986 to 2.9 per 10^5 population in 1991. Poliovirus isolates of the year 1986 were selected as the starting point. The epidemiological data pointed out that in recent years poliovirus type 1 predominated in Bombay, as compared to the earlier alternating pattern of type 1 and type 2. Hence poliovirus type 1 strains were selected for the study. As this work forms the first attempt to characterize poliovirus isolates at the genomic level, it was essential to prove the ability to demonstrate genetically related strains. Hence poliomyelitis cases occurring in a limited area (for e.g. a municipal ward) of known high transmission rate and poliocases occurring during a short span of time, were selected.

From the sixteen municipal wards in Bombay, isolates of 3 epidemiologically important wards M, R and S were chosen. In the year 1986, 1268 cases of paralytic poliomyelitis were recorded in Bombay. Data showed that 174 cases were recorded from M ward. Poliovirus was isolated from 76, of which 58 were poliovirus type 1 isolates. Similarly S and R Ward recorded 76 and 111 cases during 1986.

The five isolates of June from M ward produced closely related fingerprint patterns. The two isolates of August however were different. These fingerprints could not be matched with the previous five.

The strains studied for S ward were from July and December months. The four isolates of July revealed fingerprints which were unrelated to each other. On the other hand, maps of three isolates of December were almost

identical. The fingerprint of one of the July isolate was closely related to the major group pattern of the December isolates. This indicated that the genotype has survived in the population for long time and the fingerprinting analysis could detect such relationships.

Results obtained from R ward isolates showed that, from the five strains of July only two produced closely similar oligonucleotide maps. Three strains were distinctly different.

Examination of all the maps together detected that the two October isolates of R ward were genomically related to the June isolates of M ward.

Thus, oligonucleotide fingerprinting analysis could determine the following information about poliovirus type 1 isolates.

1. Genomic relationship could be demonstrated between virus isolates.
2. More than one genotype circulated in each ward during the period studied.
3. Isolates of the same genotype could be identified even if they were separated by long period of time.
4. Genomic relationship between strains could be pointed out even when these were isolated from different geographic localities.
5. Migration of genotype from one place to another could be demonstrated.

Results of oligonucleotide fingerprinting will be discussed in detail in the thesis.

Analysis of the viral polypeptide patterns corroborated with fingerprinting results. Majority of the S ward and M ward strains showed similar protein profiles, where as R ward strains showed number of differences among them. This is in correlation with the fingerprint patterns obtained. Polypeptide profiles could suggests the relationship among poliovirus strains. However, this analysis was not sensitive enough to detect the genomic differences among the strains. The method may be utilized in the preliminary screening of the isolates for oligonucleotide fingerprinting when large number of strains are to be studied.

References :

1. Basu R.N. Poliomyelitis in India. *Indian Pediat.* 28 (1991) 589.
2. Nakano J.H., Gelfand H.M., and Cole J.T. The use of modified Wecker technique for the serodifferentiation of type 1 polioviruses related and unrelated to Sabin's vaccine strain. II. Antigenic segregation of isolates from specimens collected in field studies. *Amer J Hyg* 78 (1963) 214.

3. Crainic R., Couillin P., Blondel B., Cabau N., Boue A., and Hordiniceanu F. Natural variation of poliovirus neutralization epitopes. *Infect Immu* 41 (1983) 1217.
4. Wenner H.A., Archetti I., and Dubes G.R. Antigenic variations among type 1 polioviruses. A study of 16 wild type strains and 5 variants. *Amer J Hyg* 20 (1959) 66.
5. Mc Bride W.D. Antigenic analysis of polioviruses by kinetic studies of serum neutralization. *Virology* 7 (1959) 45.
6. Van Wezel A.L. and Hazendonk A.G. Intratypic serodifferentiation of poliomyelitis virus by strain specific antisera. *Intervirology* 11 (1979) 2.
7. Ferguson M., Magrath D.I., Minor P.D. and Schild G.C. WHO collaborative study on the use of monoclonal antibodies for the intratypic differentiation of poliovirus strains. *Bull WHO* 64 (1986) 239.
8. De Wachter and Fiers W. Preparative two dimensional polyacrylamide gel electrophoresis by ³²P labeled RNA. *Anal Biochem* 49 (1972) 184.
9. Harris T.J.R., Robson K.J.H., and Brown F. A study of the level of nucleotide sequence conservation between the RNAs of two serotypes of foot and mouth disease Virus. *J Gen Virol* 50 (1980) 403.
10. Young J.F., Desselberger U and Palese P. Evolution of human influenza A viruses in nature : sequential mutations in the genomes of new H1N1 isolates. *Cell* 18 (1979) 73.
11. Banerjee K., and Randive S.N. Oligonucleotide fingerprint analysis of Japanese encephalitis virus strains of different geographical origin. *Indian J Med Res* 89 (1989) 201.
12. Lee Y.F., Kitamura N., Nomoto A., and Wimmer E. Sequence studies of poliovirus RNA. Nucleotide sequence complexities of poliovirus type 1, type 2 and fingerprint of poliovirus type 3 genome. *Gen Virol* 44 (1979) 311.
13. Nottay B.K., Kew O.M., Hatch M.H., Heyward J.T. and Obijeski J.F. Molecular variation of type 1 vaccine related and wild polioviruses during replication in humans. *Virology* 108 (1981) 405.
14. Rico-Hesse R., Pallansch M.A., Nottay B.K., and Kew O.M. Geographic distribution of wild poliovirus type 1 genotypes. *Virology* 160 (1987) 311.
15. Olive D.M., Almufti S., Almulla W., Khan M.A., Pasca A., Stanway G. Detection and differentiation of picornaviruses in clinical samples following genomic amplification. *J Gen Virol* 71 (1990) 2141.
16. Hovi T. Outbreak of poliomyelitis in Finland in 1984-85. Significance of antigenic variation of type 3 poliovirus and site specificity of antibody response in antipolio immunization. *Adv Virus Res* 37 (1989) 243.

17. Otten M.W., Deming M.S., Jaiteh K.O., Fiagg. E.W., Forgie I., Sanyang Y., Sillah B., Borgan D., and Gowers P. Epidemic poliomyelitis in the Gambia following the control of poliomyelitis as an endemic disease 1. Descriptive findings. Amer J Epidemiol 135 (1992) 381.
18. Slater P.E., Orenstein W.A., Morag A., Avni A., Handsheer R., Green M.S., Costin C., Yarrow A., Rishpon S., Havkin O., Ben-zvi T., Kew O.M., Rey M., Epstein I., Swartz T.A., and Melnick J.L. Poliomyelitis outbreak in Israel in 1988 : Report with two commentaries. Lancet 335 (1990) 1192.
19. Sutter R.W., Patriarca P.A., Brogan S., Malankar P.G., Pallansch M.A., Kew O.M., Bass A.G., Cochi S.L., Alexander J.P., Hall D.B. Outbreak of paralytic poliomyelitis in Oman : Evidence for widespread transmission among fully vaccinated children. Lancet 338 (1991) 715.
20. Poliomyelitis outbreak at Netherlands. Weekly epidemiological Record, 13th November 1992 67 (66) 341.
21. Forty first World Health Assembly : Global eradication of poliomyelitis by the year 2000. Geneva WHO 1988 WHA 41.28.
22. Dave K.H., Annual Report. Enterovirus Research Centre 1990 and 1991.
23. Kew O.M., Nottay B.K. and Obijeski J.F. Application of oligonucleotide fingerprinting to the identification of viruses. Methods in Virology VIII (1984) 41.
