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*Development of Reagents for Diagnosis of
Acute Hemorrhagic Conjunctivitis
Caused By EV 70 And CA 24v Viruses.*

A

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By

Masoud M. Eslami

Haffkine Institute For Testing
Research and Training,
A. D. Marg, Parel,
Mumbai – 400 012.

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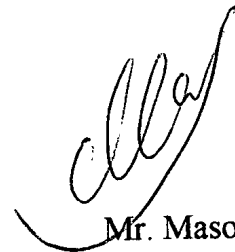
October - 2001

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Dr. S. V. Gadre,
Research Guide



Mr. Masoud M. Eslami
Candidate

FROM DARKNESS OF IGNORANCE TO ...

LIFGHTNESS OF WISDOM

**WITH MY WHOLE SOUL AND HEART, HONESTY
AND HUMBLY THIS THESIS IS DEDICATED TO
MY BELOVED PERFECT MASTER, WHO HAS
SHOWN ME THE TRUE PATH OF SCIENCE AND
HAS BLESSED MY ENTIRE LIFE.**

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PREFACE

Before the discovery in the nineteenth century that bacteria cause disease, the term *virus*, a Latin word for poisonous substances, was used to describe any disease-causing substances. Following that, this term was often used to describe microbial disease-causing agents. In the twentieth century, the term virus acquired a new and more specific meaning. Now it describes a large group of disease-causing agents that are fundamentally different from all cellular forms of life: viruses are infectious nucleic acid encapsulated in a protein coat. They may possess membranes but do not have any cytoplasm or metabolism of their own. Therefore, they must penetrate host cells where their nucleic acid directs the replication of viral macromolecular components, which are then *assembled* into new viruses.

The coming age of virology has started in the 1930s with the first studies of the nature of viruses, and it was not until about 1950 that biochemical and morphological data on virus particle composition and structure were substantial enough to influence classification efforts.

Prior to the year 1970s, adenoviruses were the major cause of epidemic outbreak of viral acute conjunctivitis and keratoconjunctivitis all over the world. Around three decades ago, when the first pandemic of acute hemorrhagic conjunctivitis (AHC) appeared in the world, the outbreak was immediately attributed to an unusually virulent strain of adenovirus. But, later the etiologic agents were confirmed as new serotypes of enteroviruses, and named as enterovirus type 70 and coxsackievirus CA 24 variant (CA 24v).

AHC virus since its first emerge in 1969 has affected millions of people in the world. These viruses periodically have invaded India.

Though, at present there is no a radical treatment for these viral infections, controlling and to suppress the spread of the AHC viruses playing very important role. Hence, identification of the etiologic agents is essential in order to control the spread of the disease in the communities.

Therefore, to know about different characteristics of these viruses is a must. The present thesis contains of two main parts. The first part as review of literature in which is included general epidemiology of enteroviruses, EV 70 and CA 24v. Also regarding the epidemic and endemic behavior of the enteroviruses, global distribution and historical background of spread of EV 70 and CA24v in the world has been discussed.

In this chapter I have discussed in detail about biological properties of EV 70 and CA 24v as well as physical characteristics and biochemical structure, antigenic structure, cellular receptor sites, strategy of replication, sequence comparison and evolutionary relations of enteroviruses.

It should be mentioned that all these data have been collected from different sources since the first emerge of AHC caused by EV 70 and CA 24v in the world, i.e. 1969 till the year 2000.

The second part of the thesis is focused on practical aspects related to scope of current study. Therefore, in this chapter all the technical parameters involved in this study have discussed in detail. They include: laboratory animals maintenance, tissue culture techniques for preparation viral antigen, immunization, bleeding of the animals schedule and also type of adjuvant and its application for increasing the immune response in animals inoculated with viruses. In this part of the thesis, a method for diagnosis based on immunological techniques has been described in detail.

It should be mentioned that to develop a rapid and easy method for diagnosis of one of the etiologic agents of acute hemorrhagic conjunctivitis (AHC) i.e. coxsackievirus A 24v (CA 24v) was successfully achieved. Failure to obtain high antibody response to EV 70 in rabbits and other animal species is also discussed.

Hence, I strongly believe that, this thesis, besides its principal development of reagents for diagnosis of AHC viruses, could also be considered as an up to date reference especially regarding virology of EV 70 and CA 24v viruses.

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CHAPTER 1

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Viruses are replicating microorganisms that are amongst the smallest of all life forms. This simplicity has led to an obligate requirement for intracellular growth and had a heavy dependence on host cell structural and metabolic components. Although attempts to understand the growth of viruses in cells has occupied much of the efforts of virologists in recent years, viruses still are major causes of human diseases. Viral diseases are some of the major scourges of mankind and include such virulent disorders as, yellow fever, rabies, poliomyelitis and HIV. Even to prevent and elimination of many of the most lethal viral diseases of humans, either acute form (e.g. respiratory and gastrointestinal infections) or chronic-infections (e.g. hepatitis and herpes).

In spite of visibility of this infection, it is a remarkable and proved fact that the vast majority of viral infections occur without overt symptoms (hence are subclinical). Thus the distinction between infection (viral multiplication in an infected cell) and disease (the illness due to viral multiplication and its resultant injury) is a critical one that must be made for all viral infections. With some viral infections, there are few, if any, cases of multiplication of the virus without overt disease (measles is an example of such a virus).

The coming age of virology started in the 1930s with the first substantial studies of the nature of viruses, but it was not until about 1950 that biochemical and morphological data on virus particle composition and structure were substantial enough to influence classification efforts. In the Early 1950s, the first grouping of viruses on the basis of common physiochemical properties emerged-the myxovirus group (Andrews et al., 1955). From the same time, the early 1950s, there was also an explosion in the discovery of new viruses of humans and animals.

Prior to the year 1970, adenovirus strains 8, 19, and 37 were the major cause of epidemic outbreak of viral acute conjunctivitis all over the world. It is not surprising therefore, when the first pandemic of acute hemorrhagic conjunctivitis (AHC) appeared in Africa (Accra, Ghana) in June 1969 (Chatterjee et al., 1970); the outbreak was immediately attributed to unusually virulent strains of adenovirus. The

conjunctivitis swept along the coastal of West, East and North Africa (Akinsete 1970; Parrot 1971; Nejmi et al, 1974), and subsequently to major cities in India (Bharucha et al, 1982; Wadia et al, 1972; Pramanik, 1971; Singha, 1972; Roy et al, 1972), many Southeast Asian countries (Salim, 1972; Lim and Yin-Murphy, 1972; Chang et al, 1977) including Japan (Kono et al, 1973; Jones, 1972) and a few European countries in 1970 and 1971.

Acute hemorrhagic conjunctivitis, variously known as Apollo 11 disease, Singapore epidemic conjunctivitis, Joy Bangla disease, picornavirus epidemic conjunctivitis, epidemic hemorrhagic keratoconjunctivitis, affected tens of millions of people during the 1969-1971 pandemic. Up to 1981, large epidemics of AHC were confined to the eastern hemisphere, especially the tropical coastal areas where crowded, unhygienic living conditions contributed to spread of the disease. The first epidemic out break in the western hemisphere, the Americas, was in mid-1981 (WHO wkly epidemic 1981) to mid-1982 the only continent to have escaped the eye affliction is Australia.

The discovery of a new coxsackievirus type A24 variant (Mirkovic et al, 1974) from the 1970 epidemic of conjunctivitis in Singapore (Lim and Yin-Murphy, 1971) and enterovirus type 70 (Mirkovic et al, 1973) and Japan (Kono et al, 1972) in 1971, triggered the search for a picornavirus etiology in other affected countries.

The universal recognition of coxsackievirus A24 variant and enterovirus type 70 as causative agents of a new disease entity- 'Acute hemorrhagic conjunctivitis'- opened a new chapter in virology and ophthalmic viral disease.

General Epidemiology of Enteroviruses

Mechanism and Route of Transmission

Humans are the only known reservoir, for members of the human enterovirus group, and close human contact appears to be the primary avenue of spread. For almost all these agents, virus can be recovered from the oropharynx and intestine of individuals with apparent or in apparent infections, and virus is generally shed for longer periods (up to a month or more) in stools than in secretions of the upper alimentary tract.

Thus, fecal contamination (through fingers, table utensils, food, and drinking water) is the usual source of infections. However, droplet or aerosols from coughing or sneezing can also be a source of direct or indirect contamination. Coxsackievirus A 21 has been in those from the throat and has been experimentally transmitted from infected volunteers by airborne aerosols produced by natural coughing (Couch et al, 1970). Enterovirus 70, the agent of acute hemorrhagic conjunctivitis (Mirkovic et al., 1973) has been found almost exclusively in conjunctival and throat specimens, though fecal isolations have been reported.

Warm weather favors the spread of virus by increasing human contacts, the susceptibility of the host, or the dissemination of the virus by extrahuman mechanisms. The viruses are most readily spread within the family, and the extent of interfamilial infection appears to be closely related to duration of virus shedding particularly by young children.

During periods of epidemic prevalence, in both rural and urban areas, houseflies (*Musca domestica*) and filth flies (*Phormia regina*, *Phenicia sercara*, *sarcophagi* species) may be found contaminated with enteroviruses and may act as mechanical carriers (Melnick et al, 1953). The importance of flies in transmission is not easily evaluated; although it is noteworthy that virus has been found in food naturally contaminated by flies (Ward et al., 1945). Virus persists in flies for weeks but dose not multiply (Melnick et al, 1973). Enteroviruses are also present in urban

sewage even during periods of subclinical infection. Sewage may serve as a source of contamination (of flies or) of water supplies used for drinking or bathing, or through its use as fertilizer (Melnick et al., 1978). Enteroviruses also have been found in aerosols produced by sprinkler irrigation using recycled wastewater (Melnick et al, 1984).

The amount of virus present in sewage is highly variable, depending on factors such as the hygienic level of the population, the prevalence of infection in the community, and the time of year (Berg et al, 1976). In the United States, peak levels of enteroviruses occur in the summer and early fall. Enteric viruses survive customary secondary sewage treatment and chlorination as commonly practiced.

In most of the world, human wastes are discharged into natural waters, with little or not treatment. Thus, there is little reduction in the initial input of viruses. However, in the developed countries a large part of sewage is processed by biological or physiochemical methods before discharge into receiving waters. Although treatment processes can result in large reductions in the concentration of viruses present in raw sewage, substantial numbers usually remain. Disinfection of treated sewage by chlorine is sometimes practiced, although this is effective in reducing bacterial pathogens, viruses are not eliminated by this treatment because of their greater resistance, particularly in the presence of organic matter. Since viruses may be detected in water free of fecal coliforms and streptococci, the adequacy of bacterial standards for evaluating the sanitary quality of water has been questioned as it relates to its potential for transmission of viral diseases.

Waterborne outbreaks due to enteroviruses are not easily recognized. Many of these viruses cause inapparent infections that are difficult to recognize as being waterborne and usual epidemiological methods are not sufficiently sensitive to detect low-level transmission through water. In most cases it is difficult to identify the point source of viruses that are detected at many distance downstream from their discharge. But, sometimes it has been done, and the results show that viruses can travel long distances from the source of contamination and still be infectious.

Although at times viruses have been isolated from conventionally treated drinking water in several countries, this does not imply that process, when properly carried out, are inadequate for the removal of enteric viruses. In many studies, the actual water quality and water treatment conditions at the time when viruses were isolated were either not known or not stated.

Into the coastal waters of the world, billions of gallons of treated and untreated sewage are discharged daily. In addition, polluted surface water discharge of virus laden sewage wastes into oceans became evident with the outbreaks of shellfish-associated hepatitis A (caused by enterovirus type 72). Presently, hepatitis A has classified in a separate group of viruses other than enteroviruses.

Oyster, clam, and mussels are filter-feeding organisms, that is, they sieve out suspended food particles from a current of water passing through the shell cavity. These animals have been found to concentrate viruses from water into their tissues. Since the enteric shellfish is often consumed raw or inadequately cooked, it functions as a passive virus carrier.

Effective control of enteric bacterial disease spread by recreational waters and shellfish has resulted from the establishment of bacteriological standards, by using total and fecal coliform indices as the adequacy of bacteriological standards to reflect a viral disease hazard, and several studies have now demonstrated the occurrence of enteroviruses in shellfish, shellfish harvesting waters, and marine recreational waters that met currently accepted bacteriological standards. Enteroviruses were detected 43 % of the time in recreational water considered acceptable as judged by coliform standards and 35 % of the time in water, which met acceptable standards for shellfish harvesting (Melnick and Cebra, 1980).

Enteroviruses have also been isolated from marine areas receiving both raw and chlorine-treated sewage. They have also been detected several kilometers from the nearest source of sewage disposal, in bathing beach waters, and in areas of sewage sludge discarded many kilometers from the coast. Enteroviruses have also been detected in marine sediment in areas of sewage and sludge disposal. The greatest portion of virus pollutants may be associated with solids. Virus has been detected in

72 % of samples of suspended solids, 46 % of fluffy sediments, and 5 % of compact sediments. In contrast, in the same study, virus was detected only 14 % of the water sample tested (Metcalf et al, 1984). Viruses adsorbed to sediment material remain infectious and survive for greater lengths of time in the marine environment than do freely suspended viruses. Viruses in sediments pose a public health problem, since the water/sediment interface is not static. Sediments can be resuspended easily in response to currents, storms, movements of boats and swimmers, dredging, or changes in water quality. Therefore the sampling of surface water alone does not give a true indication of the potential viral disease hazard.

Understanding environmental factors controlling enteric virus survival and transport in nature could be a key to defining the sudden occurrence of epidemics and the maintenance of infections with a population. Environmental conditions can greatly affect the survival and transport of viruses in nature, and there are many routes by which excreted virus may back to the human host (Figure 1).

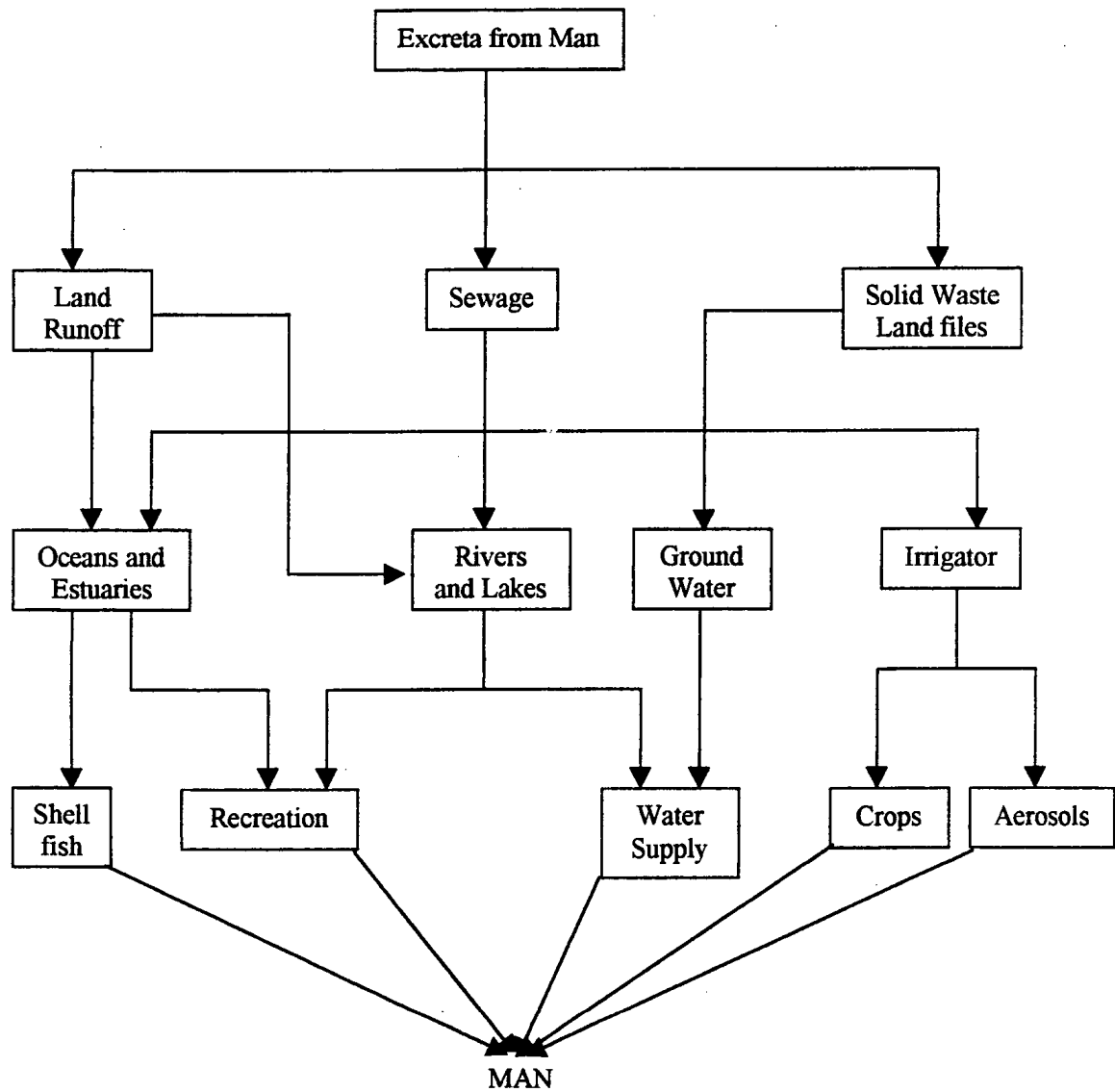


Figure 1: Routes of potential enteric virus transmission in the environment (Melnick et al., 1978).

A strain of coxsackievirus A6, originally obtained from mosquitoes in Fiji, has been found to survive in mosquitoes experimentally infected by injection or by feeding on viremic mice, and a small number of the transmission to baby mice by bite were obtained. The function of the mosquito as a true vector was not demonstrated, however, for virus did not multiply within the insect, in both fed and injected mosquitoes, virus titers never exceeded the original level (Maguire, 1970).

Epidemic and Endemic Behavior

By far the most common form of infection with any of these viruses is a mild or silent episode. Paralytic poliomyelitis remained an epidemiological enigma until knowledge of widespread inapparent infection was obtained (Cavery et al, 1896; Wickman 1905).

Recognition of the *disease* associated with the enteroviruses is based on the occurrence of a clinical syndrome sufficiently characteristic to be recognized, such as paralysis of poliomyelitis or the lesions of the hand-foot-and-mouth syndrome of echovirus 16, or on the occurrence of an outbreak of illness such as aseptic meningitis associated with the prevalence of one serotype.

The presence in the community of *infection* due to enteroviruses may be recognized by the isolation of virus from the stools of healthy persons or from sewage from the prevalence of antibody as determined by serological surveys.

The pattern of incidence and prevalence, the age at the time of infection, and the nature of the host response are the consequences of a number of interdependent variables, which include not only susceptibility to infection but also the opportunity for exposure.

The close correlation between low socioeconomic setting and the early acquisition of infection with the enteroviruses has been repeatedly emphasized in both tropical and temperate environments and reflects the general level of hygiene of the group (Grist et al, 1978).

Wide dissemination, in which overt illnesses represent only a "very small tip of large iceberg", has been repeatedly documented for a number of enteroviruses. During an epidemic in which 149 inhabitants of a city of 740,000 were hospitalized with echovirus 9 disease, approximately 6 %, or 44,000 persons, had an illness compatible with infection due to this agent (Sabin et al, 1958). Among families that have been invaded by the virus, the rate of unapparent or unrecognized infection (based on recovery of the virus) was 18 %, however, among 107 persons in families where no illness was observed, only one person yielded the virus from a stool specimen.

When outbreaks of aseptic meningitis and related illnesses due to echovirus type 30 began along the Pacific Coast in 1968, the arrival of the virus in the Washington area coincided with the initiation of the Seattle Virus Watch Program, and extraordinary opportunities were available to observe infection and illness among the regulatory studied Virus Watch families as related to the community pattern of illness (Hall et al., 1970). Some 64 such families containing 291 members underwent containing virological surveillance in this period. By virus isolation or serology or both, infection was documented in 70 (79 %) of 88 members of 18 families, in the total observed Virus Watch population, the rate was 24 %. The invaded families were of slightly larger size, include more children 5 to 9 years of age, and included only three persons who had antibody prior to the epidemic. Of the persons observed to shed virus, 47 % reported a related mild febrile illness, few of these episodes were serious enough to require medical attention, one father in an invaded family developed aseptic meningitis. Thus, there must have been many thousands of echovirus 30 infections in the area, more than half of which were without symptoms, during the period when 44 virologically confirmed cases of echovirus 30 aseptic meningitis occurred in the city (Torphy et al, 1970).

Poliomyelitis serves as the best-known example of the epidemiological behavior of enteroviruses. In the era before polio vaccine were developed, epidemics of poliomyelitis occurred with great frequency in the economically advanced countries of the world. In the United States, the first sizable outbreak was recognized in Vermont in 1984 and involved 132 cases; it was by far the largest number of cases ever reported in any one year in the world up to that time. In the first half of the

twentieth century, recurrent outbreaks involved the "developed countries" of the world, such as the United States, Canada, Australia, and Europe and Scandinavian countries.

Great reductions in epidemic incidence occurred after the introduction of killed polio vaccine in 1955. In the prevaccination period from 1951 to 1955, an average of almost 38,000 cases of poliomyelitis (approximately 21,000 paralytic) were reported annually in the United States, from 1961 to 1965, the annual average was 570 cases (460 paralytic). After live attenuated vaccines were introduced during 1962-1963, even further reductions took place. By 1971 to 1975 (WHO, 1978), an annual average of only 15 cases was occurring in the United States, and the number has remained low ever since. Similar reductions have occurred in other industrialized countries.

Outbreaks of infection with coxsackieviruses occur frequently in widely separated areas of the world, in various climates and conditions, and at different time intervals. Enterovirus isolations reported through the WHO virus reporting system were extensively analyzed in 1978 (Grist et al., 1978). The most frequently reported types for the 8-year period from 1967 to 1974 were coxsackievirus types A9, A16, B3, and echovirus types 4, 6, 9, 11, and 30. These serotypes generally continue to be commonly reported, although in any single year their predominance may be more marked, or another type may exceed them in frequency for a time. For example, in 1980. Coxsackieviruses A 9 and A 16 again predominated, with a record high number of A16 isolations reported, coxsackievirus B4 in 1984 somewhat exceeded B3 and B5 in frequency. Echovirus 30 has had an increasing trend since 1977 and may be heading toward a peak higher than that seen in 1974. In 1980 this type accounted for more than 2400 isolates, out of a total about 5800 isolations of all echovirus types reported. Of the echovirus 30 reports that included clinical information, 76% were from cases involving the central nervous system, especially in the older age groups. Of all patients 15 to 24 years of age. Echovirus 9 and 11 were also frequently isolated in 1980, but echovirus 7, previously less commonly reported, accounted for more than 500 of the isolates-more than other type 30. Echovirus 22 isolations also have increased in recent years, and more than half of the isolates were from infants less than 6 months old. It is suggested that the prevalence of echovirus 9 may fluctuate in

3- to 4-year cycles and that of echovirus 6 in even longer cycles. After a peak in 1975, isolations of the latter virus were still decreasing in 1980.

Enterovirus surveillance data from the United States for 1970 are informative (Moore, 1982). During the 10-year period, a total of 18,309 enteroviral agents were isolated from 18,152 patients. Echoviruses accounted for 58 % of the agents isolated, group B coxsackieviruses types 68 to 71, only 0.1%. The most frequently isolated were as follows, echovirus type 9 (more than 2,000 isolates), coxsackieviruses B5, echovirus 11, echovirus 4, and echovirus 6 (each of the foregoing accounting for more than 1,000 isolates), coxsackieviruses B2, B4, and A9, echovirus 7, poliovirus type 1, coxsackievirus B3, echovirus type 3, and poliovirus type 2 (each of this latter group accounting for more than 500 isolates). These 13 agents for accounted for 76 % of all isolates reported for the decade.

Analysis of the seasonal distribution in the United States over 10-year period presents a classical picture for enterovirus distribution in temperate climates (Figure 2). The non-polio enteroviruses were isolated chiefly during the summer and early fall. The average number of enteroviral isolations for each month from June through October was 6.6 times higher than the monthly average for the other the 7 months, and 82 % of the total isolates were isolated during these peak months. For all three groups of non-polio enteroviruses so analyzed, August was the peak month of isolation. Polioviruses, however, were isolated throughout the year, as would be expected from a nation in which live polio vaccine is administered year-round.

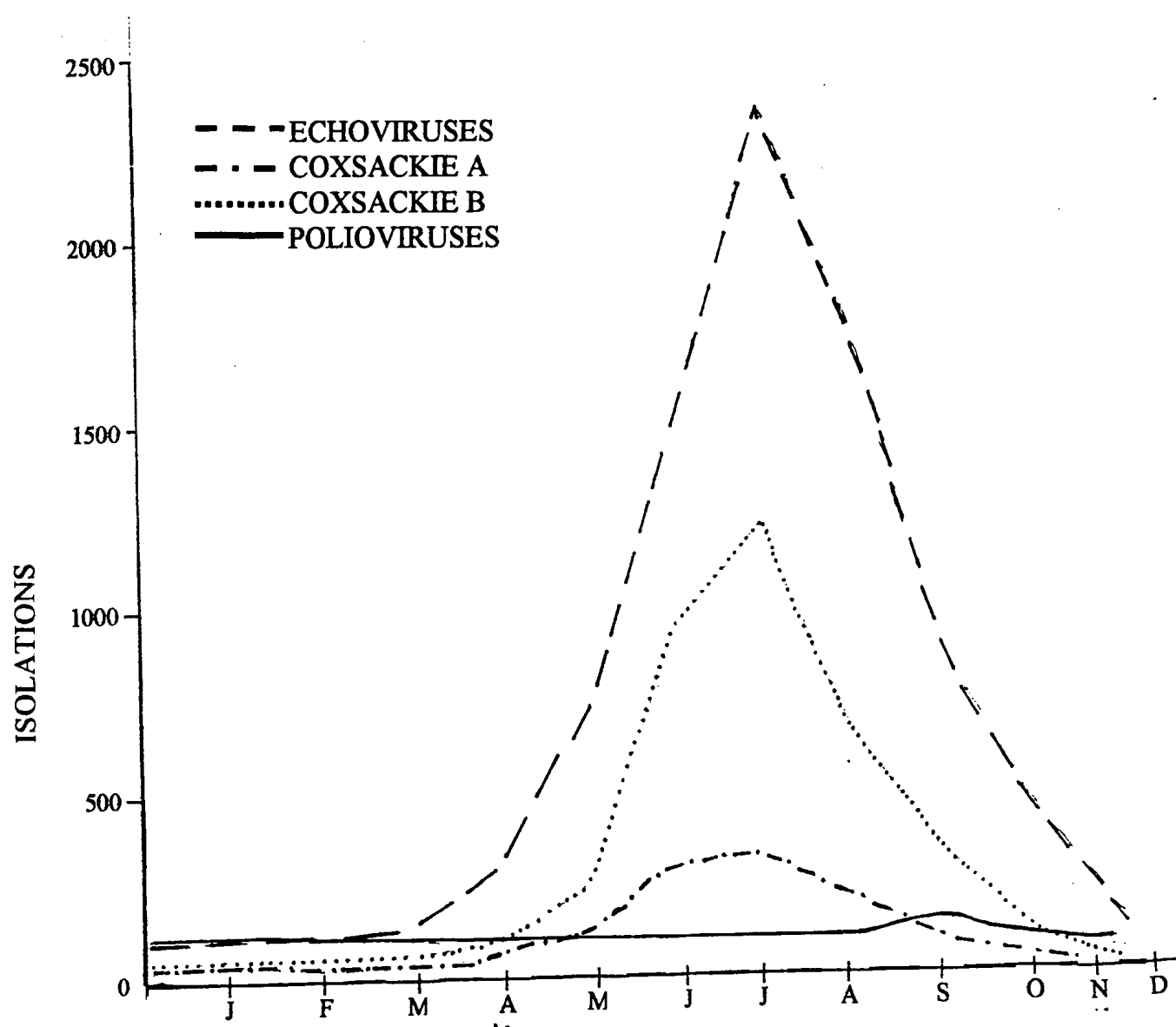


Figure 2: distribution of infections due to enterovirus in the United States by the month of the onset of illness from 1970 to 1979 (Moore, 1982).

Among the 13 most frequently isolated agents, 8 (coxsackievirus B5, poliovirus type 2, and echovirus type 3, 4, 6, 7, 9, and 11) appeared to circulate in an epidemic pattern, with many cases over shorter periods of time. The others showed no particular cyclic recurrence. From year to year the predominant enteroviruses may vary considerably. In 1981, the two most common isolates were echovirus types 9 and 30, in 1982, types 11 and 30, and in 1983, coxsackievirus B5 was most often reported.

Of special interest were the clinical syndromes associated with the isolates, from 1976 to, 1979, meningitis was recorded in 35 % of the patients, respiratory disease 21 %, encephalitis, 11 %, non specific febrile illness 6 %, illness with rash, 4%, carditis, 2%, paralytic disease, 1%, and other known syndromes, the remaining 22 % of disease reported. By comparing the distribution of isolates from all the reported cases with the distribution of serotypes associated with each clinical syndrome, clearly specific agents were over presented in certain of the syndromes. For example, polioviruses accounted for 53 % of the paralytic disease, echoviruses for 78 % of the encephalitis and meningitis cases, group B Coxsackieviruses for 63 % of the carditis cases, group A coxsackieviruses of group A and B, and polioviruses, were disproportionately low among cases of respiratory illness (15, 29, and 17% respectively).

Age distribution of the illness was also of interest; confirming and underscoring in these national data the previous views based on small epidemiological and clinical studies. Most of the reported cases were in young children (56 % of the total being under 10 years of age and 26 %, under 1 year). This disproportional of isolates from children under one year of age as compared to those 1 to 9 years old held true for all groups except group A coxsackieviruses, which were found more frequently in the older children. Adults 20 years of age and older accounted for only 16 to 20 % of all patients yielding non-polio enteroviruses.

When the age distribution of each clinical syndrome was compared with the overall age distribution of the population from whom these were obtained, adults and older children were over represented among carditis cases and also among cases of encephalitis and of meningitis. The proportion of paralysis was greater than expected among the youngest patients (10 to 4 years), as well as among those in the 20-to-24-

year old and the 35-to 39-year-old groups. Overall, however, the youngest age group (0 to 4 years old) was over represented among the milder clinical illness. This may reflect both the larger numbers of specimens collected from this age group and the actual predominance of mild rather than severe illness in young children (except for neonates).

The suggestion is made, from this and previous studies, that heavy physical exertion may predispose an infected individual to more severe illness. This is at least one of the possible factors in the recently studied outbreaks of enteroviral meningitis among high school football players (Moore et al., 1983).

A number of both coxsackieviruses and echoviruses have been involved in large epidemics, enteroviruses 70 and 71 also have been responsible for epidemics in various parts of the world. These outbreaks have been mostly localized to one area, although one epidemic of echovirus 9 infections in the late 1950s had almost world wide distribution and enterovirus 70 spread explosively in Asia and Africa from 1969 to 1973, and again in 1981, when the virus also spread to the Western Hemisphere.

Acute hemorrhagic conjunctivitis (AHC) was first recognized during 1969, in Ghana, and soon after in other African countries. The outbreak reached pandemic proportions, with tens of millions of persons being involved from 1969 to 1971. Large epidemics occurred during 1971 in Japan, Singapore, and Morocco. Serological surveys confirmed that the virus was not prevalent before the pandemic in the populations involved (Kono et al., 1975). Multiple epidemics have occurred within a 5-period in the same regions, particularly in Southeast Asia, suggesting that immunity may be short-lived (Yin- Murphy).

Until 1981, virtually no enterovirus 70 infection or disease had been found in Australia and the Americas. In more than 1,000 sera collected from 1971 to 1974 from residents of the United States, only 3 had antibodies to the virus (Hierholzer et al., 1975). Nevertheless, when the virus was introduced into the United States in 1980, secondary spread did not take place.

Early in 1981 AHC reappeared in some of the countries from which it had been absent for a number of years. The disease spread widely in Africa and Asia, and this time it spread extensively in the Caribbean area, in northern South America, and in Central America during the spring and summer of 1981 (Partriarca et al., 1983). In the early autumn, an explosive outbreak in the Miami metropolitan area, involving thousands of cases.

Enterovirus 70 is highly contagious, spreading rapidly under crowded and unhygienic conditions; warm humid coastal climates seem particularly favorable to its transmission. Some localized outbreaks, specially in developed countries, have entered on eye clinics. Interfamilial spread is common. In the explosive outbreak in the Mumbai metropolitan area, school age children in school of most heavily affected section were more likely to become household index cases than were at greater risk of acquiring AHC. Marked reduction in cases was achieved after exclusion of affected children from schools and educating the public about hygienic precautions (such as separate towels, *frequent hand washing*, and avoidance of touching eyes or face of affected family members). This agrees with the earlier reports that enterovirus 70 is transmitted almost exclusively by touching the eye with contaminated fingers or fomites (Yin-Murphy, 1984).

Enterovirus 70 isolates obtained from widely separated locals (Asia and Africa) during the same pandemic period, 1980 to 1981, were closely related by ribonuclease T1 oligonucleotide fingerprinting (Kew et al., 1983). However, two isolates obtained from Japan and from Morocco during the first AHC pandemic, 1969 to 1972, although closely related to each other, differed from the current strains by many oligonucleotides. The similarities among contemporaneous suggest that only one basic genotype of this virus appears to be in circulation, world wide, at any one time.

Enterovirus type 71 has moved about the world and is a good example of an agent associated with a variety of clinical manifestations at different times and in different regions (Table 1). The variety of host responses and the difficulty of isolating some of the strains of enterovirus 71 make it probable that only a small fraction of the cases that actually occur are diagnosed and reported (Melnick, 1984).

In the severe 1975 epidemic of CNS disease that occurred in Bulgaria, which included numerous polioencephalitis cases as well as cases of meningitis, about 21 % developed paralysis, and there were 44 fatalities. Young children were the most severely affected, children under 5 years of age represented 48 % of the total cases and constituted 81 % of the paralyzed patients and 93 % of the patients who died. From specimens obtained from 65 cases, 92 virus isolations were made (Chumakov et al., 1979; Shindarov et al., 1979). Of these, 37 came from brain and medulla, 1 from mesenteric lymph nodes and tonsils, and 44 from feces. All the Bulgarian isolates belonged to a single antigenic type, antigenitically related to the American and Swedish strains of enterovirus 71 (Melnick et al., 1979). Antibody to enterovirus 71 was found in 72 % of 392 patients with a history of poliomyelitis-like disease that year, and tests for a rise in antibody titers against poliovirus in convalescent sera collected during the epidemic were uniformly negative. This supports the etiological association of enterovirus 71 with the epidemic and excluded any possible role of poliovirus. In the very high incidence of paralytic cases, the epidemic in Bulgaria differed considerably from other epidemics. Virus was widely distributed during the epidemic year, infecting a high proportion of the population, but then disappeared. To date, the disease has not reappeared in Bulgaria.

Table 1: Enterovirus 71 outbreak, 1969-1979

Year	Location	No. of patients	Clinical findings
1969-1973	California	20	Aseptic meningitis Encephalitis
1972	New York State	11	Aseptic meningitis Encephalitis Hand-foot-and-mouth disease
1972	Australia	49	Aseptic meningitis Rash Acute respiratory infection Polyneuritis
1973	Sweden	195	Hand-foot-and-mouth disease Aseptic meningitis
1973	Japan	>3200	Hand-foot-and-mouth disease Aseptic meningitis
1975	Bulgaria	705	Aseptic meningitis Encephalitis and some with acute myocarditis
1977	Rochester, N Y	12	Aseptic meningitis Hand-foot- and-mouth disease
1978	Hungary	1550 ^a	Aseptic meningitis Encephalitis Polio-like paralytic disease with persistent flaccid paralysis
1979	Lyon, France	5	Acute respiratory infection with CNS involvement

^a A mixed epidemic of tick-borne encephalitis (chiefly in adults) and enterovirus 71 disease (chiefly in children)

In the large mixed epidemic of CNS disease that occurred in Hungary in 1978 (Nogy et al., 1982), clinical manifestations predominantly aseptic meningitis, but there were 826 cases of aseptic meningitis and 724 cases of encephalitis with 45 deaths, the encephalitis cases included 13 polio-like cases with flaccid paralysis. All the polio-like paralytic cases were in children between 8 and 36 months of age. At least two agents were involved: a tick-borne encephalitis virus for the majority of cases in adults and enterovirus 71 for the cases in children regardless of the clinical form of their disease. In the polio-like cases, only enterovirus 71 was isolated, and poliovirus infection was excluded by neutralization tests.

In the following years, enterovirus 71 isolations in Lyon, France, were associated with a small outbreak in children 5 to 9 years old. Their disease was chiefly influenza-like, with CNS involvement only in the most severe cases (Sohier, 1979).

With most non polio enteroviruses, the pattern of infection for a specific serotype in a particular locality resembles that of poliomyelitis: (a) There may be local serotypes that are endemic, constantly circulating among the few non-immune mostly very young children, since virtually all the older children and adults already have protective antibody from previous infections. (b) or specific serotypes may be completely absent from a particular locality or very limited in dissemination for a number of years, a population of a susceptible builds up, and a wave of wide and rapid spread of the virus may occur, reaching a large proportion of all age groups. Dissemination of different serotypes may occur in waves, with one predominant type in an area succeeding another from one year to the next or even within the same summer-fall season (Grist et al., 1978).

Global Distribution

Enteroviruses are found in all parts of the world. In tropical and semitropical regions, they are widely distributed through the year. In temperate climates, they are present at low levels in winter and spring, but are encountered far more commonly during summer and fall. Some outbreaks of enteroviral infection have continued from fall into winter months, winter outbreaks have been recorded, but they are rare.

Climate appears to be an important factor in the circulation and prevalence of enteroviruses (Melnick et al., 1979). Even within the climate range represented in the continental United States, healthy children in Southern cities harbor a greater abundance of enteroviruses and a wide variety of antigenic types than do those in comparable age in Northern cities. Repeated tests on the stools of 136 healthy preschool children in Charleston, West Virginia, over a period of 29 months indicated that 90 % of the cytopathogenic enteroviruses were recovered during the summer and the autumn months and that the incidence was 3 to 6 times higher in the lower socioeconomic than in the middle to upper class districts, 52 % of the viruses recovered belonged to the echovirus group. In areas farther south, such as Phoenix, Arizona (Melnick, 1975), and Louisiana (Gelf et al., 1957), the prevalence of enteroviruses among healthy children was more evenly distributed throughout the year but still markedly higher from May to October. Subsequent studies on enterovirus excretion rates in young children in Seattle, San Francisco, Minneapolis, Buffalo, Atlanta, and Miami (Froeschle et al., 1966; Gelfand et al., 1963) have confirmed and extended the earlier results: Higher levels of endemicity and longer periods of prevalence were found in the Southern cities.

Among the Atlanta and Miami children studied from 1960 to 1963 and the earlier investigations in Charleston and Phoenix (Melnick, 1957), average annual virus excretion rates were 7 to 14 %, and far larger numbers of enterovirus serotypes were commonly prevalent than in Northern cities. In the normal population under study in the New York Virus Watch Program (Kogon et al., 1969), only four to six serotypes of cytopathogenic enteroviruses were prevalent at any given period, and the enterovirus isolation rate in fecal specimens from young children (0 to 5 years of age) was only 2.4 %.

Among children living in warm climates and poor hygienic conditions, the incidence of infection with one or more enterovirus serotypes may exceed 50%, and mixed infections are common (Cooney et al., 1972; Parks et al., 1976). In a study of infants in Karachi, Pakistan-almost all of whom was less than 2 years of age approximately 80% of those tested yielded at least one enterovirus. Mixed infections may also occur in children living under good socioeconomic circumstances in temperate areas, but they are less frequent.

In some isolated groups, such as Eskimo communities (Banker and Melnick, 1951), the whole population may lack antibodies to some serotypes (Figure 3). In the survey depicted, not even the old persons, up to 72 years of age, had any serological evidence of past infection with either coxsackievirus B1 or coxsackievirus B2. Coxsackievirus A1 apparently had been present or had been in the very recent past.

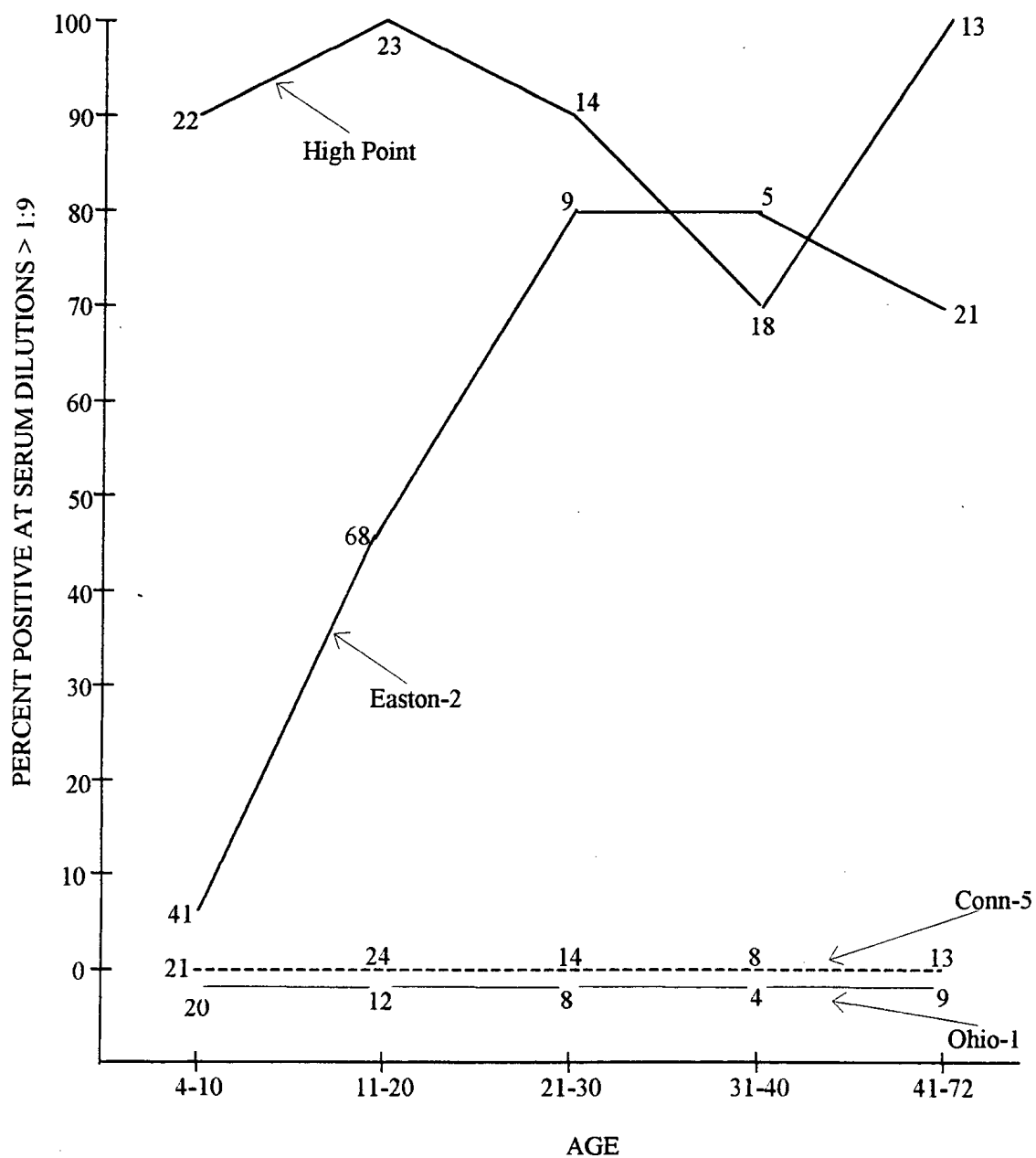


Figure 3: Results of neutralization tests with local Eskimo sera and coxsackieviruses expressed in terms of percentages of sera positive against five coxsackievirus strains (Banker and Melnick, 1951). The serotypes represented are coxsackievirus A24 (High point), coxsackievirus A 10 (Alaska), coxsackievirus A1 (Easton-2), coxsackievirus B1 (conn-5), and coxsackievirus B2 (ohio-1). The numerals at each point show the number of sera tested in the indicated age group.

However, a study conducted in the Accra of Ghana, at a time before polio vaccination was introduced in that country to any significant extent, indicated that infants and young children were experiencing widespread infections with all that of polioviruses (Pacsa and Werblinska, 1971), a pattern typical for a dense population living under poor hygienic conditions. Even among children less than 3 years old, 77% had antibody to poliovirus type 1 and only slightly lower portions had antibody to type 3, 80% were immune to all three types by the age of 6 years. In the same children, coxsackievirus A9 antibody developed at almost the same rates as poliovirus antibodies, 79% were infected by 3 years of age and 94% by the age of 6. Coxsackievirus B3, however, was less widespread, infecting only 52 % of the children by 4 to 6 years of age.

The beginnings of a global of enterovirus prevalence were drawn by Assaad and Cockburn, (Assaad and Cockburn, 1972), who analyzed the reports received by WHO for the 4-year period from 1967 to 1970 from laboratories around the world that participated in the WHO reporting system. This analysis was extended to cover 8 years, from 1967 to 1974 by Grist et al., (1978). Chiefly temperated regions were represented in these reports, which for the 8-year analysis included about 5,200 Coxsackieviruses of the B group, and more than 23,000 echoviruses. It was recognized that coxsackievirus A isolates were relatively few because few laboratories include mice in their isolation systems.

Most of the infections reported from 1967 to 1974 (Grist et al., 1978) were in children under 15 years of age; 85% of the coxsackievirus isolates, 79% of the coxsackievirus B, and 79% of the echovirus came from this age group. These overall percentages were virtually identical in both the 4-year periods, the total 8-year data changing by only 1 or 2 percentage points from those reports for 1967 to 1970.

With all the coxsackieviruses (both A and B groups) that were isolated in sufficient numbers in the 8-year tabulation, and with most of the echovirus serotypes also, the number of isolates from younger children (0 to 4 years) was greater than that for the 5-to 14-year-old group and constituted 62% of all the coxsackievirus A isolations reported, 54% of the coxsackievirus B, and 45% of the echovirus types 4, 6, 9 and 30, the younger children accounted for a smaller share of the infections,

whereas the 5-to 14-year-old group had 44, 40 and 50% respectively, of the isolations of these types, and the adults, 34, 22, 24 and 28%. Adults and other children also had a significant share of the coxsackievirus B infections reported (types 1 to 5), with B3 and B5 the most frequently isolated.

No regular early pattern, world wide, was observed in the recurrence of specific types. With each serotype analyzed, the number of reported isolations fluctuated greatly from year to year, and even larger fluctuations were observed for individual countries, e.g. of 154 coxsackievirus A9 infections reported by Japan from 1967 to 1970, all but were concentrated into 1967.

In this world wide survey, which tended to focus on virus isolations usually included aseptic meningitis, respiratory disease, skin eruptions, undifferentiated febrile illnesses, or gastroenteritis. In the combined reports for the 8-year period from 1967 to 1974, disease of the CNS—commonly aseptic meningitis was associated with 28% of the coxsackievirus A infections (and with nearly 50% of those identified as A9 infections), with 34 % of the coxsackievirus B infections (42% of those identified as infections with B5), and with 56% of the echovirus infections with much higher percentages of the infections with types 4, 6, 9 and 30: 81, 63 and 83% respectively. The previously recognized associations of specific syndromes to specific serotypes were also observed, e.g. Bornholm disease (pleurodynia) and myocarditis associated with Coxsackie B viruses particularly in persons over 15 years of age. Although cardiac involvement was more frequently associated with coxsackievirus B as a group, representing 3% of the coxsackievirus B isolations reported, a few cases of cardiac illness were reported with all the coxsackievirus A type tabulated and with most echovirus types, including 3% of the isolates of the echovirus 22. Skin eruption was the most frequent disease associations of coxsackievirus A isolates (especially hand-foot-and-mouth disease, seen mainly in children under 5 years of age), 33 % of the coxsackievirus A group infections generally, but 82% of A16 infections, 44% of A5 and 41% of A10 infections were associated with disease of the skin or mucosa or both. Respiratory illness accounted for about 20% of the coxsackievirus B isolations, but only about 12% of the coxsackievirus infections, however, with echoviruses 1, 11, 13 and 22, about 25 % of the isolation were associated with respiratory disease. There

were also small numbers of case of paralytic CNS disease reported in association with many of the types or each group.

Irrespective of the virus type, the largest number of cases of CNS disease was in children under 15 years of age, although substantial numbers of adults also had neurological involvement. The largest number of cases of respiratory illness was in children under 5 years of age. Children under 5 years of age predominated among the patients with serious CNS disease in the large outbreaks of enterovirus 71 (Bulgaria in 1975, Hungary in 1978), (Nagy et al., 1982; Shindarov et al., 1979).

Epidemiology of Enterovirus 70

A. Mode of Transmission

Transmission of conjunctivitis can take place by direct person-to-person contact or by indirect contact with inanimate objects such as face cloth, bath towels, handkerchiefs and bedding contaminated with feces, ocular and respiratory droplets via aerosols, and by fecal-oral route. In eye clinics, cross infection can occur by instruments such as forceps, tonometers, public basins used for washing purposes, and spring water have been implicated (Guadin et al., 1977; Arnow et al., 1977).

B. Distribution by Environment, Age, Sex, Ethnic Group, and Socioeconomic Status

There is no apparent seasonal influence on outbreak of AHC. In tropical countries, epidemics have occurred through the year. In countries where the climate is marked with seasons, epidemics have been reported in autumn, spring, summer and winter. Extensive epidemic involving tens of thousands of people occur mainly in developing countries where over crowding, unhygienic conditions, an hot and humid climate probably contribute to spread of disease. Epidemics in more developed countries, like those in Europe, involved a comparatively small number of people.

More males than females were affected with a predominance of older children and young adults. People of all ethnic groups and socioeconomic status were affected. Infection rate is densely populated areas.

The majority people contracted infection at home, others at work, schools, and a few from unknown sources (Chatterjee et al., 1970; Diallo et al., 1971; Quarcopome, 1973; Bagar et al., 1979; Nejmi et al., 1974; Metselaar et al., 1976; Chaturvedi et al., 1975; Yin-Murphy, 1972; Kono, 1975).

A survey carried out by Goh et al (1980), during the 1980 epidemic in Singapore showed that close contact was an important factor in transmission of conjunctivitis of 82 out of 118 home contacts who contracted conjunctivitis, 67.1% shared personal effects such as face-cloths, bath towels, handkerchiefs, pillows and bedding. The remaining 32.9% that did not share any personal articles also contracted infection. Hospital cross-infections were reported in London, England (Jones, 1972).

Historical Background

A. The First Wave: Distribution in 1969-71

The first pandemic of acute hemorrhagic conjunctivitis due to enterovirus 70 (EV 70) or Apollo 11 disease

In June 1969, Chatterjee et al (Chatterjee et al., 1970a; Chatterjee et al., 1970b) reported an epidemic of a new type of conjunctivitis in Accra, the capital of Ghana. They named it "epidemic hemorrhagic conjunctivitis" but it was nick named locally "Apollo 11 disease" because it coincided with the time of the Apollo 11 landing on the moon. Tree years later a similar disease reached Japan where ophthalmologists called it "acute hemorrhagic conjunctivitis (AHC)" since the incubation period is as short as 24 hours, the clinical course is rapid and subconjunctival hemorrhage is the most prominent and characteristic symptom (Sugiura et al., 1972). This disease was later found to be identical with the so-called Apollo 11 disease (Mitsui et al., 1972).

When the first epidemic wave of AHC hit Tokyo, Mie and Sapporo near the end of 1971, a team of Japanese investigators isolated a new picornavirus (Mirkovic et al., 1973) from conjunctival scrapings of patients with AHC. They reported that AHC virus, a causative agent, has the general characteristics of an enterovirus but is not neutralized by antisera in the intersecting pools of enterovirus immune sera (Chobel et al., 1973).

Since 1969 AHC has spread throughout the world except the Americas and Australia in pandemic fashion and that the same antigenic type of AHC virus has been isolated from patients with AHC in Asia, Africa and Europe (Yin- Murphy, 1973; Chombel et al., 1973; Jones, 1972; Higgins, 1973). It was named as enterovirus type 70 under the collaboration of WHO International Reference Center for enteroviruses (Mirkovic et al., 1973).

Pandemic spread in the world

The global spread of AHC is shown in Figure 4, apparently, there were two foci in the pandemic: Ghana and Java. The following information is based on reports from WHO and its regional offices, on published papers and on the Kono's observations in pandemic areas of Africa and Asia. Table 2 summarizes available incidence data for several geographic areas. The information is incomplete because reporting of non-fatal diseases such as AHC cases exceeded by far the figures shown in the table, and EV 70 may have affected several millions of people (Kono, 1975).

Table 2: Number of patients with AHC reported or treated and rough estimates of actual number of cases in different part of the world.

<i>Name of Country or City</i>	<i>Period</i>	<i>No. of Cases*</i>	
		<i>Reported or Treated</i>	<i>Rough estimates of the whole*</i>
Africa			
Accra, Ghana	June-Oct 1969	13,664	NA
Lagos, Nigeria	Sep-Dec 1969	12,799	Over 100,000
Morocco	Dec 1970 – May 1971	137,991	NA
Tunisia	Feb 1971	NA	Over 50,000
Sana, Yemen	Feb 1972	NA	30,000
Asia			
Singapore	Sep-Oct 1970	60,180	NA
Singapore	Jun-Dec 1971	38,150	NA
Hong Kong	Aug 1971	6,420	NA
Bangkok, Thailand	Jan-Nov 1971	2,105	NA
Japan	Aug 1971 – Feb 1972	NA	30,000?
Bombay, India	Mar-Sep 1971	NA	500,000
Calcutta, India	May-Jul 1971	NA	1,000,000

* - Data from published papers and WHO information

NA – Figures not available

? – Data is not confirmed

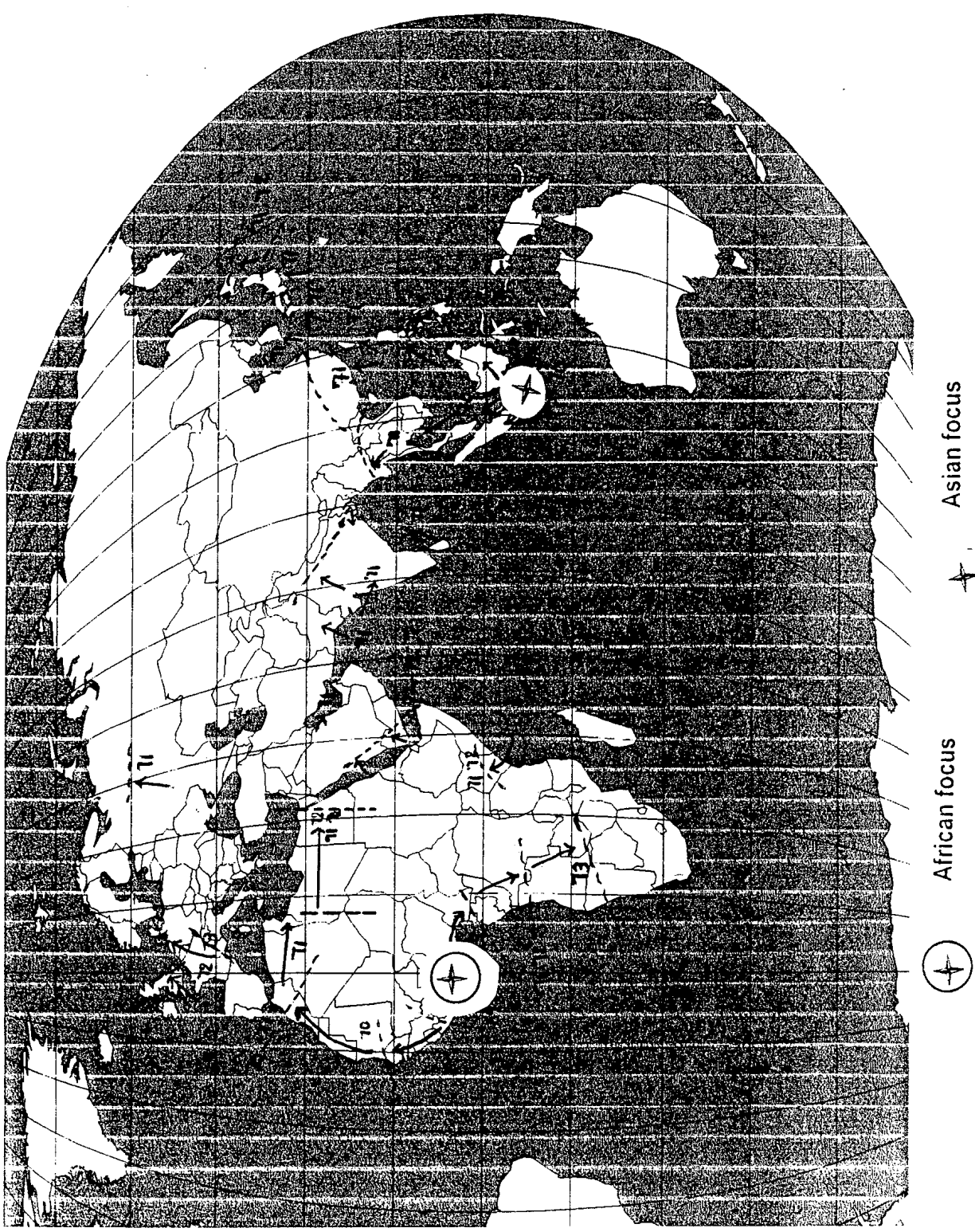


Figure 4: Trends of global of actual hemorrhagic conjunctivitis (1969-1973) indicating years of epidemic in different areas.

Africa: AHC appeared in June 1969, in the east suburb of Accra, Ghana (Chatterjee et al., 1970a; Chatterjee et al., 1970b). and spread along the coastal line of West Africa during 1969 and 1970. During 1969 the disease spread to the Ivory Coast, Nigeria and the Cameron (Akinsete, 1970; Akinsete, 1972; Parrot, 1971; Animashaun, 1970), while in Morocco the peak of the epidemic was December 1970 to 1971 (Bourdeeu, 1972). Epidemic periods for other countries in 1970 were Togo. Dahomey, Liberia, Sierra Leon, Gambia (October), (Akinsete, 1972), and (September to December), (Diallo et al. 1971).

AHC was first appeared in Kinshasa, Zaire in 1972 (unpublished data) from Morocco it went through Algeria, Tunisia, Libya and probably the UAR where, although not reported, local ophthalmologists indicated that there had been an epidemic in 1971 and 1972. In East Africa, AHC appeared in a coastal city of Kenya in 1971, spread to the inland and reached Nairobi near the end of 1971 and the beginning of 1972 (Manku, 1972). Apparently, the epidemic was imported by sea.

Asia: The Asian focus seems to have been Java, Indonesia, and the disease apparently spread from there to other parts of Asia. There was an unconfirmed rum of that AHC already existed elsewhere in the Country in Djakarta and Bali and also in Vietnam in 1969 but epidemiological confirmation has not been possible (Mitsui et al., 1972). However, there is no doubt about the epidemic witnessed by ophthalmologists in Djakarta (April to September), Bali and Surabaya in northern Java (July to December) in 1970, (Mitsui et al., 1972). These outbreaks are considered to have had a comparable scope and extent with those outbreaks considered to have had a comparable scope and extent with those in West Africa. AHC then invaded Singapore in September and October of 1970 (Lim and Yin-Murphy, 1971; Ray et al., 1973).

In 1971, the pandemic spread widely in Asia including all Indonesia, Burma, India, Pakistan, Sri Lanka, Malaysia, Vietnam, Cambodia, Laos, Philippines, Korea and Japan. The speed of its pandemic spread is comparable to that of Asian influenza.

In India AHC was first recognized in Mumbai in March 1971 (Wadia et al., 1973) it spread throughout India and Sri Lanka within that year (Wadia et al., 1973;

Bahrucha and Monndkar, 1972; Singha, 1972; Saxena et al., 1972; Ray et al., 1972; Pramanik, 1971; Bujarbura et al., 1972). Because the outbreak seems to have occurred earlier in the western part of the country it is doubtful if it came in from Indonesia and is believed to have been brought into Bengal by refugees from the Bangladesh war (Pramanik, 1971). Accordingly it was called "Joy Bangla Disease" or "Bangladesh conjunctivitis", (Pramanik, 1971). In Calcutta alone the number of cases is reported to have reached one million (Ray et al., 1972).

In other South Asian and Far Eastern countries, AHC appeared during August to December 1971 except on Bangkok, where it started in June (Dummavibhat et al., 1973). In Japan the first epidemic began in August 1971 in Kyushu in the southeast and spread northward, the peak was reached by October to December in Tokyo and December to January of the next year in Sapporo (Sugiura et al., 1972; Mitsui et al., 1972; Uchida, 1973; Kimura, 1973). China was also involved in the pandemic of epidemic of AHC in 1970-1971. Virus isolated in Shanghai.

Middle East: Although the Middle East is located geographically between Africa and Asia, it has been relatively spread by the epidemic. The first report of AHC came from Bahrain in 1971 but the epidemic in Yemen and Jeddah, Saudi Arabia, were reported in February of 1972 and in early 1973, respectively.

America and Oceania: North and South America seemed to be free at that time. However an AHC outbreak in Numea, New Caledonia, was reported was reported to have occurred from 1972 to 1974 (Pattgay, referred by Kono, 1975). Therefore, Oceania is no longer free of AHC.

Europe: In Europe, small outbreaks were noted in London (Jones, 1972; Higgins, 1973), Rotterdam, and Moscow (Maitchuck et al., 1973) in 1971. Particularly since 1972 AHC has been prevalent in France and Benelux, and rather isolated outbreak in Mustra-Sobota, Yugoslavia, 1973.

B. The Situation in 1972-1979

Most countries affected by the 1969-1971 pandemic continued to experience sporadic and periodic epidemic outbreaks of the first time. Collaborative investigations between laboratories confirmed the importance of CA 24v and EV 70 in AHC.

Year 1972: Countries or cities which reported epidemics in 1972 were Sana's (Yemen), (Maitchuk, 1975; Kono, 1975), Zaire (Desmyter, 1972) and Indonesia in January-February (Higgins et al., 1974, Thailand (Jayavasu et al., 1975), Singapore in June (Yin-Murphy et al., 1974) and Tunisia at the end of 1972 (Dawson et al., 1974; Whitcher et al., 1976). EV 70 was in these epidemics with the exception of the one in Singapore, where adenovirus 11 was dominant causative agent and EV 70 was confirmed in only a few cases.

Year 1973: Outbreak were reported in Jeddah, Saudi Arabia, in February (Melnick, 1975; Kono, 1975), in Lyon, France (Mirkovic et al., 1973), and in Dunkirk, Belgium, in the summer of 1973 (Desmyter et al., 1974). Outbreaks also occurred in Yugoslavia (Likar et al., 1975), Algeria (Gaudin et al., 1977) and Japan (Minami et al., 1976). EV 70 was implicated in all these epidemics with the exception of AHC in Dunkirk where adenovirus 19 was incriminated.

Year 1974: Epidemics were reported in Rome, Italy, in March (Muzzi et al., 1975), Kota Kinabalu, Sabah (Malaysia), in May (Kan et al., 1977), Thailand in July (Thongchareon et al., 1978; Phuapradit et al., 1976) and Japan in 1973 (Aoki et al., 1979). EV 70 was again implicated with the exception of Italy where adenovirus 4 was incriminated. A small outbreak of AHC caused by adenovirus 19 was reported in Seattle, Washington (Taylor et al., 1975).

Year 1975: The CA 24 variant resumed its dominance over EV 70 in epidemics from years 1975-1979. Extensive epidemics caused by CA 24 occurred in Singapore (Yin-Murphy et al., 1976; Lim and Yin-Murphy, 1977; Doraisingham, 1975), Hong Kong, Chang et al., 1977, Bangladesh, Sri Lanka (Higgins and Chapman 1977), Brunei (Bahrain et al., 1976) and Bangkok (Thongcharoen et al., 1978), in the

second half of the 1975. Vellor had a concurrent outbreak of CA 24v and EV 70 in SUMER (Christopher, 1977), and Lucknow an EV 70 conjunctivitis epidemic in July (Mathur et al., 1977). A localized outbreak of mixed adenovirus 11 and EV 70 conjunctivitis occurred among some Vietnamese in May (Arnow et al., 1977).

Year 1978-1979: No report of major AHC epidemic is available for 1976 and 1977. CA 24v reappeared in Malaysia in May (Tan et al., 1980), Singapore in August and Sri Lanka in November, 1978, Poona in May, Bombay in August (Gogate et al., 1981), and Vellor in September 1979 (Christopher et al., 1982).

C. Distribution in 1980-1982

Year 1980: EV 70 was implicated in epidemics in Nairobi, Kenya, in April (WHO Wkly epidem 1980), Singapore in July (Goh et al., 1981), and Malaysia in November 1980 (Tan and Lee, 1981). A small localized outbreak among Southeast Asian refugees occurred in Oakland, California, in July (CDC, 18).

Year 1981-1982: expectation of a pandemic spread of AHC was feared when rapid and extensive outbreaks appeared in the Americas, EV 70 was responsible for epidemics in Lagos in January, New Delhi, Calcutta, Madras, Bombay, Vellor, and Pakistan in May (John et al., 1981) and Zaire in June (Desmyter et al., 1981). Democratic Yemen in August (WHO Wkly epidem 1982), and the United Emirate in September 1981. Several strains of EV 70 were isolated from Pakistan. Serodiagnosis showed neutralizing antibody to EV 70 in patients in the remaining countries.

The first epidemic in the Americas occurred in Brazil, it rapidly spread to Colombia, Belize, Guyana, Panama, Costa Rica, Nicaragua, Honduras, El Salvador, Guatemala and Mexico in South and Central America, Florida and North Carolina in the United States, Trinidad, Tobago, Barbados, Antigua, Dominican Republic, Bahamas and Cuba in the Caribbean in June-September 1981 (CDC MMWR, 1981), then to the Pacific Islands, Fiji, Tonga, Samoa, American Samoa, Guam, Saipan, Solomon, Cook, Wallis, Niue, Tokelau, Tuvalu, Nauru, French Polynesia, and many imported cases in Hawaii (WHO Wkly epidem 1982), at the end of 1981 to the beginning of 1982.

Outbreak of AHC have also been reported in New Zealand in January 1982 (WHO weekly report. The majority of epidemics, which occurred during 1981-1982, were assumed to be caused by EV 70 on clinical evidence.

In one of the in Lagos, Nigeria covering 31,366 children from 20 primary schools, 4372 had AHC from the second week of October to December 1969. The morbidity rate of the 5 to 6 year-old group was 9% (8.6% in males and 9.5% in females), it increased to 15.3% (14.6% in males and 16.1% in females) in the 11 to 12 year-old group and reached 34.6% (32.6% in males and 37.6% in females) in those over 13 years old (Salami, 1971)

Salami's study indicated that the younger age-groups are less likely than older children to develop clinically recognizable AHC are. Female morbidity rates were usually higher but Salami suspected that these might be due to underreporting of boys.

As shown in Table 3 children under 10 constituted a smaller percentage of cases than older age groups. The highest proportions were in the 10 to 20 year-old groups in Bangkok and Singapore and in the 20 to 30 year-old groups in India, Nairobi and Japan. A relatively higher proportion of older patients was observed in Japan. These differences may reflect community wide spread in tropical areas and eye clinic-centered ~~indogenic~~ spread in Japan (Sugiura et al., 1972; Manku, 1972; Lim and Yin-Murphy, 1972; Bujarborua et al., 1972; Uchiday, 1973; Kimura, 1973).

Table 3: Age distribution of patients with AHC under treatment at ophthalmologic clinics in the first outbreak of the different countries

Age groups	Singapor 1971, 159 cases * (%)	Bangkok 1971, 2105 case † (%)	Gauhati, India 1971, 409 cases ‡	Nairobi, 1971-72, 150 cases ¶	Age groups	Tokyo, Singapore 1971-72 cases % ≈
10 or under	2.5	13.1	6.4	8	0-9	4.0
11-20	33	30.5	21.8	9.3	10-19	9.5
21-30	20	24.4	38.1	40	20-29	21.8
31-40	20	14.2	19.8	16	30-39	18.5
41-50	12.5	9.3	8.5	16	40-49	18.5
51-60	10.6	4.5	3.7	4	50-59	9.5
61-70	1.2	2.9	1.0	5.3	60-69	10.5
71 over		1.0	1.0	1.3	70 or over	8.5

* Lim and Yin-Murphy, 1973

† Dumavibhat et al., 1973

‡ Bujarborua et al., 1972

¶ Manku, 1972

≈ Suigura et al., 1970; Uchida 1973; Kimura 1973

In contrast to the above, post-epidemic antibody prevalence was highest in children under 9 years of age in Pontianak, West Borneo (Kono et al, 1975), as well as in Ghana, Africa (Gear, 1955).

These observations suggest that infants and children may experience higher infection but lower clinical attack rates. Typical hemorrhagic conjunctivitis was very rare in babies, even in a household outbreak, and those clinical symptoms were milder in children (Kono R, 1975). This pattern of differing susceptibility to infection with age resembles that seen in a "virgin soil" outbreak of poliomyelitis (Gear, 1955) and in the 1918-1919 pandemic of "Spanish" influenza (Burnet, 1960).

Family outbreak was very common in the tropical developing countries. A house-to-house survey by Bargar cited by Akinsete, (1972) identified 582 patients in 154 households (3.8 cases per family) among 1544 inhabitants in Free Town, Sierra Leone. Family attack rates were found to be proportional to the number of persons per room: 20.3% in households with 1.9 persons per room but 48% in those with more than 3.5 persons per room. These data shows that over crowding favors spread of AHC virus. Parrot reported an attack rate of 24% among Nigerian junior staff, laborers and other low echelon personnel but rate of only 6% and 2% respectively, among Nigerian and expatriate managers in the United America Co. Outbreak in Lagos (Parrot, 1971). Indeed, in all epidemic areas, attack rates were highest in the lower socio-economic classes (Saxena et al., 1972). Chatterjee et al reported that stringent hand washing was successful in preventing infection of hospital staff and nurses (Chatterjee et al., 1970). Apparently, both individual and environmental sanitary conditions exert an important influence on spread of the disease.

It is highly likely that the main mode of transmission is person-to-person contact via contaminated hand by eye discharge (Pramanik and Bangla, 1971), but indirect transmission may be possible through contaminated vehicles such as towels. In eye clinics the most probable source of infection seems to be the contaminated hands of ophthalmologists, ophthalmologic instruments may also contribute to the spread. A fecal-oral route of transmission is considered to be unlikely at present, since there is no definite proof that alimentary tract supports massive multiplication of EV 70 virus. The possible role of swimming pools does not appear as incident as in the

case of pharyngo conjunctival fever due to adenoviruses. However, it has been reported that holy water regarded as curative of eye diseases and a public basis for face washing transmitted AHC in some tropical countries (Agarwal and Mahajan, 1972).

There seems to be no consistent seasonal pattern since AHC was not only prevalent in the tropical climate but also in the winter Hokkaido, Japan, where the outbreak continued during subzero temperatures.

Consideration of the possible origins and vehicles by which the AHC pandemic spread

Influenza apart, there is no recent parallel to this emergence of a new virus causing so large a pandemic. It is very likely that, at the beginning, there were two epidemic foci, Ghana and Java, located about 13,000 km apart, from which the virus spread in a pandemic fashion to other African and Asian countries. A conceivable common denominator of the two foci is Islam. One suggestion is that the Moslem pilgrimage may have played a part in the initial spread and thus the two foci have emerged. It is possibility that AHC was imported to India by Haj pilgrims (Singha, 1972; Agarwal et al., 1972; Pramanik, 1971).

As it is unlikely that an antigenically unique virus has emerged at two remote places about the same time independently, it must have originated at another focus before the Ghanaian and Indonesian epidemics in 1969 and 1970. Two possibilities could be assumed: a) AHC might have existed endemically in Saudi Arabia and spread to the second focus during pilgrimages, and b) somewhere either in West Africa or in South East Asia there might be an original focus. The first possibility seems to be difficult to defend since country-to-country spread of AHC was so obvious early (1969-1970) in West Africa with subsequent appearance as late as 1972 and 1973 in the Middle East. If the pilgrimages played a role in AHC spread, Saudi Arabia might be nothing but a point of junction.

With regard to the second possibility, viewed from chronological aspects and the scope of the initial outbreaks, the Ghanaian outbreak in 1969 was earliest; a

comparable one ensued in Java and Singapore the next year. Thus it may be more reasonable to attempt to trace the original focus of AHC to West Africa. One possible solution may be obtained by seroepidemiologic investigations of human and animal sera collected in West Africa and Indonesia before and after the pandemic.

The finding that animal sera, cattle and horse sera so far examined, containing neutralizing substance to AHC virus, suggests that an AHC-like virus may exist in animals (Kono et al., 1975). Polio and other enteroviruses have often been recovered from domestic animals (Grew et al., 1970) and it has been shown that the poliovirus neutralizing substance in sera of cattle and horses consists of both specific antibodies and nonspecific inhibitors (Usawa et al., 1968). Thus, analogous to the postulated origin of pandemic influenza strains, such an enzootic animal picornavirus might have been "humanized", creating a pandemic eye disease (Webster and Laver, 1972).

Like pandemic influenza originating from Southern China, pandemic eye disease has often started from Africa and Middle East, and been brought to Europe by repatriated soldiers or Mohammedan invaders in the past. Infectious eye diseases, viral, bacterial, fungal or parasitic, are prevalent in this area. Obviously, the environmental conditions must be favorable for prevalence of eye diseases: sand storms, strong sunshine, hot weather, poor sanitation, close proximity to domestic animals and abundance of flies could be predisposing factors. Exposure of the new born to animals, a condition that is considered to be necessary for "humanization" of animal virus (Kaplan, 1972) would also be likely. The following historical compilation seems to support the view of West African origin of AHC.

Egyptian ophthalmia during the time of the Napoleonic Campaign in Egypt is the most notable among pandemic eye diseases. By review of the old literature it is suggested that Egyptian ophthalmia was not a simple disease and consisted of at least three kinds of eye afflictions (Meyerhof, 1932; Law, 1939; Tower, 1963), a) acute conjunctivitis believed to be caused by Koch-Weeks bacilli, b) gonorrheal ophthalmia, and c) Trachoma. The first category, acute conjunctivitis due to Koch-Weeks bacilli might involve acute hemorrhagic conjunctivitis.

Another interesting episode occurred in 1819 and is known as "the voyage of the *Rödeur*" (Synder, 1964). The *Rödeur* was a French ship carrying slaves from

Nigeria to the West Indies when a severe eye disease occurred abroad. The disease spread from a Nigerian slave to companions and crew. Record show that she met another slavy ship of Spanish nationality named "Leon" and a similar eye disease disabled that crew. The episode tells us that a highly contagious eye infection probably existed in Nigeria at the time and has been brought abroad the two ships by the native people. It is impossible to establish the true etiology now. The clinical features and outcome described in these early reports seem to be more severe than present-day AHC, but it might take such a severe clinical course if bacterial infection complicated the illness. Akinsete reported that AHC took an unusually severe course in some patients who received improper medical treatment (Akinsete, 1970). The pandemic of AHC raises many interesting problems of epidemiologic significance and reminds us that constant of lance is still necessary against the existence of new virus disease other than influenza.

Some Biological Properties of EV 70 and CA 24v

Cytopathogenicity for Cell Cultures

CA 24v and especially EV 70 produced cytopathogenic effects in a variety of tissue cell cultures. For isolation from clinical specimens, cells of human origin are preferred to cells of simian origin. However, once established in human cell lines, some strains of CA 24v and EV 70 can be adapted to grow in simian cell cultures (Nejmi et al., 1974; Lim and Yin-Murphy 1973a; Yin-Murphy, 1973b; Thongchareon et al., 1978; Chang et al., 1977; Higgins and Chapman, 1977; Bahrain et al., 1976; Tan et al., 1980; Tan and Lee, 1980; Hatch et al., 1981; Yin-Murphy and Lim, 1982). Higgins and Scott isolated several strains of EV 70 in human fetal organ cultures derived from conjunctival, nasal, and tracheal epithelium, whereas primary monkey kidney, HeLa, human fibroblastic lung (W138) and human embryonic kidney cell cultures when inoculated with clinical specimens failed to yield virus (Higgins and Scott, 1974). It has been reported that EV 70 strains grew well and caused cytopathogenic effects in corneal conjunctival cell cultures prepared from monkey, rabbit and mouse but CA24 strains replicated and caused cytopathogenic effects in monkey corneal conjunctival cell cultures only (Langford and Stanton. 1980). Very recently Maiteryi et al., reported primary isolation of EV 70 using Hep-2 cells (1999),

also in another case research using the same cell has been reported (Shulman et al., 1997).

Antigenic Variation in Cell Cultures

Strains of CA 24 and EV 70 established in HeLa-T subline when passed into simian cell cultures undergo antigenic variation. The simian-cell-derived strains were poorly neutralized by homologous antibody raised against the 'parent' virus grown in HeLa, due to an antigenic broadening of the simian-cell-derived virus. Invariably, the neutralizing antibody titers for both CA 24 and EV 70 strains grown and tested in HeLa-T cells were 8-to 16-fold higher than for strains passaged and tested in Vero cells (Mirkovic et al., 1973; Yin-Murphy and Lim, 1982). Consequently, strains adapted in simian cells may escape identification with antisera prepared with strains established in HeLa when the antisera are used at the screening dilution of 1/20.

Plaque Formation

CA 24 produced plaques of 3-4 mm diameter in monolayers of HeLa cells with agar overlay whilst EV 70 produced minute irregular plaques of 1-2 mm diameter (Higgins et al., 1974). Esposito and et al, grouped EV 70 into strains with three plaque sizes, 1.0, 2.5, and 0.5 mm diameter, in human embryonic lung fibroblast cells overlaid with Eagle's minimal essential agarose medium (Esposito et al., 1974). Addition of zinc chloride to infected tissue cell cultures inhibited virus plaque development and interfered with post-translational cleavage (Esposito and Obijeski, 1976).

Effect of Temperature on Virus Replication

Stanton et al. found that both CA 24 and EV 70 grew best at 36°C normal temperature of the eye and body. Replication of both viruses were inhibited approximately 90% at 37-39 C, depending upon the cell type. Both EV 70 and CA 24 viruses can adapt to replicate at higher temperatures. When isolates were grown at 37-40.5 C, a virus population was selected that grew better at these temperatures and poorly at 33 C. These investigators suggested that the ability of these viruses to adapt

to replication at higher temperatures may explain their rare spread to the central nervous system where they may give rise to neurological symptoms (Stanton et al., 1977).

Pathogenicity for Laboratory Animals

Intracerebral Inoculation of Mice

Adult mice do not succumb to intracerebral inoculations with CA24 and EV 70. Whereas EV 70 strains are non pathogenic for suckling mice, strains of CA 24 varied in pathogenecity for suckling mice (Yin-Murphy, 1973a; Yin-Murphy, 1973b; Yin-Murphy, 1973c; Wei et al., 1974; Jayavasud et al., 1975; Gogate et al., 1978; Higgins and Chapman, 1977; Higgins et al., 1974). Furthermore, mouse colonies reared in different laboratories varied in susceptibility to CA 24 (Yin-Murphy, 1973; Higgins et al., 1974). It is reported that virus was present in the brain and torso of mice paralyzed between 7 and 21 days of inoculation (Higgins et al., 1974). Also loss of striation and fragmentation of fibers in the voluntary muscles of mice inoculated with CA 24 has been observed. The muscle fibers were found to be eosinophilic and infiltrated with mononuclear cells, polymorphs and eosinophils as seen in a typical Coxsackievirus A infection (Gogate et al., 1978).

Intraspinal and Intrathalamic Inoculation of Monkeys

Kono and co-workers reported that monkeys inoculated with EV 70 intraspinally and intrathalamically developed paraplegia or monoplegia of lower limbs. Histopathological examination of the lumbar and cervical cords showed varying degrees of neuronal destruction and prevasculumbar cuffing. The lesions were most severe in the anterior horn of the lumbar cord. Although the virus could not be re-isolated from the central nervous system and other tissues, the morphology showed neutralizing antibody rise to EV 70. These findings agree with neurological manifestations following AHC observed in man (Kono et al., 1973).

Conjunctivitis in Experimental Animals

EV 70 instilled into the eyes of rabbits and monkeys gave rise to conjunctivitis. No hemorrhage or corneal involvement was observed. The

conjunctivitis resolved within 10 days. One monkey developed muscular weakness after the conjunctivitis had subsided, and died 28 days after inoculation (Jayavasu et al., 1978). In contrast, CA 24 instilled into the eyes of monkeys, hamsters, guinea pigs and mice showed no ill effects (Gogate et al., 1978).

Hemagglutination

Higgins et al., reported that CA 24 did not hemagglutinate human group 'O' and fowl red blood cells at 4° C, room temperature and 37° C (Higgins et al., 1974). Esposito and co-workers found EV 70 strains unable to hemagglutinate rhesus and African green monkey, rat, guinea pig and human 'O' red blood cells at 4°, 20° and 37° C and at pH 5.8 and 7.2 (Esposito et al., 1974). It has been stated that EV 70 hemagglutinated human 'O' guinea pig and chicken red blood cells at 4° C and room temperature. A sufficiently high concentration of the virus was said to be critical for hemagglutination to take place (Kono et al., 1978).

Tear Interferon and Sensitivity to Interferon

Interferon (IF) ranging from $10^{1.5}$ to $10^{3.3}$ U/ml was detected in tears samples on the 1st to 3rd day after onset of conjunctivitis by CA 24v (Langford et al., 1980). The IF activity is completely neutralized by antiserum to human fibroblast IF. Like the human fibroblast and leukocyte interferons produced in vitro, the IF activity in tears protected human and rabbit but not mouse cells. It was found that the sensitivity of CA 24v and EV 70 strains to fibroblast IF was 0.0 to 16 times as sensitive to the vesicular multiplicity of infection (Stanton et al., 1977).

Immunity Following Natural Infection

To diagnose 4-fold or greater neutralizing antibody rises to CA 24v and EV 70, it was frequently demonstrated that, the collected sera 3 to 4 week post-conjunctivitis was indicated to contain low neutralizing antibody titers i.e. 1:20 or 1:40, without a 4-fold rise. Despite repeated epidemic and sporadic outbreaks of CA 24 and EV. 70 conjunctivitis in Singapore, the sera sampled at the acute stage of conjunctivitis did not have neutralizing antibody titer i.e.(less than 1: 20) to either

enterovirus (Yin-Murphy and Lim 1972; Goh et al., 1981). The absence of neutralizing antibody in acute serum samples was also observed in other countries with recurrent AHC outbreaks. A serological survey carried out by Chang et al., in late 1975 showed that despite an extensive AHC epidemic in the summer of 1975, there was a low prevalence of immunity to both enteroviruses in the local population (Chang et al., 1977).

AHC and Post-Conjunctivitis Neurologic Complications

Rare cases of radiculomyelitis following AHC were observed during the 1971 and 1981 epidemics in India (Wadia et al., 1972; Wadia et al., 1973; Wadia et al., 1981; John et al., 1981; Bharucha et al., 1982), the 1971 epidemics in Taiwan (Hung et al., 1976; Green et al., 1975), and the 1974 epidemics in Thailand (Phuapradit et al., 1976; Kono et al., 1977). Neurological symptoms most commonly developed from 2 days to 1 month after conjunctivitis. However, a few cases of neurological symptoms developed whilst the conjunctivitis was still present. There were also radiculomyelitis patients with no history of AHC but who had been in contact with household members who had AHC. With few exceptions, neurological complications involved adult patients.

The initial neurological manifestations were radicular pains and acute flaccid motor paralysis or paresis. The onset, distribution of flaccid paralysis and early development of muscular wasting indicated involvement of the anterior horn cells of the spinal cord. This contention was supported by electromyelography and nerve-conduction studies. Paralysis affected the lower limbs more commonly and more severely than upper limbs. Recovery was gradual, and symptoms in some cases persisted for several months. In severe cases, permanent incapacitation of lower limbs resulted from muscular atrophy and paralysis in the affected proximal muscles. Pleocytosis ranging from 11 to 270 per cubic millimeter was demonstrated in the majority of patients when the cerebrospinal fluid was examined within the first 3 weeks post-infection up to the 7th week or later (Bharucha et al., 1982; Wadia et al., 1972; Hung et al., 1976; Phuapradit et al., 1976; John et al., 1981; Wadia et al., 1973; Wadia et al., 1981; Green et al., 1975; Kono et al., 1977; Kono et al., 1981).

The pre-paralytic phenomena, the prognosis of flaccid motor paralysis, and the changes in the cell count and total protein level of the cerebrospinal fluid are said to be reminiscent of acute poliomyelitis.

Other neurological complications associated with AHC are palatal paresis, observed in Lucknow in 1971 (Saxena et al., 1972), and facial palsy resembling Bell's palsy, seen in Bombay in 1981 (Wadia et al. 1981). A 12-year-old girl developed Bell's palsy temporarily following AHC in North Carolina, United States, in 1981 (CDC, 1981).

Neurological complications have so far been associated only with conjunctivitis caused by EV 70. Although no virus has been isolated from the conjunctiva or cerebrospinal fluid of these patients, neutralizing serum antibody titers of 1:16 to 1:512 to EV 70 were observed, and in some cases, 4-fold antibody rises were demonstrated (Wei et al., 1974; Wadia et al., 1981; Green et al., 1975; Kono et al., 1977; Kono et al., 1981). John et al., (1981) reported the presence of neutralizing antibody in the blood serum and also in the cerebrospinal fluid of patients with poliomyelitis-like neurological illness during the 1981 epidemic in Vellore. Similar observations were made by Kono et al., (1981) in Bombay. The presence of neutralizing antibody in the cerebrospinal fluid was said to be a further proof that the virus did invade the nervous system.

During the 1981 Indian EV 70 conjunctivitis epidemic, Katyar and co-workers studied on 79 patients with neurological complications were evaluated by clinical, electro-physiological, histopathological and virological investigations. The above events occurred in an orderly sequence. The paralysis affected the cranial nerves, limbs or both. The clinical course closely simulated poliomyelitis, without any evidence of radiculopathy or myelopathy.

The electrophysiological and histological studies pointed towards the affection of anterior horn cells. The raised neutralizing antibody titers to enterovirus 70 confirmed the etiology of the disease. On the available data, the investigators believe that this nosological entity should be considered under the general title of "polio-like syndrome" following EV 70 conjunctivitis (Katyar et al., 1983).

Laboratory Diagnosis of EV 70 and CA 24v

A. Isolation of Virus

CA 24 v and EV 70 can be isolated from conjunctival swabs and scrapings, tears, throat swabs and gargling, rectal swabs and feces. Samples collected in Hank's balanced salt solution (HBSS) with 1M Mg Cl₂ and antibiotics can be stored frozen at -20° C.

For primary isolation, tissue cell cultures of human origin are preferred to simian cell cultures. A HeLa subline known to be susceptible to both CA 24 and EV 70 would be the obvious choice. Human embryonic lung and human kidney cells have been used with some success. When these cell cultures fail, one may have to resort to less easily available human fetal organ cultures derived from conjunctival, nasal, and tracheal epithelium. For isolation and identification, the procedures recommended for enteroviruses by Melnick et al should be adapted (Melnick et al., 1979).

On the whole, isolation of CA 24v is more easily achieved than that of EV 70. The EV 70 strains may require several passages in cell cultures before cytopathic effects become apparent. Frequently, EV 70 strains produce cytopathic effects in the initial tissue culture passage but fail to replicate in subsequent passages. Difficulty in isolating the causative agent of AHC is exemplified by the 1981-82 epidemics in the Americas where apart of few strains of EV 70 from Key West and Miami, Florida, isolates from many other affected areas are still not available (Yin-Murphy, 1984).

B. Serological Tests

Neutralization test

Paired sera are taken, at the acute and convalescent stages of infection. The diagnostic 4-fold or greater rise in neutralizing antibody to CA 24 or EV 70 is more readily observed when the first serum is taken at the onset of conjunctivitis and the second serum is obtained 21-28 days after.

Demonstration of neutralizing antibody is performed by the conventional tissue culture tube neutralization test (Melnick et al., 1979). The micro-metabolic inhibition test is more economical and suitable for coping with large numbers of sera during an epidemic (Yin-Murphy, 1976). All sera should be tested against CA 24v and EV 70 to confirm the etiology of AHC. Where a picornavirus etiology is excluded, sera can be screened against adenovirus 11, 19, and 4, which are known to give rise to AHC syndromes clinically indistinguishable from those caused by CA 24v and EV 70.

Hemagglutination-inhibition test (Kono et al., 1978), *immunofluorescence test* (Pal et al., 1988), *enzyme linked immunosorbent assay (ELISA)* (Philip et al., 1985), *immuno-electron microscopy (IEM)* (Ramia and Arif, 1990), *monoclonal antibody* (Yousef et al., 1987) and *immunohistochemistry* (Deshpande 1980) are used as serodiagnosis methods.

Treatment

There is no specific treatment for conjunctivitis caused by CA 24v and EV 70. Local application of broad-spectrum antibiotics and sulfonamide drugs are used to eliminate secondary bacterial infections. Hydrocortisone is used to reduce the inflammatory condition and to relieve discomforts in the eyes (Lim and Yin-Murphy, 1971; Dumavibhat et al., 1973; Tan and Lee, 1981). Complications such as chronic conjunctivitis and corneal uncertain have been observed following treatment with corticosteroids (Akinsete, 1970; Bagar et al., 1979). It is reported that although corticosteroids gave quicker relief, relapses of conjunctivitis around 4 to 5 times in some patients (Pramanik, 1971). He suggested that corticosteroids did not allow immunity to develop.

5-Iodo-2-deoxyuridine, recommended for treatment of adenovirus and herpesvirus conjunctivitis, is not effective on CA 24v and EV 70 (Maitchouk, 1972; Higgins et al., 1974).

Symptomatic relief is given for treatment of radiculomyelitis following AHC. Corticosteroids diminish the inflammatory reaction to other drugs. It has, however, no

obvious therapeutic effect on the paralytic symptoms (Pramanik, 1971; Saxena et al., 1972).

Enteroviruses and antigens associated with them are resistant to all known antibiotics and chemotherapeutic agents. Alcohol (70%), 5% Lysol, 1% quaternary ammonium compounds (Roccal), or similar laboratory disinfectants are not effective. These viruses are intensive to ether, deoxycholate, and various detergents that destroy other viruses (such as arboviruses, orthomyxoviruses, or paramyxoviruses). Treatment with 0.3% formaldehyde, 0.1 N HCl, or free residuale chlorine at a level of 0.3 to 0.5 ppm causes inactivation, but the presence of extraneous organic matter protects the enterovirus from inactivation (Task et al., 1945). Therefore, caution must be exercised in using the results of laboratory findings on chlorination of enteroviruses, either in purified or raw form under natural conditions.

A further cautionary investigation regarding echovirus type 1, which has been treated with chlorine, indicated that a significant part of the lost infectivity titer was regained while the pH of the medium adjusted to 4.5. Therefore, inducing aggregation of the virus was occurred. Apparently individual virions were not repaired, rather, there was a multiplicity-related increase in infectivity when the host cell received several damaged virions in a clump.

Viral Inhibitory Elements

2-(α -Hydroxybenzyl) benzimidazole (HBB) inhibits the cell culture propagation of most cytopathogenic enteroviruses. Exception are group A Coxsackieviruses 7, 11, 16 and 18 and echoviruses 22 and 23 (Tamm and Eggers, 1962). Guanidine also is a potent inhibitor of poliovirus synthesis (and the synthesis of other enteroviruses in cell cultures). However, viral progeny growing in the presence of guanidine become resistant to the drug (Melnick et al., 1961), and further passage results in selection of strains that are drug dependent. HBB and guanidine can act synergistically to protect newborn mice from a fatal infection (Eggers, 1976).

Enteroviruses are thermolabile. Exposure of these viruses to a temperature of 50°C destroys them rapidly. However, in the presence of molar magnesium chloride, only partial inactivation occurs in 1 h at 50°C (Wallis and Melnick, 1961). Enteroviruses are stable at freezing temperatures for many years, and they remain viable for weeks at refrigerator temperatures and for days at room temperature. Their inactivation at all environmental temperatures is inhibited by magnesium chloride, this property has led to the wide spread use of MgCl_2 as stabilizer of oral poliovirus vaccine (OPV) (Melnick et al., 1961).

Enteroviruses are rapidly inactivated by ultraviolet light and usually by drying unless special conditions are observed. Vital dyes (neutral red, acridine orange, proflavine), when incorporated into the structure of these viruses, render them readily susceptible to visible light (Schaffer, 1962; Wallis and Melnick, 1965).

Prevention

To prevent intrafamilial and epidemic spread, public education should be given on the mode of transmission of the disease, and good personal hygiene should be advocated. Exclusion of affected persons from schools, offices, factories, amusement and recreational centers, and use of public swimming pools, would help interrupt the chain of transmission. Nosocomial transmission can be prevented by segregation of patients with infectious eye disease, through hand washing before and after treatment of patients and proper sterilization of instruments used for the eye.

VIROLOGY

Classification

Of the more than 55 families of viruses recognized by the International Committee on Taxonomy of viruses (ICTV) in 1973, 17 contain viruses of animals and humans (Mathews, 1982). In addition, three more families have been proposed but not yet approved. An unresolved problem concerns the order of listing of the virus families; this problem again points out the arbitrariness of weighting virion characteristics for taxonomic purposes. The listing of the families containing human and animal viruses in Table 4(a) is in the order now used by the ICTV. Nucleic acid types, presence or absence genome, genome replication strategy, positive or negative sense genome, and genome segmentation. The latest classification of the Picornaviridae family in which originally based on physical properties (particle density and pH-sensitivity), serological relatedness, and more recently based on nucleotide sequence. The most recent revision of virus taxonomy has erected 9 genera within the family Table 4(b).

Table 4(a): The family Picoranviridae^a

Genus	Number of serotypes	Members
Rhinovirus	110	Human rhinovirus 1A-100, 1B, "Hanks"
	3	Bovine rhinoviruses 1, 2, 3
Enteroviruses	3	Human polioviruses 1, 2, 3
	23	Human Coxsackieviruses A1-22, 24 (A 23-echovirus 9) ^b
	6	Human Coxsackieviruses B1-6 ^c (swine vesicular disease virus is very similar to Coxsackie B5 virus)
	30	Human echoviruses 1-7, 9, 11-27, 29-34 Echo 8 is echo 1 ^b , Echo 10 is reovirus, type 1 ^{b,d} Echo 28 is human rhinovirus 1A ^b
	4	Human enteroviruses 68-71
	1	Vlyuisk virus
	18	Simian enteroviruses 1-18
	2	Bovine enterovirus 1, 2
	8	Porcine enteroviruses 1-8
Aphthovirus	7	Foot-and-mouth disease virus 1-7 (serotypes A, C, O, SAT-1, 2, 3, Asia-1)
Cardioviruses	2	Encephalomyocarditis (EMC) virus ^a , Theiler's murine encephalomyelitis (TME) virus (TO, GDVLL)
Hepatoviruses	1	Human hepatitis virus Af
Unassigned	3	Equine rhinoviruses 1, 2, cricket paralysis virus

^a The so-called feline picoraviruses are now classified in the family Caliciviridae

^b vacated numbers are now unused, Echovirus 22 is atypical in that it shows little sequence relationship to other picornaviruses (Hyypia et al., 1992).

^c Coxsackieviruses are named after Coxsackie, New York, the town from which the initial isolates were made.

^d Echo is an acronym for enteric cytopathic human orphan (Melnick JL, 1983).

^e Also mengovirus, Maus Elberfeld (ME) virus, Columbia SK virus, MM virus

^f Formerly classified human enterovirus 72.

Table 4(b): The family Picornaviridae as the latest classification

Family	Genus	Serotype	Type species	Hosts
Picornaviridae	Enterovirus	89[64H+25NH]	Poliovirus	Vertebrates
	Rhinovirus	103[100H+3B]	Human Rhinovirus A	Human, Bovine
	Hepatovirus	3	Hepatitis A virus	Vertebrates
	Cardiovirus	3	Encephalomyocarditis virus	Murine, Human
	Aphthovirus	8	Foot-and-mouth disease virus	Mainly cloven footed animals
	Parechoviruses	2	Human parechovirus	Human
	Erbovirus	1	Equine rhinitis B virus	Equine
	Kobuvirus	10	Aichi virus	Human
	Teschovirus	10	Porcine teschovirus	Porcine
	Unassigned	>25		Various species
	Total	At least 255		

The Picornaviridae are among the smallest riboviruses known, hence their name *Pico* (very small) *rna* virus. They also comprise one of the largest and most important families of human and agricultural pathogens. Foot-and-mouth disease virus, the most important single pathogen of livestock, was in fact the first animal virus to be recognized when Loeffler and Frosch in 1898 (Loeffler and Frosch, 1898) discovered that the causative agent passed through Berkfeld filters and was therefore much smaller than other microorganisms then known to transmit disease. That poliomyelitis was also caused by a virus was announced about a decade later by Landsteiner and Popper (Landsteiner and Popper, 1909), but it was not called poliovirus until around 1955 (von Magnus et al., 1955).

Picornavirus virions are non-enveloped, have icosahedral symmetry, and are 22 to 30 nm in diameter. Virions have 60 subunits in a T=1 arrangement. The genome consists of one molecule of positive sense infectious ssRNA of MW 2.5×10^6 . The viruses have four major polypeptides. Replication involves translation of a large precursor polyprotein and post translation of a large precursor polyprotein and posttranslational cleavage into functional polypeptides. Replication and assembly take place in the cytoplasm, and virus is released via cell destruction. The viruses have a narrow host range.

Studies on the molecular biology of picornavirus infection were ushered in by discovery that poliovirus could be propagated in cultured cells (Enders et al., 1949). Other crucial advances were the development of the plaque assay for infectivity (Dulbecco and Vogt, 1954) and of methods for purification and crystallization of poliovirus (Schaffer and Schwerdt, 1955; Steere and Schaffer, 1958), thus opening the way for structural analysis by the methods of X-ray crystallography. Determination of the nutritional requirements of cultured cells (Eagle, 1955) was also important. With defined nutrient media it became practical to radiolable viral macromolecules in synchronously infected cultures (Darnel, 1958) using precursors of known specific activity (Darnel and Levintow, 1961).

The discovery that isolated picornaviral RNA is infective (Alexander et al., 1958; Colter et al, 1957) facilitated the demonstration that the susceptibility of cells is correlated with the presence of specific receptors (Holland and Mc Laren, 1959).

Three additional discoveries, with important implications for molecular biology as a whole, were:

- (a) the demonstration of the first RNA-dependent polymerase (Baltimore et al., 1963)
- (b) the identification of a helical base-paired double stranded replicative form of viral RNA (Montagnier and Sanders, 1963)
- (c) The discovery that poliovirus synthesizes its gene products by proteolytically cleaving a large polyprotein (Summers et al, 1965).

Recognition that this cleavage was a special mechanism for dealing with the monocistronic character of eukaryotic mRNA as distinguished from the polycistronic character of many prokaryotic mRNAs (Jacobson and Baltimore, 1968).

The quest for effective vaccines, particularly against FMDV (Brooksby JB, 1982) and poliovirus (Bodaian and Hortsman, 1965; Cherry, 1988; Melnick, 1983; Paul, 1971), led to the discovery of a host of other polio like viruses, now numbering in excess of 255 serotypes [Table 4(b)].

The Picornaviridae family is currently divided into five genera: the rhinoviruses, the enteroviruses, the aphthoviruses (FMDVs), the cardioviruses, and hepatoviruses. Comparison of genetic organization, cleavage patterns, and sequence relationships suggests that this classification require further revision. Human rhinoviruses for example bear many sequence similarities to human enteroviruses and might well be classed as subgenera of a single group I (Figure 1). The murine polioviruses (Theiler viruses) are closely related in sequence and polyprotein structure to encephalomyocarditis viruses and are now classified as cardioviruses (Figure 1). Members of these five genera are morphologically indistinguishable by electron microscopy. However, they do exhibit distinctive surfaces at the higher resolution afforded by X-ray crystallography.

The enteroviruses, so-called because most inhabit the alimentary (enteric) tract, include the polioviruses (three serotypes), but also the coxsackieviruses (group

A and B), the echoviruses (31 serotypes), human enteroviruses 68-71 and number enteric viruses (Melnick 1976; Melnick 1983).

The human rhinoviruses, so-called because of their special adaptation to the nasopharyngeal region, are the most important etiologic agents of the common cold in adults and children (Dick and Inhorn, 1992; Gwaltney 1989; Kapikian 1969). Officially there are now 102 serotypes (Hamparian et al., 1987), including human rhinoviruses 1B and 'hanks' which was not assigned a number.

Most of the picornaviruses isolated from the human respiratory system are acid labile, and this liability has become a defining characteristic of rhinoviruses (Kapikian 1967; Melnick 1974; Melnick 1983; and Tyrrell and Chanock, 1963).

Aphthoviruses (FMDVs) infect cloven-footed animals, especially cattle, goats, pigs, and sheep, and rarely infect even humans. Seven immunotypes have been identified: types A, C, O, Asia-1, and the South African Territory types SAT-1, SAT-2, and SAT-3. Within these seven types, at least 53 subtypes have been designated by complement-fixation tests. The serological characteristics of the subtypes are sufficiently different to cause difficulty in classification and vaccination. The aphthoviruses are highly labile, being rapidly inactivated at pH less than 7 or 6.

The cardioviruses are represented by two serotypes. The encephalomyocarditis (EMC) viruses (encephalomyocarditis SK virus, ME virus, and mengovirus) represent one serotype. They are generally regarded as murine viruses, although their host range includes humans, monkeys, pigs, elephants, and squirrels, among others. The second serotype includes the Theiler's murine encephalomyelitis viruses (TMEV). They are divided into two groups, typified by strains called GDV1 and TO. The GDV2 viruses cause an acute polio like disease in mice. The TO viruses are less virulent and cause a chronic demyelinating disease resembling multiple sclerosis and have thus become important models for study of this and other motor neuron diseases (Lipton and Rozhon, 1986). Vilyuisk virus may be a divergent Theiler virus (Pritchard et al., 1992).

Physical Characteristics of the Virus Particle

The virion is a largely featureless, symmetrical particle, approximately 27 nanometer (nm) in diameter when examined electron microscopically. Non infectious empty capsids, unlike virions, are penetrated by strain, revealing that the virion shell is approximately 2.5nm thickness. The essentially spherical appearance of the particle is consistent with a virus with icosahedral symmetry, and this has been broadly confirmed by particle shadowing which produces pointed and blunt shadows. The sedimentation coefficient of the intact virus on centrifugation is 155-160 s, while that of empty capsid is 70-80s. The buoyant density of the virion in cesium chloride gradients is 1.34 gm/ml (Fout et al., 1984). A particle with the chemical composition of an enterovirus virion would be expected to have a density of 1.47 gm/ml if freely permeable to cesium ions; the low density of the enteroviruses implies that the virion is essentially impermeable consistent with the failure of electron microscopic stains to penetrate infectious particles. In the past, the behavior of the viruses on isopycnic centrifugation has been used as a criterion for differentiating enteroviruses from rhinoviruses and aphthoviruses, both of which have a higher buoyant density in caesium chloride.

Biochemical Structure of the Virus Particle

The infectious particle consists of a shell of 60 copies, each of four proteins, VP1, VP2, VP3, and VP4, arranged with icosahedral symmetry about a genome composed of a single strand of RNA of messenger sense. Like many eukaryotic messenger RNA molecules, the genomic RNA terminates in a sequence of 40-100 adenylic acid residues at the 3' end (the poly (A) tract). At the 5' end, however, it bears small protein (VPg) approximately 22 amino acids long, covalently linked to the RNA by the hydroxyl group of a tyrosine residue. The virion is believed to be entirely devoid of carbohydrate, and lacks a lipid membrane, although as described below lipid molecules form an integral part of the structure. The particle has a molecular weight of 8×10^6 , of which the RNA provides 32% and the protein 68% by weight. The four principal structural proteins of the virion have molecular weights of approximately 30,000 (VP1), 27,000 (VP2), 24,000 (VP3) and 7,000 (VP4). VP2 and

VP4 are formed by the cleavage of a precursor protein, VP0, as the last stage in the maturation of a virus particle, probably in an autolytic process resulting from the encapsidation of the nucleic acid genome.

The defined structure of the virus particle resulting from the absence of a lipid layer has made it possible to obtain crystals suitable for X-ray diffraction studies, and the molecular structures of poliovirus (Hugle et al., 1985), rhinovirus type 14 (Rossmann et al., 1985), and CVB3 (Muckelbauer et al., 1995) have been resolved such that the interactions involved in maintaining the structural features of the infectious virion are now clear. The three largest virion proteins VP1, VP2, and VP3 have a very similar core structure, in which the peptide backbone of the protein loops back on itself to form a barrel of eight strands held together by hydrogen bonds (the β barrel). This core forms a wedge shape, and the amino acid sequences between the sequences forming the β barrel, and the sequences at the N- and C- terminal proteins of the protein contain elaboration, which include the main antigenic sites involved in the neutralization of viral infection.

The proteins are arranged with icosahedral symmetry, with VP1 molecules at the pentameric apices of the icosahedron oriented such that the pointed end of the wedge-shaped protein points towards the apex. The other two proteins, VP2 and VP3, alternate about the center of the triangular face of the icosahedron (the three fold axis of symmetry). There are extensive interactions between the three large proteins, and also with the fourth protein, VP4, which is internal. In particular, the N terminus of VP1 lies under VP3, and the N terminus of VP3 under VP1. The apical structure formed by VP1 is separated from the plateau formed by VP2 and VP3 by a cleft or canyon; the peak of VP1 structure is 16.5 nm from the virion center whilst the plateau made up of VP2 and VP3 is approximately 15 nm from the center. The base of the cleft is of the order of 11 nm from the center, and it has been postulated that this cleft may contain the region of the virus to which the cellular receptor attaches. The interactions between the component proteins are such as to suggest that the most stable unit of the structure is a pentamer made up of five copies each of VP1, VP2, VP3, and VP4, it seems likely that this is the basic unit from which the virus is assembled.

In addition to the protein components of the virus, lipid molecules are also found in the structure. Myristic acid is found covalently attached to the N terminus of the smallest capsid protein VP4 (Chow et al., 1987). The aliphatic chain penetrates the apex of the icosahedron, and it is possible that this forms a structural support of the virion. Myristic acid has been found to be attached to virion proteins in other viruses including retroviruses and rotaviruses. Another lipid moiety has not yet been identified, but has been shown to be present in the structures of poliovirus type 1 and type 3. It has been a 16-carbon aliphatic chain that lies in the pocket formed by the beta barrel of VP1. The other end of the molecule has not been observed. Rhinovirus 14 has no such insert, and it is not yet clear how general a feature of enteroviruses this lipid molecule is. However, certain antiviral compounds insert into the corresponding region of rhinovirus 14, and in so doing prevent uncoating. It is therefore possible that lipid plays a part in uncoating or assembly of enteroviruses.

Antigenic Structure of the Virus

The antigenic properties of the enteroviruses provide one of the main methods of differentiating them. In most cases specific antiviral antisera neutralize only homologous virus, but this is not always so. For every picornavirus studied in detail, it has been shown that empty capsids prepared under appropriate conditions present major antigenic determinants different from those of the infectious virus. Moreover, the infectious virus can be readily denatured by relatively mild conditions, such as treatment with ultraviolet light, heating to 56° C for 10 minutes or attachment to a plastic surface in enzyme-linked immunosorbent assay (ELISA) Tests. The infectious particle is said to express D or N antigenic character, and the empty capsid or denatured virus C or H antigenic character. C antigen is less specific to a particular virus than is D antigen. Thus there is evidence for cross-reaction of C antigen-specific monoclonal antibodies with poliovirus of type 1, type 2 and type 3, whereas cross-reaction by neutralizing monoclonal antibodies or those reacting with D antigen is rare. However, monoclonal antibodies able to neutralize both type 1 and type 2 poliovirus have been reported (Uhlig et al., 1990) and priming for a secondary response to type 1 or type 3 by previous injection with type 2 is documented (Katrak et al., 1991). Similarly there is evidence for cross-reaction of echoviruses 11, 16 and

22 with poliovirus with appropriate C-specific antibodies. The difference between particles expressing D and C antigen is due to the conformation of the proteins, rather than to the loss of a protein. These antigenic characteristics suggest that methods based on virus neutralization by antibody may give different results concerning the identity of enterovirus strains to methods based on immunochemical techniques such as ELISA or immunoblot methods.

The antigenic structures involved in the neutralization of picornavirus infectivity have been the subject of much study and have been clarified by the resolution of the molecular structures of poliovirus and rhinovirus outlined above. The similarities in the structures, which have been found to be important in neutralization of these two types of virus, suggest that homologous features may be involved in all picornaviruses. Sites have been identified by the isolation of antigenic variants, which are resistant to monoclonal antibodies able to neutralize a parental strain, and then characterized by sequencing the genomic RNA. This has been revealed four major sites to which neutralizing antibodies bind. For type 3 poliovirus they include: (a) a sequence of VP1 about one-third of the way in from the N terminus involving residues 89-100, together with sequences from three other loops from VP1, including amino acids 142, 166 and 25; (b) a complex site involving residues 286-290 of VP1 and 58-60 of VP3 (Minor, 1990). The fourth site involves residues in different pentamers and is therefore only found in the intact virion, while the other sites are also found in some viral subunits. For poliovirus type 1, the site located at residues 89-100 of VP1 has been implicated in virus neutralization, although to a far lesser extent than for type 3 where the homologous region is the principal target for neutralizing antibodies in sera from immunized animals. The site involving VP3 is the next most common target of antibodies, including most of the antibodies showing extreme strain specificity for poliovirus, whilst the site involving VP2 is the least common.

The antigenic structure of type 2 virus has been less well characterized. The sites to which cross-reactive antibodies bond appears to involve sequence around 200-210 of VP3 and 239-245 of VP2, and may be linked to the strain-specific site involving VP1 and VP3 (Uhlig et al., 1990).

Cellular Receptor Sites

A growing number of picornavirus receptors have been characterized, and it appears that most are molecules involved in cell-cell interactions or in the regulation of cell function (Bergelson and Fiberg, 1993; White and Littman, 1989). Molecules belonging to the immunoglobulin (Ig) superfamily serve as receptors for the major-group human rhinoviruses [intercellular cell adhesion molecule-1, ICAM-1 (Greve et al., 1989; Staunton et al., 1989)], for poliovirus (Mendelson et al., 1989), and for a variant of encephalomyocarditis virus (Hubber, 1994). Echoviruses 1 and 8 attach to integrin VLA-2 a known surface molecule (Bergelson et al., 1992; King et al., 1995) and $\alpha_3\beta_3$ integrin is reported to be the receptor for Coxsackievirus A9, echovirus 22 (Roivainen et al., 1994), and foot-and-mouth disease virus (Berinstein et al., 1995). Minor-group human rhinoviruses bind to cells via members of the low-density lipoprotein receptor family (Hofer et al., 1994).

Enteroviruses attach to and enter cells by specific receptor sites. This was first shown for poliovirus by the observation that purified genomic RNA was infectious for rabbit cells whereas the virus itself was not. Competition experiments between different viruses have shown that, for example, all three poliovirus serotypes utilize the same cellular receptor, which is distinct from that for the Coxsackie B viruses. This phenomenon has been confirmed in a number of cases by the isolation of monoclonal antibodies, which will block specifically the attachment of certain types of virus by reacting with the host cell rather than with the virus (Minor et al., 1984).

The enteroviruses and the picornaviruses in general are responsible for a range of diseases with different target organs. These include the heart (Coxsackie viruses), eye (enterovirus type 70 and Coxsackievirus A24variant), and others as well as the intestinal tract. It is possible that the expression of the receptor sites in specific tissue plays a part in the tropism of the virus, although it is unlikely to be the only factor. This in turn may lead to a novel chemotherapeutic approach to viral diseases; for example, by the use of chemically synthesized receptor analogues.

The genome encoding the cellular receptor for poliovirus has been identified (Mendelsohn et al., 1989) and is a previously unknown three-domain protein of the immunoglobulin superfamily. Transgenic mice carrying the gene for the human receptor for poliovirus have been prepared by two groups (Ren et al., 1990; Koike et al., 1991) and are susceptible to poliovirus infection with a pathology similar to that shown by primates. Mouse cells are not normally susceptible to infection with poliovirus or most other human enteric viruses. When stably transfected with the human gene for the poliovirus receptor, however, they are rendered sensitive, and can thus be used to identify polioviruses in the presence of the other viruses. This is of value in clinical studies related to program to eradicate poliomyelitis.

The Receptor Site for EV 70

The receptor for enterovirus 70 is DAF (decay accelerating factor), (Karnauchow et al., 1996). Human DAF is a 70-75 KDa membrane glycoprotein involved in protecting cells against lysis by homologous complement (Nicholson-Weller and Wang, 1994). The molecule has five extra cellular domains (Lublin and Atkinson, 1989; Medof et al., 1987); four contiguous short consensus repeat (SCR) domains of approximately 60 amino acids each, followed by a serine/threonine-rich, heavily O-glycosylated C-terminal domain. A glycosylphosphatidylinositol (GPI) anchor attaches the molecule to the outer leaflet of the cell membrane.

Timothy and co-workers demonstrated that DAF is also the human (HeLa) cell receptor for EV 70 (Timothy et al., 1996). A monoclonal antibody (MAb), EVR1, which blocks infection of HeLa cells by EV 70, was produced. MAb ERV1 reacts with a 75-KDa cell surface glycoprotein that shares a number of properties with DAF, including sensitivity to phosphatidylinositol-specific phospholipase C (PI-PLC). Several anti-DAF MAbs also protect HeLa cells against EV 70 infection and interfere with virus binding. The ability to bind virus is conferred to NIH 3T3 cells following transient surface expression of human DAF support that DAF is the HeLa cell receptor for EV 70.

Strategy of Replication

The genome of the virus is a single positive-strand RNA molecule, and thus acts as a messenger RNA for the synthesis of the protein found in infected cells. The genome can be divided into four regions, comprising a sequence of about 750 bases with non coding function, followed by a large open reading frame of which the first half codes for the structural protein found in the virus article, while the second half encodes proteins necessary for virus replication, such as the RNA polymerase. The fourth portion of the genome is a second short sequence with no known coding function. This arrangement is summarized in Figure 5. The non-coding regions are well conserved between related viruses, and there is thus reason to believe that they are of important in virus replication (Rueckert and Wimmer, 1984).

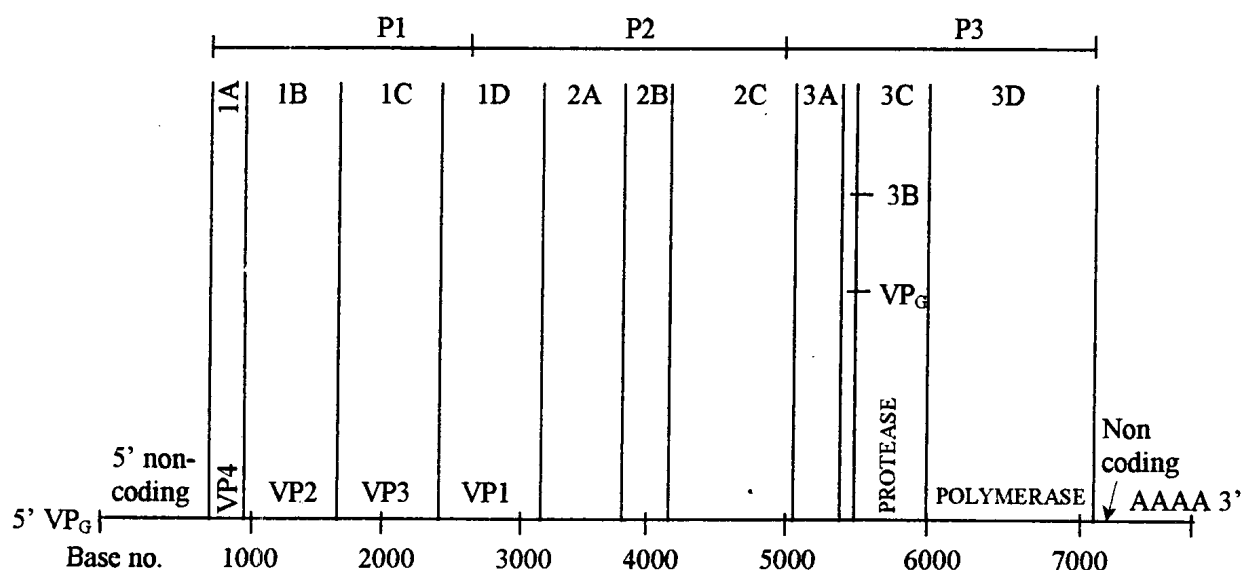


Figure 5: Organization of the genome of poliovirus, a typical enterovirus showing the two non-coding regions and the location of the regions coding for the various proteins. In the formal nomenclature (Rueckert and Wimmer, 1984), the P1 region encodes the structural capsid proteins, and the P2 and P3 regions encode non-structural proteins found only in infected cells.

The 5' non-coding region of eukaryotic messenger RNA usually terminates in a methylated guanosine cap, which is important in binding ribosomes and thus translation of the RNA into protein. Thus most eukaryotic messenger RNA molecules require a free 5' terminus, and initiation of translation internally is not possible. However, it has been shown that the 5' capped terminal structure (Pelletier and Sonenberg, 1989).

The mechanism of internal initiation is believed to involve binding of as yet ill-defined factors to complex secondary and tertiary structures formed by the 5'-non-coding region. Both the 5' and 3' non-coding regions must also be involved in the attachment of the RNA polymerase during RNA replication.

The viral proteins are synthesized as one large protein from a single open reading frame. The precursor protein includes two known sequences that act as proteolytic enzymes and digest the protein at specific positions as it, and they, are in the process of being synthesized. The first (P2 A) acts to cleave the structural from the non-structural proteins, whilst the second completes all the other processing of the protein, except the final maturational cleavages of VP0 to VP2 and VP4. A part from these two functions the non-structural proteins include a polymerase and the small genome-linked protein VPg, and a number of proteins of unknown but important function. Protein 2C is known to be virtually identical in all the polioviruses, for example, implying that it can not be altered readily without affecting virus viability.

The synthesis of RNA is less well understood than that of protein. The positive-sense copy, which in turn acts as a template for more positive-sense strands. The positive-sense RNA is used both for translation and for packaging into virus and is the major species made after the early stages of infection. All nascent strands carry a 5' terminal molecule of VPg. The polymerase activity indicated in Figure 5 is also able to elongate nascent RNA *in vitro* but is unable to initiate RNA synthesis on its own. The mechanism of initiation is not yet fully known.

The assembly of virus particle involves the synthesis of VP0, and VP1 and VP3 as a single unit, which is then assembled into pentamers, followed by the

association of 12 pentamers into the virion, a process which involves host factors. The X-ray crystallographic structure of the virus implies that the final maturation cleavage of VP0 to VP2 and VP4 is autocatalytic. Thus a serine residue is found close to the N-terminus of VP2. A number of serine protease's are known, and it may be concluded that as soon as the proton donor, such as the genomic RNA, is brought into the vicinity of the serine residue the final cleavage will take place. The virus is in general released from the cell by lysis.

Sequence Comparisons and Evolutionary Relations of Enteroviruses

Sequences of the genome of a number of enteroviruses and other picornaviruses have been determined (Stanway, 1990). Comparison of such sequences indicates the evolutionary relationship of the different viruses and a surprisingly close relationship between the enteroviruses and rhinoviruses where the genomes show a high degree of homology. In contrast hepatitis A has no significant homology with the archetypal enteroviruses (*poliovirus type 1*) at the nucleic acid level. *Echovirus 22* is also unique in sequence, showing no homology with other enteroviruses (Minor et al., 2000).

The human enteroviruses can be further divided into two groups on the basis of sequence comparisons of the 5' non-coding region (Pöyry et al., 1996; Hyypiä et al., 1997). One includes polioviruses, *Coxsackie virus A21* and *A 24* and *enterovirus 70*, while *Coxsackie B*, *Coxsackie virus A9*, *A 16*, *enterovirus 71*, *Echovirus 11*, *Echovirus 12* and all partially sequenced enteroviruses form the second. Similar sequence comparisons of the coding region and 3' non-coding region result in four subdivisions of the human enteroviruses. The first group comprises the polioviruses and *Coxsackievirus A1*, *A11*, *A13*, *A17*, *A18*, *A21*, and *A24*; the second group includes only enterovirus type 68 and 70 so far; the third includes *Coxsackievirus B1-6*, *A9*, all enteroviruses except types 22 and 23, and enterovirus 69. *Coxsackievirus A2*, *A3*, *A5*, *A7*, *A8*, *A14*, *A16* and enterovirus 71 form the final group.

Sequenced animal enteroviruses, such as bovine enteroviruses, form a group distinct from the human enteroviruses when either the 5' non-coding region or the

remainder of the genome is considered. Different regions of the genome vary in their homology between enteroviruses and other picornaviruses. Certain sections in the 5' non-coding region of the genome are identical in all enteroviruses examined, except for *Echovirus 22*, implying some definite functional constraint; this identity may carry over into the rhinoviruses, but it is possible to identify sequences which are apparently enterovirus specific (Pöyry et al., 1996). It is further possible to identify regions such as the 3' non-coding region, which are strongly conserved for a particular species, such as a poliovirus. Finally, certain regions including areas coding for the coat protein VP1, but also some sequences in the 5' non-coding region, are extremely strain specific. In principle, it is possible to exploit these various types of region to devise genomic probes or polymerase chain reaction (PCR) primers that are specific for enteroviruses in general, for a particular species of enterovirus, or for a particular strain of a particular species. Such probes are valuable in clinical and epidemiological studies. They also imply a major function for regions of the genome such as the 5' and 3' non-coding regions where they are strongly conserved, although such functions have not yet been fully resolved.

Complete Nucleotide Sequence of EV 70 and CA 24v

EV 70 has a number of features that are unusual amongst picornaviruses; it is a naturally occurring temperature-sensitive virus and has a wide range (Langford and Stanton; 1980). EV 70 will grow in various non-primate cell cultures (Yoshii et al., 1977) and in endemic areas anti-EV 70 antibodies have been detected in the sera of cattle, sheep, swine, chickens, goats, dogs and wild monkeys (Kono et al., 1982).

EV 70 represents, therefore, a clinically significant human pathogen possessing a number of unusual features. The virus provides a unique opportunity to study RNA virus evolution in the field, having arisen some 30 years ago, with a comprehensive collection of documented epidemic strains being available. Martin et al., (1996) reported complete nucleotide sequence of the EV 70 prototype strain (J 670/71), illustrated in Figure 6. Comparisons of these sequences with those of other enteroviruses are provided and evolutionary relationships within the groups are examined.

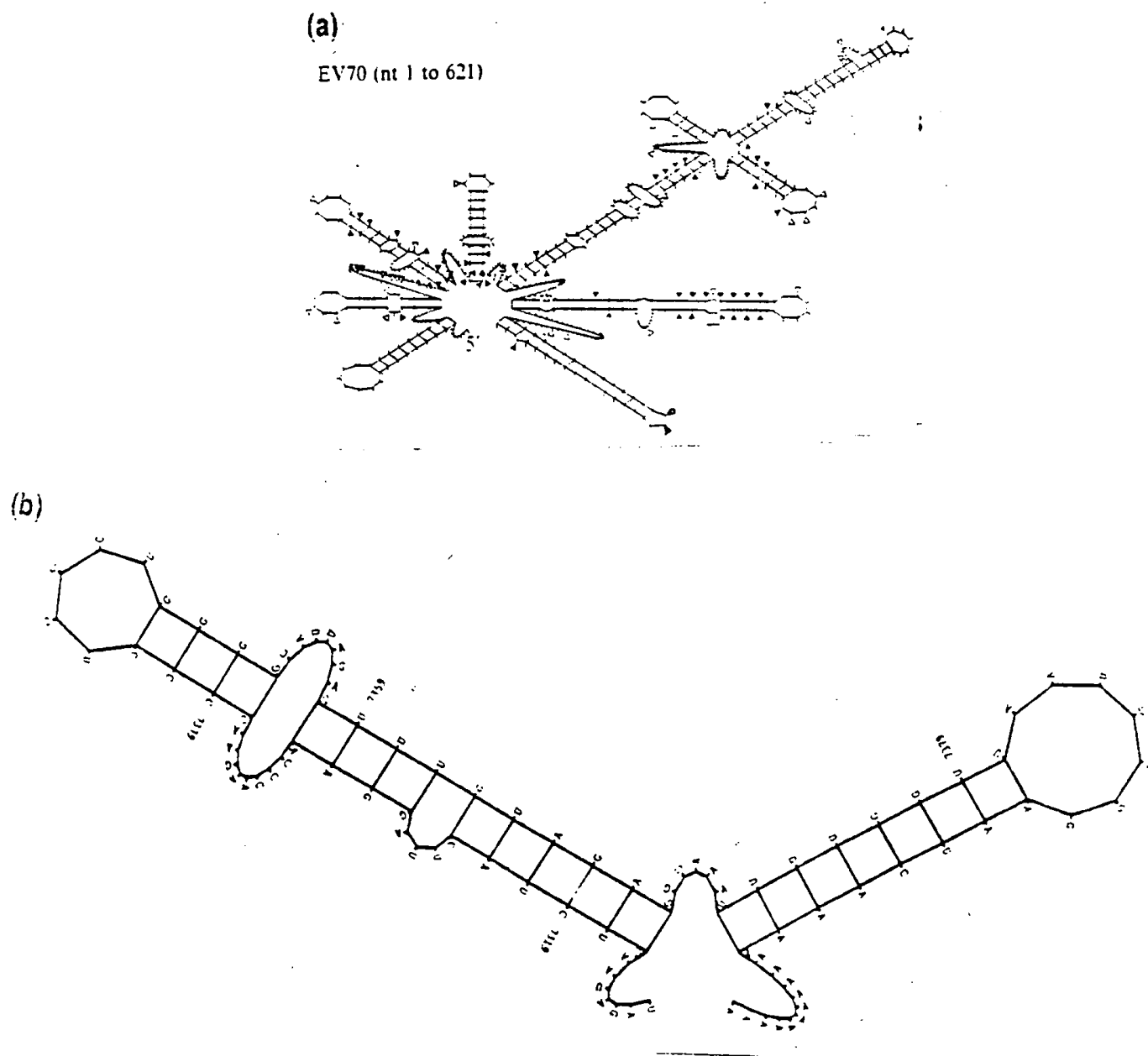


Figure 6: Secondary structure of the 5' NCR of EV 70 genome (a), (Martine et al., 1990) and predicted secondary structure of 3' NCR of EV 70 (b), (Tuija et al., 1996).

The complete nucleotide sequence of the genome of EV 70 has been determined (Martin et al., 1990). The genome consists of a 5' non-coding region of 726 nucleotides (nt), a long open reading frame and the poly (A) tract. Comparison of the nucleotide sequence and the predicted amino acid and sequence of the polyprotein with those published for other enteroviruses reveals sufficiently high similarity to predict antigenic regions and polyprotein cleavage sites. The P1 region of EV 70 is as similar to those of the entero- as to those of the rhinoviruses, whereas the P2 and P3 regions are more closely related to the Coxsackie B and swine vesicular disease viruses than other entero-or rhinoviruses.

The criteria used to place enteroviruses within groups often proves to be unsatisfactory and recently discovered enteroviruses are classified numerically (EV 68 to 72). Cluster analysis of the similarities between the EV 70 5' non-coding region with those of other entero- and rhinoviruses showed this region to cluster closely with the polioviruses and CA 21 whereas the capsid protein region clustered slightly more closely with the bovine enteroviruses than with the polio-, Coxsackie- or rhinoviruses. The P2 and P3 regions tended to cluster along the BEV/SVDV/Coxsackie B virus groups of viruses. In general, EV 70 did not cluster closely with any representatives of the 'classical' entero or rhinovirus grouping, but seemed to form a new branch somewhat more closely related to the bovine enteroviruses (Martin et al., 1990).

A series of subgenomic cDNA clones spanning 7390 base pairs were produced (Martin et al., 1990). Overlapping cDNA clones representing the entire EV 70 genome were selected and cDNA was subcloned into M13 vectors-prior to study dideoxynucleotide sequencing. Comparison of the sequence with those published for other enteroviruses shows a sufficiently high degree of similarity to allow prediction of the genetic organization and biochemical map of this virus. The genome is composed of 3' non-coding region of 726 nucleotides (nt), a single long open reading frame (ORF) of 82 nt prior to a poly (A) tract is 7390 nt in length and has a base composition of A (31.2 %), G (21.9 %), C (20.9 %) and U (26.0 %).

The 5' non-coding region

The long 5' non-coding region (NCR) of picornaviruses has been shown to contain an internal ribosome entry sequence (IRES) involved in mediation an internal ribosome entry sequence RNA in a cap-independent manner (Pelletier and Sonenberg, 1988). Indeed, the 5' non-coding region of EV 70 contains no fewer than 11 AUG triplets before the AUG (nt 727) codon the long open reading frame is encountered initiating (Rivera et al., 1988; Skinner et al., 1989; Pilpenko et al., 1989). The overall similarity between EV 70 and poliovirus type 3 (P3/Leon) in this region (nt 1 to 620) is high 85.4 % (Table 5) and a comparison of the structure proposed for the 5' non-coding region of P3/Leon with that predicted for EV 70, folded using identical constraints, showed almost identical structures (Figure 5), approximately 43% of nucleo-base-pairing. An alignment and analysis of the distribution of sequence differences between EV 70 and P3/Leon show that they occur with equal probability in regions predicted to be involved in base-pairing. An alignment and analysis of the distribution of sequences between EV 70 and P3/Leon show that they occur with equal probability in regions predicted to be involved in stem-loop structure (46 differences) to those regions, eight disrupt base-pairing, 14 do not disrupt base-pairing and at 24 occur as 12 different base-pairs, pressure for conservation appears, therefore, to act at the level of secondary structure rather than primary sequence.

Table 5: Nucleotide sequence similarity (%) between the 5' non-coding region of EV 70 and other entero-and rhinoviruses.

	PV1*	PV2*	PV3*	CA9	CA21	CB1	CB3	CB4	SVDV*	BEV	
nt 1-624	89	87	85	74	87	73	74	72	73	58	
nt 625-726	35	30	32	39	39	35	37	35	37	20	
nt1-726	81	79	78	69	81	68	69	67	67	53	
	HRV1B		HRV2		HRV9		HRV14		HRV85		HRV89
nt 1-619 ^t	61		61		62		63		60		60

* Similarities shown represent an average value derived from all members of the serotype.

^t Alignments of rhino-and enterovirus sequences have shown that rhinoviruses have a large deletion (about 125 nt) relative to the enteroviruses following domain III (Pilpenko et al., 1988).

The long open reading frame (ORF)

The initiation codon at nt 727 is part of the sequence AUA AUG G, almost on optimal translation initiation environment (Kozak et al., 1986). Translation proceeds in this reading frame until the termination codon at nucleotide 7309, encoding a polyprotein of 2194 amino acids. Alignments of sequences permit confident prediction (in all but one case) of polyprotein cleavage sites. The sizes of the predicted mature products of polyprotein processing are shown in Table 6. Which of the three alternative amino acid cleavage pairs Q/S (1A residue 235/6), Q/A (242/3) or Q1 (247/8) forms the 1C/1D cleavage site is not known. However, for the purposes of M_r calculations, the Q/S pair is assumed to be used, as this amino acid pair probably is used at 2B/C cleavage site. The similarity of the predicted amino acid sequence of EV 70 proteins with those of other members of existing groupings within the enteroviruses is shown in Table 7.

Table 6: Calculated M_r of EV 70 proteins based on predicted sites.

Protein	Amino acids	M_r (K)
P1	871	95.7
P1 A	69	7.3
P1 B	250	27.5
P1 C	235*	25.8*
P1 D	317*	35.1*
P2	527	72.4
P2 A protease	143	15.8
P2 B	99	18.8
P2 C	330	37.8
P3	751	84.5
P3 A	89	9.9
P3 B	22	2.4
P3 C protease	183	20.3
P3 D polymerase	457	51.9
Complete polyprotein	2194	252.6

* Molecular weights of P1C and P1D are calculated on the assumption that the Q-S amino acid pair is utilized in polyprotein processing.

Table 7: Amino acid sequence similarity (%) between the protein of EV 70 and representative viruses of enterovirus groups.

PROTEIN	1A	1B	1C	1D	2A	2B	2C	3A	3B	3C	3D
PV1	62.3	52.9	42.8	35.9	53.4	58.1	62.6	56.8	81.8	60.1	75.8
PV2	60.8	51.2	42.4	36.0	52.2	56.1	63.2	58.0	77.3	60.1	75.6
PV3	62.3	53.5	43.2	35.7	54.1	57.1	63.2	56.8	72.7	60.1	75.6
CA21	62.3	54.0	41.8	35.5	50.0	60.25	56.8	56.8	77.3	57.4	75.0
CA9	55.1	54.1	43.6	38.5	58.1	59.6	67.5	62.9	86.4	63.9	75.6
CB1	53.6	56.6	44.0	38.8	57.4	60.6	66.9	64.0	86.4	63.9	75.0
CB3	53.6	55.9	44.4	38.6	54.7	60.6	66.9	64.0	81.8	62.3	75.4
CB4	55.1	54.9	46.1	39.0	58.1	59.6	66.3	64.0	86.4	64.0	75.0
SVDV	53.6	56.1	45.2	39.2	55.4	59.6	66.6	61.8	81.8	64.0	75.2
BEV	53.5	64.7	52.2	38.8	54.1	56.6	59.1	46.1	36.4	54.1	65.8
HRV1A	55.1	53.7	44.6	37.2	-	-	-	-	52.4	58.6	-
HRV1B	55.1	53.9	44.9	35.2	45.8	47.4	44.2	39.8	47.6	48.1	57.2
HRV2	53.6	52.9	43.0	37.1	43.8	46.4	43.9	36.1	47.6	47.5	57.2
HRV9	56.5	51.9	44.4	34.0	45.1	44.3	43.6	33.7	47.6	47.5	57.9
HRV14	53.6	52.3	42.1	37.2	41.8	55.1	52.7	48.3	59.1	50.0	62.5
HRV85	55.1	53.9	43.6	35.8	45.2	47.4	44.2	36.1	47.6	47.0	57.4
HRV89	55.1	52.3	46.1	37.8	42.3	45.4	48.9	39.0	42.9	48.1	55.7

Structural proteins

The primary polyprotein cleavage product P1 forms the precursor of the capsid protein 1A, 1B, 1C and 1D. As observed previously, capsid protein 1A is the most highly conserved amongst the rhino- and enteroviruses. The EV70 1A protein is more closely related to that of CA21 and the polioviruses than the other entero- or rhinoviruses (Table 7). This protein is not exposed on the surface of picornaviruses (Hogle et al., 1985; Rossmann et al., 1985) and does not appear to elicit neutralizing antibodies. In the case of other three structural surface proteins 1B, 1C and 1D, a familiar pattern of sequence conservation is observed. Regions of EV 70 sequence which align with those known to be involved in the formation of the eight-strand, anti-parallel β -barrel basic structure common to all three capsid proteins shows much higher conservation than those EV 70 sequences forming surface loops or 'puffs', some of which form important antigenic determinants (Minor et al., 1986; Rossmann et al., 1985). The capsid of EV 70 might be expected to have a slightly smoother surface than that of poliovirus, as antigenic site 2 is apparently a smaller surface feature. Alignments indicate that EV 70 has a relative deletion of 18 amino acids at the position analogous to that of poliovirus antigenic site 2a of protein 1D. The EV 70 region corresponding to poliovirus site, however, has an insertion of three amino acids and, therefore, might be expected to be an important antigenic determinant. The C-terminal region of EV 70 protein 1D is 18 residues longer than that of poliovirus, a region that has also been shown to be involved in antigenicity (Minor et al., 1986). Generally, EV 70 proteins 1B and 1C are more closely related to those of the bovine enteroviruses than to those of other human entero or rhinoviruses, and protein 1D shows a broadly equivalent (low) similarity with the entero- and rhinoviruses (Table 7).

A major function of the capsid is that of cell attachment, probably influencing the host range and tissue specificity of the virus. In both respects EV 70 is highly unusual, virus replication has been demonstrated in L (mouse), BHK21 (hamster), IB-RS-2 (porcine), RK13 (rabbit) and BK1 (primary bovine) cell cultures (Yoshii et al., 1977) and the presence of EV 70 neutralizing IgM demonstrated in cattle, sheep, pigs, chickens, goats, dogs and wild monkeys (Kono et al., 1981). The receptor site for the

virus has been shown to be sensitive to neuraminidase treatment and free N-acetylneuraminic acid inhibited the hemagglutinating activity of the virus (Utagawa et al., 1982).

Non-structural proteins

The non-structural proteins encoded within the P2 and P3 regions of the polyprotein are concerned with replicative functions. As one might expect, sequence conservation of the P2 and P3 proteins of EV 70 with other enteroviruses (Table 7). Functions have not been determined for proteins 2B, 2C or 3A, although the functions of proteins 2A and 3C are known. The amino acid sequences of EV 70 2A and 3C are sufficiently similar to those of poliovirus to predict that these are cysteine-type proteinase (Bazan and Fletterick, 1988). The specificity of EV 70 proteinase 2A is apparently the same as that of poliovirus, cleaving at its own N-terminus between an Y-G amino acid pair. EV 70 proteinase 3C, however, appears to be less specific than poliovirus 3C, cleaving between Q-G, Q-S and possibly a Q-A or Q-I amino acid pair. EV 70 proteins 3B and 3D are also sufficiently similar to those of poliovirus to predict they are the genome-linked protein VPg and RNA-dependent RNA polymerase, respectively. Interestingly, EV 70 is a naturally occurring temperature sensitive virus with optimal growth at 33° C (Miyamura et al., 1974), which may correlate with the lower temperature of the primary site of replication, the eye. This temperature sensitive lesion has been located at the uridylation of VPg (Takeda et al., 1989). Sequencing of high-temperature-adapted mutants may identify the virus protein(s) involved in the VPg uridylation function.

The 3' non-coding region

Polyprotein translation is terminated at nucleotide 7309 by the UAG stop codon, which is followed by a 3' non-coding region of 82 nt prior to the poly (A) tract. Sequencing alignment of this region was difficult and similarities were hard to interpret. The function of this region is unknown, although it is thought to be involved in the control of genome replication. Comparisons amongst the 3' non-coding region

of enteroviruses indicate: (a) some conserved blocks of sequence which maintain the stem-loop structure (Ryan et al., 1985; Jenkins et al., 1987).

Evolutionary relationships

The criteria used to place enteroviruses within groups often prove to be unsatisfactory and recently discovered enteroviruses are classified numerically (EV 70 to 72). Cluster analysis of the similarities between the EV 70 5' non-coding region with those of other entero- and rhinoviruses showed this region to cluster closely with the polioviruses and CA 21 whereas the capsid protein region clustered slightly more closely with the bovine enteroviruses than with the polio-, Coxsackie- or rhinoviruses. The P2 and P3 regions tended to cluster along with the BEV/SVDV/Coxsackie B virus groups of viruses. In general, EV 70 did not cluster closely with any representative of the 'classical' entero- or rhinoviruses groupings, but seemed to form a new branch somewhat more closely related to the bovine enteroviruses (Martin et al., 1990).

An RNAase T1-resistant oligonucleotide mapping study led to the conclusion that EV 70 strains have recently evolved from a hypothetical ancestral virus (Miyamura et al., 1986). The presence of neutralizing anti-EV 70 antibodies in a range of animal sera both in Japan and West Africa collected before 1969 (Sasagawa et al., 1982) indicated that the same, or a very similar, virus was circulating in animal hosts before the appearance of AHC in humans. It is possible, therefore, that EV 70 may represent a zoonotic picornavirus that has extended its host range to encompass humans. Interestingly, another enterovirus, a prime variant of Coxsackievirus A24, also caused ocular disease in the Far East (although not elsewhere) at the same time that is in the year 1970 in Singapore (Lim and Yin-Murphy, 1973). No neurological complications have been reported for this variant of CA24, and serological and hybridization studies suggest CA24 to be quite distinct from EV 70 (Natori et al., 1984).

Molecular Evolution of the Major Capsid Protein VP1 of Enterovirus 70

EV 70 is uniquely suitable as a subject for studying the evolution of RNA viruses in nature, because of its recent emergence (Takeda et al., 1994). The availability of isolates collected over a decade since very close to the time of its appearance and the precise record of the date and place of virus isolation made it possible to trace the molecular events occurring in the virus genome during transmission in nature (Takeda et al., 1994).

In one study done by the same investigators (Miyamura et al., 1990) on the evolution of EV 70 by oligonucleotide mapping using 16 isolates obtained from various parts of the world revealed that all of the isolates had evolved rapidly at a constant evolutionary rate from a common ancestor which was estimated to have appeared in humans around 1967. In further study, Takeda and colleagues determined the nucleotide sequences of VP1 of eighteen EV 70 different isolates from the early epidemics in various areas (Table 8), so as to analyze nucleotide substitutions rate during evolution and estimate more reliably the time of emergence of the virus. For comparison, Takeda and colleagues have chosen VP1, the major capsid protein, because of the region with least homology in both nucleotide and amino acid sequences (Palmenberg, 1984; Ryan et al., 1990). Comparison of the VP1 nucleotide sequences of the isolates showed a phylogenetic relationship consistent with that previously reported by oligonucleotide mapping (Miyamura et al., 1986).

Table 8: Enterovirus 70 isolates

Strain	Place of collection	Date of collection	Reference
R6/71	Rabat, Morocco	Jan, 1971	Nejmi et al.,1974
R20/71	Rabat, Morocco	Jan, 1971	Nejmi et al., 1974
J648/71	Mie, Japan	16 Nov. 1971	Kono et al., 1972
J670/71	Hokaido, Japan	25 Dec. 1971	Kono, et al., 1972
SEC32/71	Singapore	1971	Yin-Murphy
ENG/71	London, England	1971	Jones, 1972
M8/72	Mie, Japan	25 Sept. 1972	Sakuri et al.,1975
I/72	Indonesia	1972	Kono et al., 1975
G10/72	Gifu, Japan	27 Nov. 1972	Kawarnoto et al., 1976
T62/73	Tunisia	1973	Whitcher et al., 1976
FB/73	France	July 1973	Chomel &Aymard, 1975
T260/74	Thailand	15 Dec. 1974	Kono et la., 1977
M51/76	Mie, Japan	16 Feb. 1976	Sakuri et al., 1989
HP85/78	Hokkaido, Japan	18 July 1978	Aoki et al., 1980
TW266/81	Kahosiung, Taiwan	14Apr. 1981	Chen et al., 1983
HP/85/81	Hokaido, Japan	26 May 1981	Chen et al., 1983
V1635/81	Karachi, Pakistan	21 July 1981	Ghafoor et al, 1984
V1250/81	Honduras	6 Aug. 1981	Kew et al., 1983

Takeda et al., estimated the putative ancestral sequence of EV 70 VP1 and superimposed each nucleotide substitution of the isolates from the ancestral sequence on the branches of the phylogenetic tree. This method made it possible to analyze the nucleotide substitution patterns among four nucleotides, the proportion of transitional and transversional substitutions, and the frequency of parallel nucleotide substitutions independently occurring at the same site of different evolutionary lineage's. On the basis of the results it is concluded that from the comparison of nucleotide sequences of capsid protein VP1 in eighteen isolates of EV 70, Takeda and colleagues inferred a phylogenetic tree (Figure 7) which was quite consistent with that previously constructed by oligonucleotide map analysis of entire genome RNA (Miyamura et al., 1986).

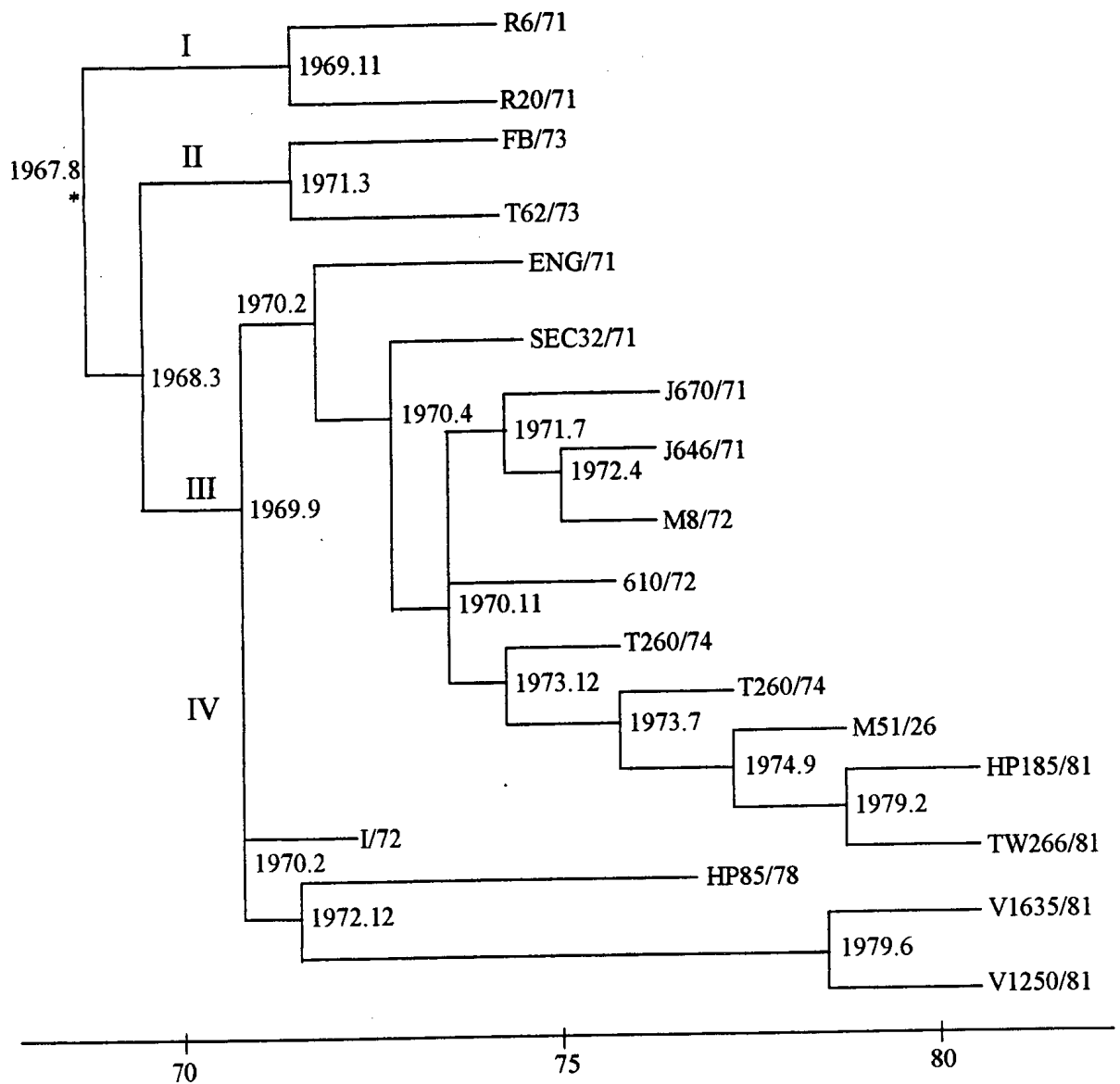


Figure 7: Phylogenetic tree of EV 70 inferred by UPGMA. These isolates are positioned at the time of virus isolation on the horizontal scale. The time of branching between strains (shown by year and month in the tree) was calculated by using the genetic distance between isolates on the base of the time of the strains and the evolutionary rate. The star indicates the hypothetical ancestral virus.

Complete Nucleotide Sequence of CA 24v

It is not clear whether CA24v appeared through accumulation of the nucleotide substitution on the genome of human enterovirus or by another mechanism, such as recombination, as has been suggested in Coxsackievirus A21 (CA21) sequencing analysis (Hughes et al., 1989).

To find clues to identify the origin of the virus, as well as to know the evolutionary relationship among other picornaviruses, Supanaranond and co-workers have determined the complete nucleotide sequence of the standard strain of CA24v, the EH24/70 strain (Supanaranond et al., 1992).

The total length of the genome excluding the poly (A) tract is 7461 nucleotides. Following 750 bases of the 5' non-coding region, a long open reading frame that terminated at 7392 was observed. This must serve to synthesize a polyprotein that encodes 2214 amino acids. The 3' non-coding region contains 69 nucleotides. The base composition of the CA24v RNA genome excluding the poly (A) tail is 2206 A (29.57%), 1733 G (23.23%), 1690 C (22.65%), and 1832 U (24.55%), respectively, representing the common characteristic of a relatively high adenine content, which has been observed in several enteroviruses. By analogy with polioviruses (Kitamura et al., 1981; Nomoto et al., 1982; Racaniello and Baltimore, 1981; La Monica et al., 1986; Toyoda et al., 1984; Stanway et al., 1983; Stanway et al., 1984) and CA21 (Hughes et al., 1989), protein alignments were made and the deduced amino acid sequence of EH 24/70 viral proteins and the predicted proteolytic cleavage sites were compared with four enteroviruses (Table 9 and 10). Through the polyprotein, the EH24/70 was found to be the most closely related to CA21 or polioviruses, rather than EV 70 (Takeda et al., 1989; Ryan et al., 1990) or CB1 (Iizuka et al., 1987).

Table 9: The predicted proteolytic cleavage site of CA 24v, the EH/70 strain

Boundary	PV1	CA21	CA24v	EV70
VP4/VP2	N/S	N/S	N/S	K/S
VP2/VP3	Q/G	Q/G	Q/S	Q/G
VP3/VP1	Q/G	Q/G	Q/S	Q/A
VP1/2A	Y/G	F/G	F/G	A/G
2A/2B	Q/G	Q/G	Q/G	Q/G
2B/2C	Q/G	Q/G	Q/G	Q/G
2C/3A	Q/G	Q/G	Q/G	Q/G
3A/VPg	Q/G	Q/G	Q/G	Q/G
VPg/3C	Q/G	Q/G	Q/G	Q/G
3C/3D	Q/G	Q/G	Q/G	Q/G

Table 10: Nucleotide sequence homology of 5' and 3' non-coding regions and amino acid sequence homology of viral-encoded proteins.

	PV1	CA21	EV70	CB1
5'	80.9	79.9	80.3	73.0
3'	97.1	95.7	63.8	65.5
1A(VP4)	82.6	81.2	63.8	69.6
1B(VP2)	74.0	84.2	54.0	57.4
1C(VP3)	72.5	85.8	44.0	56.1
1D(VP1)	56.7	70.8	41.1	45.2
2A	75.2	82.6	55.2	56.3
2B	82.5	79.4	55.6	53.5
2C	83.6	87.2	60.7	62.7
3A	74.7	70.0	57.3	50.6
3B(VPg)	90.9	86.4	81.8	77.3
3C	95.6	92.3	59.6	61.2

Supanaranond and co-workers have sequenced the entire RNA genome of the EH 24/70 strain using two cDNA clones. The clones designated pCA 24v (106) and pCAv (66), were isolated and represented 99.4% of the entire genome sequence. Translation of the nucleotide sequence in all three reading frames allowed them to identify a single large open reading frame of 6642 nucleotides with initiation codon at position 751 and termination codon at position 7393.

The overall structure of the RNA genome of the EH 24/70 has substantial similarities in nucleotide and amino acid sequences with other enteroviruses and has all the characteristics shared by enterovirus genome. The genome organization is in accordance with the L434 nomenclature system (Ruckert and Wimmer, 1984).

The 5' non-coding region is the most conserved region among enteroviruses, and even rhinoviruses share the homologous sequences, suggesting an important function in viral replication. It has been shown that the picornavirus 5' non-coding region contains an internal ribosome entry sequence that mediates cap-independent translation (Pelletier and Sonenberg, 1988). A previous study on the nucleotide sequence alignment of the 5' non-coding region revealed several conserved sequence regions, implying a biologically functional role (Rivera et al., 1988). The 5' non-coding region of the EH 24/70 is 750 nucleotides in length and showed the highest homology with poliovirus (PV) (Table 2). The first 48 nucleotides are identical with to all three poliovirus serotypes and CA 21. It was found that many compensatory nucleotide substitutions occurred at the first 650 bases, suggesting the secondary structure, as previously described (Rivera et al., 1988).

The precursor protein P1 is the cleavage product of primary polyprotein and forms the four-capsid proteins 1A (VP4), 1B (VP2), 1C (VP3), and 1D (VP1). The capsid protein 1A (VP4), which has been appeared to be an internal structural protein of the virion, is the most conserved among the enteroviruses. The 1A (VP4) of the EH 24/70 and four other enteroviruses have the same 69 amino acids. The amino acid sequence of the EH 24/70 1A(VP4) is closely related to PV1 (82.6%) and CA21 (81.2%), rather than EV70 (63.8%) and Coxsackievirus B1 (CB1) (69.6%). The amino acid sequence of 1B (VP2) is mostly related to CA21 (84.2%), whereas EV70

and CB1 contain a very different region (at amino acid position 130-190). In fact, EV70 has a relative deletion of as many as 17 amino acids when compared with other enteroviruses. 1C (VP3) and 1D (VP1) from surface loops and puffs, some of which show immunodeterminant antigenic epitopes for neutralization. In viral proteins in each virus, 1D (VP1) shows less homology, allowing us to predict possible neutralize epitopes. The amino acid sequences 95-107 of 1D are unique to the EH 24/70 and correspond to a PV1 neutralization epitope (Hoghle et al., 1985).

The comparison of homology P2 and P3 proteins among four enteroviruses ranged from 50.6% in 3A of EV70 to 98.3% in the 3D polymerase gene of poliovirus type 1 (PV1). It has been postulated that picornavirus 3C is a cysteine proteinase and that cysteine at amino acid position 147 functions as an active site. The conserved amino acid sequence GQCGGV at residues 145-150 in the EH 24/70 3C is also found in four other enteroviruses.

The 3' non-coding region is very similar to that of the PVS or CA21 with two or three base deletions, respectively, and the nucleotide sequence homology is as high as 97.1% and 95.7%, respectively. The sequence of the PV1 and CA21 at the 3' terminus is ...GGAG(A)_n; while that of the EH24/70 is ...UUGGG(A)_n.

Finally, a striking homology with PV1 was found in the amino acid sequence 3B (PVg), 3C and 3D, together with the 3' non-coding region. This result is interesting, because in case CA21 a remarkable similarity in the P3 region and the possibilities of recombination between less related viruses are unknown.

A variant of coxsackievirus A 24 (CA 24v) is one of the agents causing acute hemorrhagic conjunctivitis (AHC), which was first recognized clinically in 1969. The virus was isolated during an epidemic in 1970 in Singapore (Yin-Murphy, 1972). Since CA 24v emerged as a new human pathogen from a single focus (Miyamura et al., 1986), the virus has been causing epidemics almost every 5 years in Southeast Asian countries (Chang et al., 1977; Thongcharoen et al., 1978; Christopher et al., 1982; Yin-Murphy et al., 1986).

Molecular Comparison of Coxsackie A Virus Serotype

Human enteroviruses, which belong to the picornavirus family, are subgrouped into polioviruses, Coxsackie A viruses (CAV), Coxsackie B viruses (CBV), echoviruses, and enteroviruses 68-71. Current subgroup division is mainly based on the pathogenicity of the virus serotypes in experimental animals. Polioviruses are recognized by their ability to paralyze primates, whereas CAVs cause flaccid paralysis in newborn mice in contrast to CBVs, which induce spastic paralysis. Accordingly, CAVs affect striated muscle while CBVs replicate in several tissues including central nervous system (Hyypiä et al., 1993). Echoviruses do not cause disease in experimental animals. More recently isolated enteroviruses have not been divided into these subgroups but are numbered in the order of their identification.

There are 23 immunologically distinct serotypes in the CAV subgroup. They all are human pathogens and cause diseases like common cold, rash, meningitis, and paralytic illness. To date, four of the CAV genomes have been completely sequenced: CAV 9 (Chang et al., 1989), CAV 16 (Pöyry et al., 1994), CAV 21 (Hughes et al., 1989), and CAV 24 (Supanaranond et al., 1992). Surprisingly, these serotypes were found to belong to three different genetic clusters when compared to other enteroviruses, indicating remarkable diversity among CAVs (Pöyry et al., 1994).

Coxsackie A viruses were discovered in 1948 by Dalldorf and Sickles. They were isolated from fecal specimens from children during a poliomyelitis outbreak and were recognized on the basis of their capability to cause detectable signs in newborn mice. In spite of their importance as causative agents of human disease, relatively little is known about molecular characteristics of CAVs. In one study, Pulli and co-workers sequenced four serotypes (9, 16, 21, and 24) represent three different genetic clusters, suggesting remarkable heterogeneity among CAVs (Pulli et al., 1995). To elucidate relationships between the members of the subgroup and other enteroviruses, and to correlate molecular findings with pathogenic properties, Pulli et al., carried out partial sequencing of four different genomic regions of the CAV prototype strains.

According to comparisons in the coding region, CAVs could be divided into four genetic clusters: cluster A includes serotypes 2, 3, 7, 8, 10, 12, 14, and 16 and

enterovirus 71. CAV 9 is the only representative of the subgroup in cluster B while the rest of the CAVs serotypes 1, 11, 13, 15, 17, 18, 19, 20, 20a, 21, 22 and 24) belong to cluster C together with polioviruses, enteroviruses 68 and 70 form a distinct cluster (D) and enterovirus 69 belongs to cluster B. Similar relationships were observed in the VP2 and 3D regions but the differences within the groups were smaller in 3D, evidently because the RNA polymerase is functionally a very important protein in the life cycle of the viruses and cannot be mutated to such a degree as can the capsid proteins. Furthermore, nonstructural proteins are not under selection pressure caused by host antibodies. Analysis of the partial 3D sequences using the DNAPARS program of the PHYLIP package (Felsenstein, 1991) gives rise to similar clustering as is seen in these comparisons, indicating that phylogenetic relationships closely correlate with the genetic grouping. Since diagnosis of enterovirus infection is increasingly based on PCR, genotypic rather than phenotypic subgroup division would be desirable. The four clusters formed in the coding region of human enterovirus genomes could form a basis of this classification.

When sequences from the 3' UTR were compared, clustering similar to that seen in the VP2 and 3D regions was observed, indicating close functional relationship of this part of the genome with the coding region. Different secondary structures have been predicted for the 3'UTR among the clusters (Hyypiä and Stanway, 1993; Pöyry et al., 1994) and during genome replication this region is recognized by viral proteins thus explaining adjacent coevolution.

Phylogenetic analysis of sequenced enterovirus genomes shows that 5' UTR and the rest of the genome involve in a different manner (Pöyry et al., 1994; Dahllund et al., 1995). Two genetic clusters [polioviruses, CAV 21 and 24, and enterovirus 70, CAV 9, CBVs, echovirus (EV) 11 and 12, and enterovirus 71] are formed in the 5' UTR. These are further separated in the coding region (poliovirus, CA 21 and CA 24; CAV9, CBVs, EV11 and 12; CAV16 and enterovirus 71; enterovirus 70). These correspond well to the ones observed in Pulli's study (1995) while more heterogeneous clustering was observed in the 5' UTR, evidently because a short partial sequence was used for comparison in the analysis of complete genome, bovine enteroviruses form

an additional cluster in both these regions while swine vesicular disease virus is closely related to CBVs.

Mutation, recombination, and selection are the mechanisms playing roles in picornavirus evolution. The lack of a proof reading mechanism during picornavirus RNA replication gives rise to a high mutation rate. It is also known that recombination's frequently occur in poliovirus infections either between the vaccine strains or in combination with wild-type viruses (Zheng et al., 1993). According to Pulli's results, the same viruses are found in the same genetic clusters when sequences from regions coding for the capsid proteins, the RNA polymerase, and the 3' UTR are compared. This suggests that any recent recombination's in these regions between viruses in different clusters have not evolved from one virus by mutations and possibly also recombination.

An important factor from pathogenic and thus phenotypic point of view is the recognition of the cellular receptor. Cluster C contains viruses with different clinical characteristics (polioviruses and some CAVs causing common cold). The cellular receptor utilized by polioviruses is a member of the immunoglobulin superfamily, which is not recognized by the CAVs. On the other hand, CAVs in this cluster seem to use ICAM-1 (Table 11), a specificity shared with the major group of rhinoviruses (Colonno, 1987; Greve et al., 1989; Staunton et al., 1989). From an evolutionary point of view, this difference should be understood by recombination of poliovirus ancestors with rhinoviruses or by mutations, which have allowed an alteration in receptor specificity.

Table 11: Conclusion of the Results of the CAV sequences (Pulli et al., 1995)

CAV serotype	VP4-VP2 ^a	3D ^a	3'UTR ^b	ICAM-1 ^c
CAV1	C	NK	S	NK
CAV2	A	A	I	-
CAV3	A	A	I	-
CAV4	NK	NK	I	-
CAV5	A	A	I	NK
CAV6	NK	NK	NK	NK
CAV7	A	A	I	-
CAV8	A	NK	I	-
CAV9	B	B	L	-
CAV10	A	A	NK	-
CAV12	NK	A	I	-
CAV13	C	C	S	+
CAV14	A	A	I	-
CAV15	NK	C	S	+
CAV16	A	A	I	-
CAV17	C	C	S	NK
CAV18	C	C	S	+
CAV19	NK	C	S	NK
CAV20	C	C	S	+
CAV20a	NK	C	S	NK
CAV20b	C	C	S	NK
CAV21	C	C	S	+
CAV22	NK	C	S	NK
CAV24	C	C	S	NK

^a coding region, A, CAV cluster ; B, CBV cluster; C, poliovirus cluster, including certain CAVs.

^b S, short; I, intermediate; and L, long 3' UTR fragment.

^c Blocking of infection with monoclonal antibody against intercellular adhesion molecule 1.

While in terms of mouse pathogenicity CAV9 is clearly a member of Coxsackie A virus (Hyypiä et al., 1993) it differs genetically from all other members of the subgroup and represent CBVs rather than CAVs. It contains a carboxyl-terminal extension to its VP1 polypeptide and this extension has been shown to play a role in the cell surface binding of the virus. It has been proposed that during evolution the extension has been obtained as a result of recombination with cellular sequences (Chang et al., 1992).

A cellular receptor(s) for the cluster A is not currently known but its identification would significantly clarify the correlation between genotype division and pathogenicity. Since pathogenicity in newborn mice, inspite of different receptors recognized in cultured primate cells, is a common property for CAVs, it is possible that a distinct molecule is utilized for virus entry into murine tissues. In addition to receptor recognition, determinants for tissue tropism include specific parts of the genome recognized by cellular factors. As soon as these also become known, a more accurate correlation between genotypic and phenotypic properties can be made.

Since many CAV strains grow poorly in cell culture and require isolation in newborn mice present knowledge of their disease pattern may still be rather incomplete. Hand-foot-and-mouth disease (a vesicular rash) is usually caused by CAV 16 but cases caused by CAV5 and 10 as well as by enterovirus 71 have also been described. These viruses belong to the same genetic cluster (A). The disease pattern of CAV9 resembles that of CBVs since meningitis and carditis are often observed during the infections. On the other hand, a CAV24 variant has been responsible for large epidemics of acute conjunctivitis which are similar to those caused by enterovirus 70, but similarities in epidemiology cannot be directly correlated with molecular characteristics, since these viruses belong to different genetic clusters. Herpangina (painful vesicles in the mouth and throat) is classically associated with CAV serotypes 1, 2, 3, 4, 6, 8, 10 and 22. Again, representatives of two clusters can be found although most of these viruses belong to the A genotype. In cluster C, polioviruses are causative agents of epidemic paralysis but wide ranges of clinical manifestations including mild respiratory symptoms are observed during the infection. CAVs in this cluster often cause common-cold-like symptoms, which corresponds to their receptor specificity (ICAM-1). It seems at that direct clinical

association of molecular characteristics is not evident in spite of certain similarities in genetic grouping and disease patterns of some CAV serotypes. Additionally, more associations, such as receptor specificity, the presence of tissue-specific replication factors, and individual variation between patients, are likely to play a role in the clinical outcome of infection.

CHAPTER 2

SCOPE OF THESIS

SCOPE OF THESIS

Till present day, two pandemics of acute hemorrhagic conjunctivitis (AHC) and several epidemics caused by enterovirus type 70 (EV 70) and coxsackievirus virus A 24variant (CA 24v) swept through many parts of the world and affected millions of people. Through most countries affected by the 1969-71 pandemic continued to experience sporadic and periodic outbreaks.

The outbreak of AHC first emerged from Ghana (west Africa) and subsequently spread to other parts of Africa and Asia, including India.

The first serologic evidence of EV 70, infection in India came from Bombay (western India) in 1971-72, and the first isolation of CA 24v was reported from a single case during a small outbreak in southern India in 1975. In 1984 an outbreak due to EV 70 and CA 24v, occurred again in the same part i. e. Madras.

In north India, also two outbreaks caused by EV 70 were reported from Delhi during the years 1981 and 1986. In 1996, another outbreak of AHC occurred in Delhi and the etiologic agent was confirmed as EV 70.

Present diagnostic methods for detection of the etiologic agents of acute hemorrhagic conjunctivitis are based on molecular technology, immunoelectron microscopy method and virus neutralization antibody test a part from isolating infection virus from conjunctival samples.

From the epidemiological point of view, AHC mainly has emerged in communities with low-economic status and poor hygiene. Therefore, an early detection of etiologic agent could allow control measures prevent unnecessary treatment with antibiotics.

Hence, as the scope of the present thesis, a research strategy was planned in to develop reagent diagnosis of acute hemorrhagic conjunctivitis, considering its convenience, which could be easily, applied in most of the laboratories specially those which are in developing countries.

CHAPTER 3

MATERIAL AND METHODS

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Tissue Culture Techniques

Cell Line:

LLC-MK2: The cell line is derived from Rhesus Monkey kidney.

HeLa: The cell line is derived from human epithelial carcinoma of cervix.

The cell lines were cultured and grown in minimal essential medium (MEM, GIBCO-BRL) supplemented with 10% sheep serum (SS) and fetal bovine serum (FBS) in case of LLC-MK2 and HeLa cell respectively. Confluent monolayer of the cell line was treated with trypsin to cell suspension. Cells were resuspended in freshly prepared growth medium to yield a suspension of required cell concentration. Tissue culture flasks were seeded at a cell density of 5.4×10^6 cells (175 cm^2 flasks), and 1.2×10^6 cells (80 cm^2 flasks) and 1.0×10^6 cells (45 cm^2), and 0.7×10^6 cells (25 cm^2 flasks) according to the experimental requirements. Seeded cultures were incubated at 36°C , in an incubator with 5% CO_2 (Forma scientific). When 75% of culture flask surface was covered by cell growth (3-4 days) the culture medium was discarded and replaced with maintenance medium (MEM contains 2 % SS or 2 % FBS in case of LLC-MK2 and HeLa cell respectively). Cells were used for virus culture from 3rd day onwards. Subculture was done on day eight.

Subculture Procedure:

Subculture procedure was carried out in a Laminar Flow workstation, using sterile microbiological techniques.

1. Observe the confluent monolayer (example 80 cm² TC flask) under an inverted TC microscope (Axiovert 25-Ziess, Germany) to confirm good cell health, confluence and absence of microbial contamination.
2. Decant and discard the used culture medium from the flask. Add 5 ml PBS and gently rinse the monolayer to remove traces of medium and serum, and discard PBS.
3. Add 5ml pre-warmed (37° C) TPVG solution to the flask and gently spread over the monolayer. Laydown the culture flask on the side of the monolayer in an incubator (36° C) for about 5min. Observe the flask for detachment of the cell monolayer.
4. Decant and discard TPVG. Tap the flask several times to completely detach the cell monolayer.
5. Add 5ml growth medium using a large bore Pasture pipette (with bulb attached) gently resuspend the cells by aspirating the culture fluid several times.
6. Do cell counting using trypan blue (vital stain) and hemocytometer.
7. Resuspend the cells as per the requirements of the experiment.

Stock Virus Preparation

Standard references strains of EV 70 (J670/71), was obtained from Dr. Kono Laboratory, Tokyo, Japan, and CA 24v was obtained from Center for Disease Control (CDC) Atlanta, USA.

A high titer stock suspension of EV 70 was prepared in LLC-MK2 and CA 24v was prepared in HeLa cell respectively. LLC-MK2 and HeLa cell monolayers in 80cm² TC flasks were infected at 37° C incubator for 45 min to facilitate virus adsorption.

Cultures were then replenished with 30 ml of maintenance medium and incubated at 36° C until complete cell destruction (cytopathic effect) was observed (usually 30 h post-infection). The culture fluid (virus harvest) was collected and subjected to three cycles of freezing and thawing to rupture the cells to release the intracellular virus. The virus harvest was centrifuged at 10000 rpm for 20 min at 4° C in a Beckman JA-21 rotor to remove the cell debris. The resultant stocks were used as virus for in large quantities, for antigen preparation.

Antigen (Large Scale Virus) Preparation

Step 1: Virus Inoculation in Host Cells

Procedure:

1. Six flasks (175 cm², Nunc-Denmark) of LLC-MK2 and HeLa cell cultures are infected with 0.5 ml stock suspension of each virus.
2. Following the virus adsorption for 45 min at 37° C incubator, 30 ml of maintenance medium is added to the culture flasks.
3. The cultures are incubated at 36° C until complete cythopatic effect (CPE) i.e. cell destruction is observed.
4. Freezing and thawing 3 times. Throughout this work LLC-MK2 were used for EV 70 and HeLa cells for CA 24v.
5. The virus harvest is centrifuged at 10000 rpm for 20 min at 4° C (JA-21 rotor, Beckman).
6. The supernatant is collected and stored at -40° C till further process.

Step 2: Virus Concentration by Ultracentrifugation:

Procedure:

1. The clarified culture supernatant is ultracentrifuged at 27000 xg value for 3 ½ h at 4° C in a Beckman SW-28 rotor to pellet the virus.
2. Virus pellet is resuspended in 12 ml of PBS buffer, suspension is homogenized briefly.
3. This virus suspension is kept at -40° C for further process.

Virus Titration

Viral titers were assayed using a microtitration technique. Serial ten fold dilutions of the virus suspension were prepared in medium (MEM) containing 2 per cent SS and FBS in case of LLC-MK2 and HeLa cell respectively. 50 µl of each of the selected dilutions of virus (10^{-2} to 10^{-8}) was dispensed in ten replicate wells of a microtiter plate (Nunc-Denmark). 100 µl of cell suspension (LLC-MK2 or HeLa) containing 1×10^4 cells was seeded into each well.

Laboratory Animals

Rabbits (Belgium strain, outbreed), Guinea pigs (English short hair, outbreed), and Mice (Swiss albino, outbreed) were obtained from Haffkine Bio-Pharmaceutical Corporation Ltd., Mumbai.

Three days prior to immunization, blood collection of animals was carried out. It was considered as pre-immunization blood sample. Serum thus obtained was used as serum control.

Maintenance and Feeding of Animals

Animals were maintained in clean hygienic conditions and were fed appropriate diet of germinated Bengal gram, carrot, and Lucerne grass in case of rabbits and Guinea pigs, while mice were feed by dry pellet. The grass and carrot were treated with potassium permanganate ($KMnO_4$) for 20 min and then washed with tap water before feeding the animals.

Immunization of Animals

Most practical immunization schedules employ agents known as adjuvants to enhance the immune response of an animal. The choice of adjuvant determines to some extent the dose of antigen, route of inoculation, and the requirements for boosting.

Some adjuvants serve to form local antigen depots, at the immunization site, from which antigen leaks slowly. They can achieve this by absorbing on to themselves the antigen (this usually applies in the case of inorganic adjuvant materials) or entrapping the antigen in a water-in-oil emulsion, as is the case with the Freund's-type adjuvants. This adjuvant formulated by Freund, is the method of choice for immunizing animals in laboratory investigations.

The oil phase consists of mineral oil and emulsifier, usually mannide monooleate. This mixture is referred to as incomplete, while the complete Freund's adjuvant contains killed mycobacterium (usually *Mycobacterium butyricum*) (0.05 % w/v). The complete form is generally used for the primary inoculum. These adjuvants are usually mixed with an equal volume of aqueous solution (or suspension) of the antigen, but can be used at other ratios such as 2:1 or 9:1 (Tung et al., 1976). The complete adjuvant should be thoroughly mixed before use to ensure uniform dispersion of the mycobacteria.

Manual mixing, blending, or ultrasonication can achieve emulsification. The simplest, but possibly most tedious method is to prepare the emulsion manually. This can be achieved by repeatedly drawing the mixture into a syringe through a relatively wide-range needle (16-19 G, for example), and expelling it. In this study we prepared the emulsion manually for inoculation of antigen in animals.

Intravenous injection (iv) in rabbits and intraperitoneal injection (ip) in Guinea pigs were given without adjuvant.

Rabbits weighing 1.2-1.5 kg, injected with 1ml of concentrated virus mixed with equal volume of adjuvant i.e. complete Freund's adjuvant (DIFCO Laboratories, Cat No. 0638-59, Detroit, Michigan, USA) as the first immunization. While for the further immunizations (boosting), incomplete Freund's adjuvant (GIBCO BRL, Cat No. 15720-030, Life Technology, NY, USA) was used and emulsified. 0.5 ml of the emulsion was injected subcutaneously (sc), at four different sites on the back of the animal. Prior to virus inoculation, in order to prevent infection 70 % ethanol was applied on the site of injection. Post-injection, antibiotic powder broad-spectrum (Neosporin-Burroughs Welcome India Ltd.) was applied on the site of injection as well.

Guinea pigs weighing approximately 300 g, injected subcutaneously (sc) with 0.5 ml concentrated virus along with equal volume of adjuvant, at two different sites on the back of animal. Intraperitoneal injection (ip) was carried out using 0.5 ml of virus without adjuvant.

Mice weighing approximately 35 g received concentrated virus through intraperitoneal injection (ip) i.e. 0.2 ml of purified virus mixed with adjuvant at the ratio of 2:1.

Reimmunization (Booster)

Immunization was repeated five to seven times at two-week intervals, on the same route considering the type of the animal (Table 12). It should be mentioned that hyperimmunizing animals often raise polyclonal antibodies. Hyperimmunization refers to injection schedules in which animals repeatedly are boosted with the same antigen.

Table 12: Immunization Schedule of Laboratory Animals to produce diagnostic antisera.

DAY	ROUTE OF INJECTION			VOLUME OF DOSE OF ANTIGEN		
	Rabbit	Guinea pig	Mice	Rabbit	Guinea pig	Mice
1	sc + CFA	sc + CFA ip	ip + CFA	1.0 ml	sc 0.5 ml ip 0.5 ml	0.2 ml
14	sc + IFA iv	sc + IFA ip	ip + IFA	sc 1.0 ml iv 0.8 ml	sc 0.5 ml ip 0.5 ml	0.2 ml
28	sc + IFA iv	sc + IFA ip	ip + IFA	sc 1.0 ml iv 0.8 ml	sc 0.5 ml ip 0.5 ml	0.2 ml
42	sc + IFA iv	sc + IFA ip	ip + IFA	sc 1.0 ml iv 0.8 ml	sc 0.5 ml ip 0.5 ml	0.2 ml
56	sc + IFA iv	sc + IFA ip	ip + IFA	sc 1.0 ml iv 0.8 ml	sc 0.5 ml ip 0.5 ml	0.2 ml
70	sc + IFA iv	sc + IFA ip	ip + IFA	sc 1.0 ml iv 0.8 ml	sc 0.5 ml ip 0.5 ml	0.2 ml
84	sc + IFA iv	sc + IFA ip	ip + IFA	sc 1.0 ml iv 0.8 ml	sc 0.5 ml ip 0.5 ml	0.2 ml

iv: intravenous
ip: intraperitoneal
sc: subcutaneous
CFA: Complete Freund's Adjuvant
IFA: Incomplete Freund's Adjuvant

The resulting antibodies have many useful properties, including *class shift* sera from primary injections contain a substantial proportion of IgMs, whereas sera from hyperimmunized animals contain mostly IgG antibodies. Hyperimmune sera often have higher levels of IgG part of which is specific antibody. Levels of 1 mg/ml of antigen-specific IgG are possible. This is equivalent to 10 % of the total IgG content of serum. *Affinity maturation* and *Clonal dominance* are some other properties of hyperimmunization.

The following methods were carried out for immunization of animals:

Subcutaneous (sc) injections for rabbits

Procedure:

1. Keep the rabbit on the table and while it is hold, lightly press on the neck and end of the back (lumbago) by the palms of the assistant.
2. Starting on one side near the back of the neck, pinch the skin between the thumb and forefinger, pull the skin away from the body and insert the needle (20 G) into the space that has been created.
3. Move the end of the needle a short distance from side-to-side to ensure it is not inserted into muscle or the body wall.
4. Depress the plunger to inject the desired amount. Pause a few seconds. As the needle is withdrawn, the hole should be gently rubbed with the forefinger and the thumb to stop any of the inoculum from spacing.
5. Move to the next site and repeat. Injected is repeated in two sites per animal.

Intravenous injection (ip) for rabbits:

For most laboratory animals the intravenous injection (iv) is a practical method of delivering a secondary or later boost. As advantage of this method it could mention that antigen is delivered directly in the bloodstream will be processed rapidly by the reticuloendothelial system, primarily in the liver, lungs and spleen.

Intravenous injections are normally done in the marginal ear vein. A normal iv injection is 500-1000 μ l, that in present study 700-800 μ l of antigen was injected in each rabbit

Procedure:

1. Remove the rabbit from its cage and place in a restraining device.
2. Pull the ear out straight. The vein is on the far side edge of the ear. By pressing lightly on the base of the ear, the return of the blood to the body can be restricted and the vein will stand out strongly. Do not use xylene to help dilate the vein.
3. Insert the needle (25G) attached to a 1ml disposable syringe (insulin syringe) into the vein. When the needle appears to be in the syringe, processed with the injection. If no blood is seen, move to another location on the vein and try again.
4. Slowly and evenly inject the antigen solution in the vein. Pause for a few seconds and remove the needle. Place a piece of cotton or sterile gauze over the injection site as the needle is removed. Hold the site tightly for a few seconds to stop any bleeding.

Note:

- i) Direct iv injection carries serious risk to the host animal, as it can readily induce pulmonary embolisms or lethal anaphylactic shock in

sensitized animals. It is not safe or appropriate to use Freund's adjuvant with iv injections.

- ii) Injection by this route into immunized animals may cause an anaphylactic reaction. This can be prevented by a prior injection of an antihistamine.

Subcutaneous (sc) injection for Guinea pigs:

Procedure:

1. An assistant must hold the guinea pig's shoulders firmly with one hand whilst restraining the lower part of its body with the other, so that the injection site is exposed.
2. Injections are made under the loose skin of the back parallel with the underlying muscle i.e. approximately 0.3 ml for each sites to be injected.
3. Rub well the site of injection to prevent draining out the inoculum.

Intraperitoneal (ip) injection for Guinea pigs:

In this method it is require that the animal to be hold on its back with the abdomen uppermost. The needle (25 G) is inserted slightly to the right or left of the mid-line away from the bladder into the peritoneal cavity.

Intraperitoneal (ip) injection for Mice:

This injection is made through the lower part of the abdominal wall into the peritoneal (abdominal) cavity, slightly to one side of the mid-line to avoid piercing the bladder.

Procedure:

1. Restrain the mouse through the loose skin on the neck should be firmly grasped between the thumb and index finger.
2. Hold the tail between the third and fourth finger of the same hand.
3. The animal should be held with the abdomen uppermost and with the head titled slightly downwards so that the gut slips towards the diaphragm.
4. The skin around the abdominal area should be taut before proceeding with the injection.
5. Insert the needle (25 G) into the abdominal cavity and slowly release the antigen.

Bleeding of Animals

Blood was drawn at each two weeks interval following immunization. Rabbits were bled through ear vein, while in case of Guinea pigs cardiac puncture was the method of choice. From mice, blood as collected either by cutting the tail vein or cardiac puncture. Table 13 indicates the schedule plan of bleeding considering the type of animals.

Table 13: Bleeding Schedule of Laboratory Animals

Day	Route of Blood Collection		
	Rabbit	Guinea Pig	Mice
14	ev	—	tv
27	ev	cp	cp
40	ev	cp	tv
54	ev	cp	cp
68	ev	cp	tv
82	ev	cp	tv
96	cp	cp	cp

ev: ear vein
 cp: cardiac puncture
 tv: tail vein

In order to obtain the maximum amount of blood from rabbits as final or exsanguination of animals, we devised a rabbit bleeding apparatus and a board for rabbit bleeding adapted from Marchalonis and Warr (1982) with modification.

Tail Bleed of Mice:

Procedure:

1. Isolate the mice in a small cage or container.
2. Heat the mouse under an infra red lamp or warm its tail with moderately hot water for a few min.
3. Place the mouse in a restricted space (cage).
4. Swab a portion of the tail about 1.5-2 inches from the body with alcohol.
5. Using a sterile scalpel, nick the underside of the tail across one of the lower veins that should be visible. Collect several drops of blood in a tube and return the mouse to its cage.
6. Keep the blood in room temperature for 1h. Flick the tube several times to dislodge the blood clot.
7. Transfer to 4° C overnight.
8. Spin at 5000 rpm for 10 min at 4° C.
9. Remove the serum from the cell pellet. Discard the cell pellet and spin the supernatant a second time for 10 min.
10. Remove the serum, being careful to avoid the packed cell pellet.
11. Keep the serum frozen at -40° C.

Bleeding from Ear Vein of Rabbit:**Procedure:**

1. Place rabbit in restraining device, or wrap in a towel. Gently have a patch around the vein about two-thirds of the distance from the head of the tip of the ear.
2. Insert a sterile disposable needle (gage 20) in the vein.
3. If the rabbit is scared the normal response will be to constrict the ear artery, thus stopping the flow of blood to the ear. If this occurs, keep the rabbit warm and comfortable. As soon as the rabbit relaxes, the blood flow will start again and it can be collected (Figure 8).
4. Collect the blood by allowing it to drip into a sterile test tube. The maximum volume to be collected from a rabbit at one time is 50 ml. Do not attempt to take more, as this will make the rabbit anemic.
5. The blood flow can be stopped by gentle pressure for 10 to 20 seconds and then check to be sure the flow has stopped.
6. Prepare serum as it was mentioned earlier.

Notes:

- i) The easiest method for collecting blood from the ear is to cut the marginal vein with a sterile razor or scalpel. With either, the cut is made at a 45° angle to the vein and not directly transverse. The incision is made just through the top of the vein.
- ii) Xylene on ears is cruel. Don't do it.



Figure 8: Position for collecting blood from ear vein of a rabbit

Exsanguination of Rabbits by Cardiac Puncture

Rabbits are usually anaesthetized using Nembutal (Pentobarbital sodium) at a dose of 30 mg per kilogram intravenously. Usually three-quarters of the calculated dose is injected at once (e.g. into the ear vein) and the rest only if needed. The animal needs to be firmly secured for this injection, and if there are problems with under dosage or failure to make a clean injection, the animal will go through a phase of involuntary excitement. Some investigators inject rabbits intraperitoneally with Nembutal, but by this route adsorption is slow, dosage is uncertain, and anaesthesia ensures slowly (Marchalonis and Warr, 1982).

In present study Anèket (Ketamine hydrochloride, available from Neon Laboratories Ltd. Mumbai) was used successfully with the Belgium white rabbits

Bleeding is frequently carried out by use of a 50ml hypodermic syringe with an attached needle (1½ inch, 19 G). If this method is used, the syringe has to be detached from the needle when full and reattached after being emptied. This procedure can result in dislodgment of the needle from the heart, and is also inconvenient.

We used an alternative method, which avoids these problems, is to insert into the heart a needle attached to a flexible tube, in turn connected to a collection vessel on which a gentle vacuum can be applied. A suitable apparatus is illustrated in Figure 9. This bleeding set is connected to the collecting vessel, which in turn is connected to a 50ml syringe, which is used to apply a gentle vacuum. The syringe is connected to the collecting vessel by means of a three-way stopcock which allows the syringe to be exhausted when full of air, without dismounting the system. Using this bleeding apparatus, up to or slightly more than 100 ml of blood can be obtained from a mature rabbit.

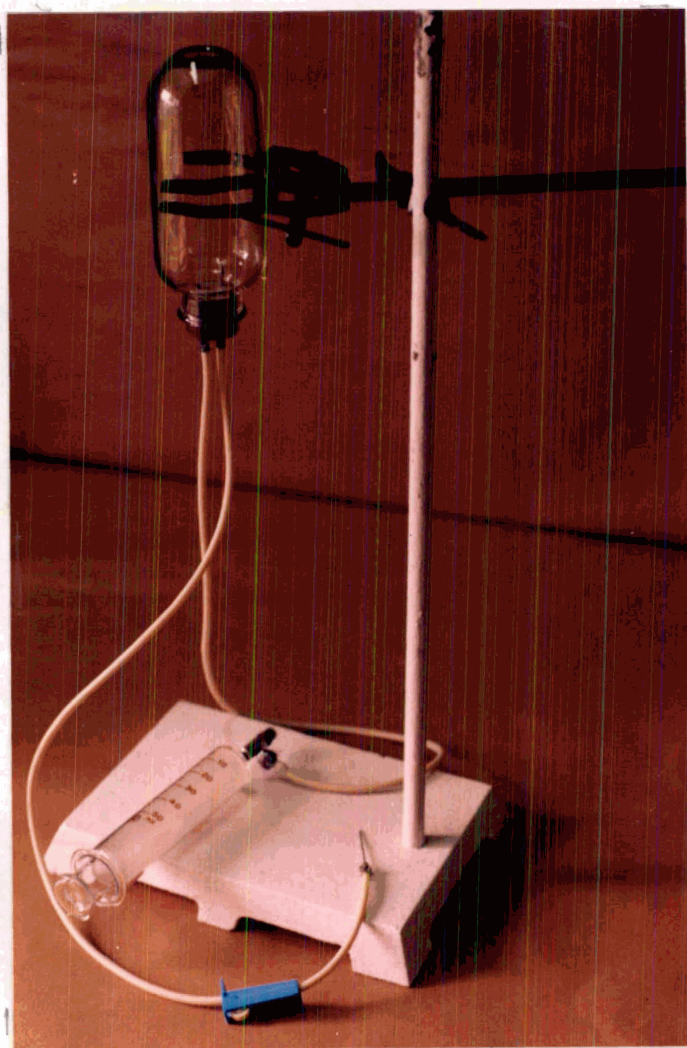


Figure 9: Rabbit bleeding apparatus with stand and clamp.

Procedure:

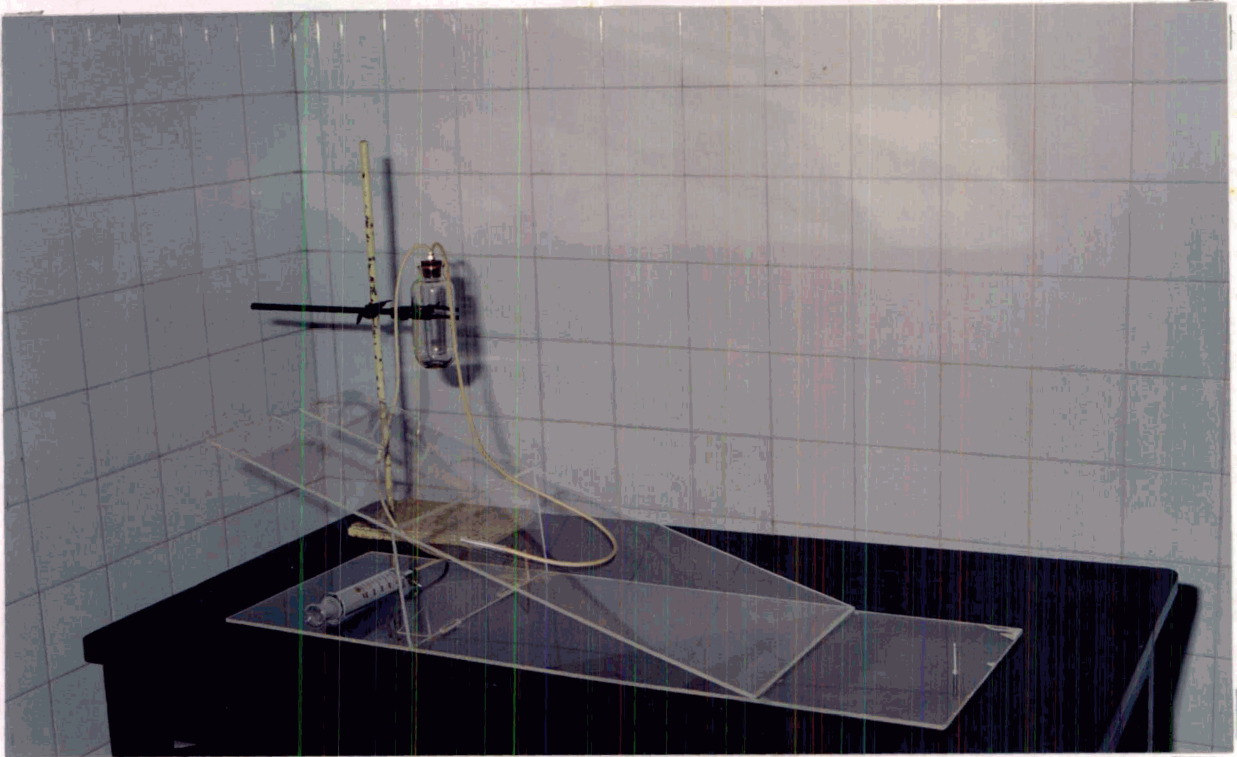
1. An injection is made intramuscularly (im) at 50 mg per kilogram body weight and anesthesia ensues within minutes (Figure 10). With this form of anaesthesia, pedal (and other) reflexes are retained, and the eyes remain open.
2. Anaesthesia is almost immediate, and can be checked by pinching a foot to test for absence of the withdrawal reflex.
3. Restrain the rabbit for bleeding. An inclined board, as illustrated in Figure 11(a). The head fits under the top of the board, with the neck passing through the slot. The hind legs are tied with a loop of string that is made fast to a peg on the board. The front legs are free, [Figure 11(b)].
4. The animal should be tied securely but not too tightly, as this can result in an alteration of the geometry of the thorax.
5. Detect the beating of the heart before penetration is achieved.
6. The needle (1½ inch, 19 G) is inserted at the angle formed between the last left sternal rib and the xiphoid process. The fur in this region is shaved, or just wetted with an aseptic (e.g. 70% alcohol), and the point for insertion of the needle is palpated. The needle is inserted, bevel up, at an angle of about 30° to the plane in which the rabbit lies.
7. Apply a gentle vacuum during this phase, results in an immediate flow of blood once the needle is correctly positioned.
8. During bleeding the operator should be prepared to adjust the position of the needle to maintain a free flow of blood.
9. After withdrawal of the maximum amount of blood, it should be ensured that the animal is dead. This can be achieved by introducing a few

milliliters of air into the heart (especially convenient apparatus described in Figure 9), by severing the spinal column with bone forceps, or by breaking the neck with a blow to the back of the head, or by placing the animal into a container filled with ether.

10. Preparation of serum is carried out, as it was mentioned earlier.



Figure 10: Intramuscular injection (ip) for anaesthesia
in a rabbit for cardiac puncture.



(a)



(b)

Figure 11: Rabbit bleeding by cardiac puncture,
Figure 11(a) shows board for rabbit bleeding and
Figure 11(b) shows the anaesthetized rabbit restrained on the board.

Bleeding through Cardiac Puncture of Guinea pigs:

The alternative method for guinea pig bleeding is cardiac puncture. Since this method is a sensitive method, up most care is must.

Procedure:

1. Animal is anaesthetized by Ketamine hydrochloride (Neon Laboratories Ltd., Mumbai) 50 mg per kilogram body weight. Injection is done through intramuscular (im) route.
2. Anaesthesia is almost immediate, and can be checked by pinching a foot to test for absence of the withdrawal reflex.
3. The injection site is cleansed with an antiseptic (70 % ethanol).
4. Animal is held securely in assistant's hands on its back with the abdomen uppermost.
5. Detect the beating of the heart by index finger before penetration is achieved.
6. The needle (25 G) which is attached to a 5ml disposable syringe is inserted, bevel up, between 3rd and 4th rib of the left chest, at the angle of about 45° to the plane in which the animal lies.
7. Collect maximum 5 ml of blood and gently take out the needle from the heart while placing a sterile gauze or cotton on the insertion site.
8. Drain out the blood into a sterile 10ml plastic vial and process for serum preparation.

Note:

- i) In order to increase the potency of the anaesthetic and reducing the side effect of medicine, 4-6 h prior to anaesthesia animals should not be given food.
- ii) Excess of saliva secretion in guinea pigs may happen.
- iii) Post-anaesthesia is very important step; animal should remain under close observation till complete recovery is occurred.

Virus Neutralization Test:

The neutralization test is based on the principle that when a specific immune serum is added to its corresponding virus, the virus is rendered non-infective or is "neutralized".

Virus neutralization test aimed at specific inhibition of cytopathic effect (CPE) in cell lines used for the estimation of antibodies in serum of virus induced immunized animals.

In the present investigation, antibody endpoints were determined by using constant dose of virus and varying dilution of serum samples.

Procedure:

1. Test is carried out in a Biological Safety Cabinet (BSC) class II type A/B3 (The Baker Company-Sanford, Main- USA).
2. The rabbit anti-CA 24v or anti-EV 70 serum was diluted in MEM containing 2 % FBS and SS in case of HeLa and LLC-MK2 respectively.
3. Virus is diluted in maintenance medium to contain 100 TCID₅₀ in a volume of 50 µl (as determined by a previous titration of the virus).
4. 50 µl of antiserum is added to designated wells of a sterile 96 well flat bottom TC plate (Microtiter plate, Nunc-Denmark)

5. 50 μ l of the diluted virus (100 TCID₅₀) were added to the wells containing antisera. The contents were mixed on a microplate shaker platform.
6. Back titration (virus control) was carried out in four wells with each virus dilution containing 100, 10, 1, and 0 TCID₅₀ of the virus.
7. The plates were incubated in a 5% CO₂ incubator at 36 C for 90 min (Forma Scientific).
8. 100 μ l of cell suspension i.e. HeLa or LLC-MK2 containing 10000 or 15000 cells were then seeded in each well.
9. Uninoculated cultures from the same batch incubated under identical conditions served as cell controls.
10. The plates were incubated at 36° C with 5% CO₂. Cultures were observed for period of 7 days using an inverted TC microscope (Axiovert 25-Ziess, Germany).

Interpretation of Results:

Manifestation of CPE in cultures inoculated with virus-serum mixture indicated the absence of neutralization. Conversely, absence of cytopathic effect inoculated with virus-serum mixture indicated presence of neutralizing antibody.

The dose of the virus in the test and the titer of neutralizing antibodies were calculated as per Kärber (1931).

Immunoenzymatic-Staining

Horseradish peroxidase (HRP) conjugated with goat-anti rabbit IgG

Followed by virus neutralization test, immunoenzymatic-staining was carried out, using antisera that showed high titer antibody. The method used in present experiment was adapted with modification from the previous investigations (Deshpande, 1980; Johnson, 1978).

The enzyme from horseradish is a glycoprotein with a hemin prosthetic group and bound Ca^{++} . The molecular weight of native horseradish peroxidase is approximately 44000 (Wellinder and Eur, 1979). Enzymes of which the most widely used in horseradish peroxidase can allow antigen tracing in both the light and the electron microscope with the resolution the later confers. Development of the reaction product of the enzyme also provides a degree of amplification while the use of soluble peroxidase-anti-peroxidase complexes in a triple bridge technique can provide a degree of sensitivity 100-1000-fold higher than immunofluorescence (Sternberger, 1972; Burns, 1975). In general, enzyme labeling has been considered superior to other markers in terms of sensitivity and specificity (Bergquist et al., 1976; Bellon et al., 1978) and because it excludes autofluorescence and the preparations are long lasting (Feteanu, 1978).

Three substrate are suitable for developing detection with enzyme-labeled reagents: chloronaphthol, aminoethyl carbazole, and diaminobenzidine (Marchalonis and Warr, 1982). The previous substrate was used in this experiment.

Diaminobenzidine (DAB, 3, 3', 4, 4' - tetraaminophenyl) is an exceptionally sensitive substrate, yielding a brown reaction product. Adding cobalt or nickel ions to the substrate solution can further enhance its sensitivity. In the presence of these ions, the product is slate black in color and more easily photographed than the unenhanced product. The draw back to DAB is that it reacts so fast that is very easy to overdevelop a high

background. The preparation of substrate solution is given in the chemical's chapter.

Procedure:

Step 1: Preparation of cell culture on serologic slides:

1. Arrange the required slides in a Petridish (25-cm diameter).
2. Add 25ml of growth medium with 10 % FBS or SS in case of HeLa or LLC-MK2 cells respectively.
3. Calculate the required number of cells to be seeded into Petridish.
4. Make a homogenous cell suspension while gently mixing the cell with growth medium.
5. Incubate the Petridish at 37° C with 5 % CO₂.
6. When complete monolayers observed change the medium and add fresh maintenance medium with 2 % FBS/SS and keep the plates at room temperature (if the virus inoculation is not done immediately).

Step 2: Virus inoculation:

1. Inoculate cells with diluted virus suspension i.e. 10⁻².
2. One slide is remained uninoculated as cell control.
3. Observe the cells till 2 CPE appear.
4. Discard TC medium in a decontaminate i.e. 5 % sodium hypochloride solution.
5. Keep the slides in 1-2 % formaldehyde solution overnight or in 10 % of the same solution for 1-2 h.

6. Discard the solution in decontaminate.
7. Rinse the slides in PBS buffer.
8. Discard the solution in decontaminate.
9. Air-dried the slides.

Step 3: Staining the slides:

1. Prepare antisera dilution in PBS 1:10 up to 1:320 respectively.
2. Add 25 μ l of different dilution into slides as designed.
3. Add 25 μ l of HRP goat-anti rabbit IgG (SIGMA-No A4914).
4. Incubate the slides in a moist Petridish at room temperature for 1 h.
5. Remove the excess of antibody three times in PBS buffer.
6. Transfer the slides to the substrate solution; allow reacting for 5 min.
7. Wash the slides for 5 min in distilled water.
8. Counter stain with 2 % aqueous methyl green for 2-5 min.
9. Rinse in distilled water.
10. Dehydrate a brief wash in alcohol.
11. Clean the slides in xylene.
12. Mounted in DPX mounting medium.

CHAPTER 4

REAGENTS AND CHEMICALS

REAGENTS AND CHEMICALS

Composition of Media and Other Reagents Used in Cell and Virus Culture:

1. Minimum Essential Medium (GIBCO BRL, Cat No. 41500-018) (With Earl's salts, L-Glutamine with non-essential amino acids without sodium bi-carbonate).

2. Phosphate buffered saline, pH 7.2 to 7.4 (PBS)

Sodium chloride	8.02 g
Potassium chloride	0.20 g
di-sodium hydrogen phosphate 12 H ₂ O	2.89 g
Potassium dihydrogen orthophosphate	0.20 g
Purified water (Milli Q) to	1000 ml

Sterilize by autoclaving at 15 ibs pressure for 15 min.

May be stored at room temperature.

3. Sodium-bi-carbonate solution (NaHCO₃)
(GIBCO BRL, 1180-025)

NaHCO ₃	7.5 g
H ₂ O	1000 ml

Sterility filtration.

30ml aliquots stored at 4° C.

L-Glutamine (GIBCO BRL, 21051-024)

L-Glutamine	3 g
Purified water (Milli Q)	1000 ml

Dispense in 5 ml volumes and store at -20° C.

4. Hydroxyethyl piperazine ethane sulphononic acid (HEPES)
(GIBCO BRL, 11344-041)

HEPES (acid)	23.8 g
Purified water	100 ml

Sterilize by filtration through 0.22 μ membrane filter.

Distribute in 30 ml aliquots and store at 4° C.

5. Penicillin and Streptomycin
(Penicillin: Alemeric Chemicals Works Company Ltd.)
(Streptomycin: Sarabhai Chemicals)

Dissolve 1×10^6 units of crystalline Penicillin G (1 vial) and 1.0 g streptomycin sulphate in 10 ml PBS; distribute in 2 ml volumes and store at -20° C.

6. Kanamycine sulphate (Alemeric Chemicals Works Co. Ltd)
Dissolve 0.5 g Kanamycin (1 vial) in 25 ml of sterile PBS, distribute into 2 ml volumes and store at -20° C.

7. Tissue culture medium

	Growth medium	Maintenance medium
MEM	85.0 ml	92.0 ml
L-Glutamine 3 %	1.0 ml	1.0 ml
NaHCO ₃	1.5 ml	2.5 ml
HEPES 1M	2.0 ml	2.0 ml
Penicillin/Streptomycin solution	0.5 ml	0.5 ml
Kanamycin	0.1 ml	0.1 ml
Foetal calf serum or sheep serum	10.0 ml	2.0 ml

Penicillin and streptomycin are the antibiotics most commonly used in cell and routine virus culture work. Kanamycin is used for prevention of mycoplasma infection.

8. Trypsin (1:250) (GIBCO BRL, 27250-018)

Trypsin (1:250)	10.0 g
PBS	500 ml

Warm the solution to 37°-40° C to facilitate the dissolution. Centrifuge at 5000g for 20 min at 4° C.

9. Ethylene diaminetertetra Acid Disodium Salt Dihydrate (GIBCO BRL, 15576-010) Versene.

Dissolve 0.2 g EDTA in 100 ml of distilled water. Sterilize by autoclaving.

10. D-glucose (Glaxo Laboratories, 3980)

Dissolve Dextrose (anhydrous) powder in distilled water to obtain a 10 % solution. Sterile by autoclaving at 1lbs pressure for 10 min.

11. TPVG

Prepare working solution of trypsin by mixing trypsin, versene as given below:

PBS	840 ml
Trypsin	50 ml
Versene (0.2 %)	100 ml
D-glucose	5 ml
Penicilin/Sterptomycin	1 ml
Phenol red (0.1 %) about	0.2 per litre

Adjust the pH with 1N NaOH to pH 7.5

Store in 30 ml aliquots at 4° C.

12. Milli Q Purified Water

Highly purified water (18 m mols) obtained by passing distilled water through Milli Q water purification system (Millipore Corporation, Milli Q ZFMQ 24004).

Chemicals for limmunization and Bleeding of Laboratory Animals:

1. Complete Freund's adjuvant (DIFCO Laboratories, Cat No. 0638-59, Detroit, Michigan, USA).
2. Incomplete Freund's adjuvant (GIBCO BRL, Cat No. 15720-030, Life Technology, NY, USA)

It should be mentioned that complete adjuvant is applied for the first inoculation into animals along with antigen, while incomplete adjuvant is used for the second inoculation onwards.

3. Ketamine Hydrochloride (Aneket), (Neon Laboratories Ltd. Mumbai, India).

Each ml contains:

Ketamine Hydrochloride I.P. (Equivalent to Ketamine)	50 mg
Benzalkonium Chloride (50 % w/v soln.) I.P. (As preservative)	0.02 % w/v
Water for injection I.P.	q.s.

Its application is for general anaesthetic. 50 mg per/kg/body weight.

Neosporin . Antibiotic Powder (BURROUGHS WELLCOME Ltd. Mumbai-India).

To be applied post-inoculation of antigen in animals, in order to prevent bacterial or fungal infection.

Chemicals for Immunoenzymatic-Staining:

1. Horseradish peroxidase (HRP) conjugated with goat-anti rabbit IgG (SIGMA, A4914).

Prepare 1:1000 dilution in PBS buffer.

2. Substrate solution

3, 3' Diaminobenzidine tetra HCl (DAB), (SIGMA, D-5637)	25 mg
0.05 moles/litre pH 7.6 Tris-HCl buffer	50 ml
3 per cent hydrogen peroxide (H_2O_2)	0.15 ml

The hydrogen peroxide is added to the DAB solution just before use.

Diaminobenzidine with metal Ion Enhancement (DAB/Metal).

1. Just prior to developing the immunoenzymatic-staining, dissolve 6 mg of 3, 3-diaminobenzidine in 9 ml of 50 mM Tris (pH 7.6). A small precipitate may form. Add 1 ml of 0.3 % (w/v) $NiCl_2$ or $CoCl_2$.

2. Filter through whatman No.1 filter paper (or equivalent).

3. Formaldehyde solution (10 %), (fixative)
Dissolve 27 ml formaldehyde in 13 ml distilled water.

4. DPX mounting medium.

5. Xylene

6. 2% methyl green (counter staining)

CHAPTER 5

RESULTS

RESULTS

Two types of enteroviruses, causative agents of acute hemorrhagic conjunctivitis (AHC) i.e. standard reference strains of enterovirus type 70 (J670/71) and Coxsackievirus A 24variant (CA 24v) were used as viral antigen in order to induce immunity of animals in term of serum antibody response. In this study, rabbits, guinea pigs, and mice (all out-bred) were used for this purpose.

Antigen preparation and virus purification of EV 70 and CA 24v was carried out after growing the viruses on host monolayer cells of LLC-MK2 and HeLa respectively. Infected cell culture harvests having viral titer in the range of $10^{6.5}$ to $10^{6.8}$ TCID₅₀ per ml for EV 70 and $10^{7.0}$ to $10^{7.2}$ TCID₅₀ per ml for CA 24v, were routinely obtained (Table 14). About 180 ml of virus suspension was used as the starting material for concentration and purification of each virus.

Table 14: Partial purification and concentration of EV 70 and CA 24v by ultracentrifugation

Steps of virus purification	EV70 TCID ₅₀ /ml	CA24v TCID ₅₀ /ml
1. Tissue culture fluid	10 ^{-6.8}	10 ^{-7.0}
2. Supernatant (10000 rpm)	10 ^{-7.0}	10 ^{-7.3}
3. Supernatant (27000 rpm)	10 ^{-5.2}	10 ^{-5.4}

The virus infected cell culture fluid was centrifuged at 10000 rpm in a JA 20 rotor to remove cell debris and large sedimental particles. The supernatant was then ultracentrifuged at 27000 rpm in a SW 28 rotor for 3½ h to pellet the virus particles.

As shown in Table 14 that the titer of EV 70 at step of culture fluid was $10^{6.8}$ centrifugation at 10000 rpm was $10^{7.0}$. Finally at the ultracentrifugation step, $10^{5.2}$ TCID₅₀ per ml was found in the supernatant.

In case of CA 24v, titration test of the same steps shows the following results $10^{7.0}$, $10^{7.3}$, and $10^{5.4}$ respectively.

Comparison between three subsequent steps of virus concentration indicates that, the maximum amount of the virus particles has concentrated in the form of pellet followed by ultracentrifugation.

The ultracentrifuged precipitated virus (pellet) was resuspended in 12 ml of buffer. Concentrated virus was not titrated to avoid loss of precious antigen.

Virus recovery was judged from the infected culture fluid (pre-centrifuged), supernatant fluid (post-centrifuged), and supernatant fluid at step of ultracentrifugation.

Immunization of laboratory animals was carried out according to schedule plan (Table 12). Booster was given in each two weeks interval along with either complete Freund's adjuvant (CFA) or incomplete Freund's adjuvant (IFA). Route of virus inoculation varies, and depends the type of animals. Rabbits were immunized through subcutaneous (sc) and intravenous (iv) injection, while Guinea pigs received antigen through subcutaneous (sv) and intraperitoneal (ip) injection, and mice inoculated with antigen through intraperitoneal (ip) injection as well.

The amount of antigen inoculum was given as follows: Rabbits received 1.0 ml concentrated virus along with equal volume of adjuvant through subcutaneous (sc) injection, and 700-800 μ l antigen through marginal ear vein. Guinea pigs immunized through injection of 0.5 ml of concentrated virus with equal volume of adjuvant through subcutaneous (sc) route, and 0.5 ml of antigen through intraperitoneal (ip) injection respectively. Mice inoculated with 0.2 ml of concentrated virus with approximately two times volume of adjuvant through intraperitoneal (ip) injection. Intravenous (iv) and intraperitoneal (ip) injections in rabbits and Guinea pigs were carried out without adjuvant (Table 12).

Blood was drawn between 12-14 days post-immunization (Table 13). Rabbits were bled through ear vein (ev) and for exanguination of animal, the blood was collected by cardiac puncture, using an apparatus functioning on the base of generation of negative pressure vacuum. Collection of blood from Guinea pigs according to their physiological conditions was carried out through cardiac puncture, that is the method of choice for collection of large sample of blood and is preferred to other methods such as the femoral vein. Mice were bled through either cardiac puncture or tail vein. Rabbits and Guinea pigs were anaesthetized by Ketamine hydrochloride through intramuscular (im) injection, while mice by ether inhalation as well.

Serum preparation was carried out and inactivation of antisera samples was followed by keeping them in a water bath at 56° C for 30 min.

Virus neutralization test was carried out with antisera samples obtained from different animals at different periods of immunization. Prior to neutralization test, 100 TCID₅₀ of the virus suspension was calculated by Kärber method. This method is explained as follows:

Kärber (1931) has reported a simple method for computing 50% end points; based on several types of reactions, such as *mortality* and is written as LD₅₀ (50 per cent lethal dose), ID₅₀ indicates the dose of *infects* 50 per cent of

the test animals, PD_{50} the dose which *paralyzes* 50 per cent of the animals. The terminology can also be applied to other host systems; for example, *tissue culture infectivity dose* ($TCID_{50}$) represents the dose that gives rise to cytopathic changes in 50 per cent of the inoculated cultures.

Kärber method is applicable primarily to a complete titration series, that is, the whole reaction range, from 0 per cent to 100 per cent mortality (or infectivity or cytopathic effect, etc), should be represented in the experimental data. However, the methods can be utilized even when these conditions are not fulfilled, provided the reactions employed. If, however, these reactions are erratic (for example, deaths irregularly scattered over a number of dilutions), the end point will be inaccurate.

The Kärber formula is as follows:

Negative logarithm of the $TCID_{50}$ end point titer = [negative logarithm of the highest virus concentration used] - $\left[\left(\frac{\text{sum of per cent mortality at each dilution} - 0.5}{100} \right) \times (\text{logarithm of dilution}) \right]$

Using the data obtained followed by cytopathic changes in the cells, or (CPE), which already has arranged according to protocol, e.g. in case of CA 24v titration becomes:

$$\begin{aligned}
 \text{Negative logarithm of the } TCID_{50} \text{ end point titer} &= -2 - [(100 + 100 + 100 + 100 + 100 + 20 - 0.5) \times (\log 10)] \\
 &= -2 - [(520 - 0.5) \times 1] \\
 &= -2 - 3.7 \\
 &= -5.7 \\
 &= 10^{5.7} TCID_{50} \text{ per } 0.05 \text{ ml} \\
 &= 10^{7.0} TCID_{50} \text{ per ml}
 \end{aligned}$$

Antibody endpoint was determined in system using constant doses of virus and varying dilutions of serum (Constant virus-varying serum method), in brief: a suitable dose of virus is selected and falling dilutions of serum are added in equal amounts to the appropriate virus dilution. The

cytopathogenicity changes of the host cell due to virus inoculation i.e. (CPE) are microscopically observed during a seven day period of time. The neutralizing end point is expressed as that dilution of serum which "protects" 50 percent of the cells against the test dose of virus used, calculation of antibody end point was used by Kärber method.

Experiment with EV 70:

The first attempt was initiated with a group of rabbits (two female) five months old, and a group of mice (three female) about three months old.

The results obtained from virus neutralization test of rabbit antisera indicated poor immune response to EV 70 (Table 15). As it is shown in this table, the maximum serum antibody titer is 1:40, which has obtained from rabbit no. 1 (R-1) and the second rabbit i.e. (R-2) showed 1:20 as the highest serum antibody titer. In case of mice, very low antibody titer i.e. 1:10 is mouse no. M-1E and M-2E, no immune response in mouse M-3E was observed (Table 15).

Table 15: Eliciting antibody response to immunization with EV 70 in rabbits and mice.

Rabbit/ Mice	Sex	Highest antibody titer
R-1	Female	1:40
R-2	Female	1:20
M-1E	Female	1:10
M-2E	Female	1:10
M-3E	Male	NIR

R: Rabbit

M: Mice

NIR: No immune response

Therefore, in the second attempt regarding production of serum antibody against EV 70, a group of rabbits including two female at the same age of the previous batch, and a group of Guinea pigs including five female (at about four months old) were selected. Virus neutralization test was carried out followed by immunization and bleeding as per schedule plan (Tables 12 and 13).

The results obtained from virus neutralization test also indicated that, the highest serum antibody titer in rabbits No. R-14 and R-15 was 1:80 respectively. Also it was shown that in Guinea pigs the highest antibody titer is 1:160 of Guinea pigs No. GP-2 and GP-3. While the titer of 1:80 belongs to GP-4 and the titer of 1:40 has obtained from GP-1, and the titer of 1:20 serum antibody of GP-5 (Table 16).

Table 16: Results of second attempt to improve antibody response to EV 70 in Rabbits and Guinea pigs.

Rabbit/ Guinea pig	Sex	Antibody Titer
R-14	Female	1:80
R-15	Female	1:80
GP-1	Female	1:40
GP-2	Female	1:160
GP-3	Female	1:160
GP-4	Female	1:80
GP-5	Female	1:20

R: Rabbit

GP: Guinea pig

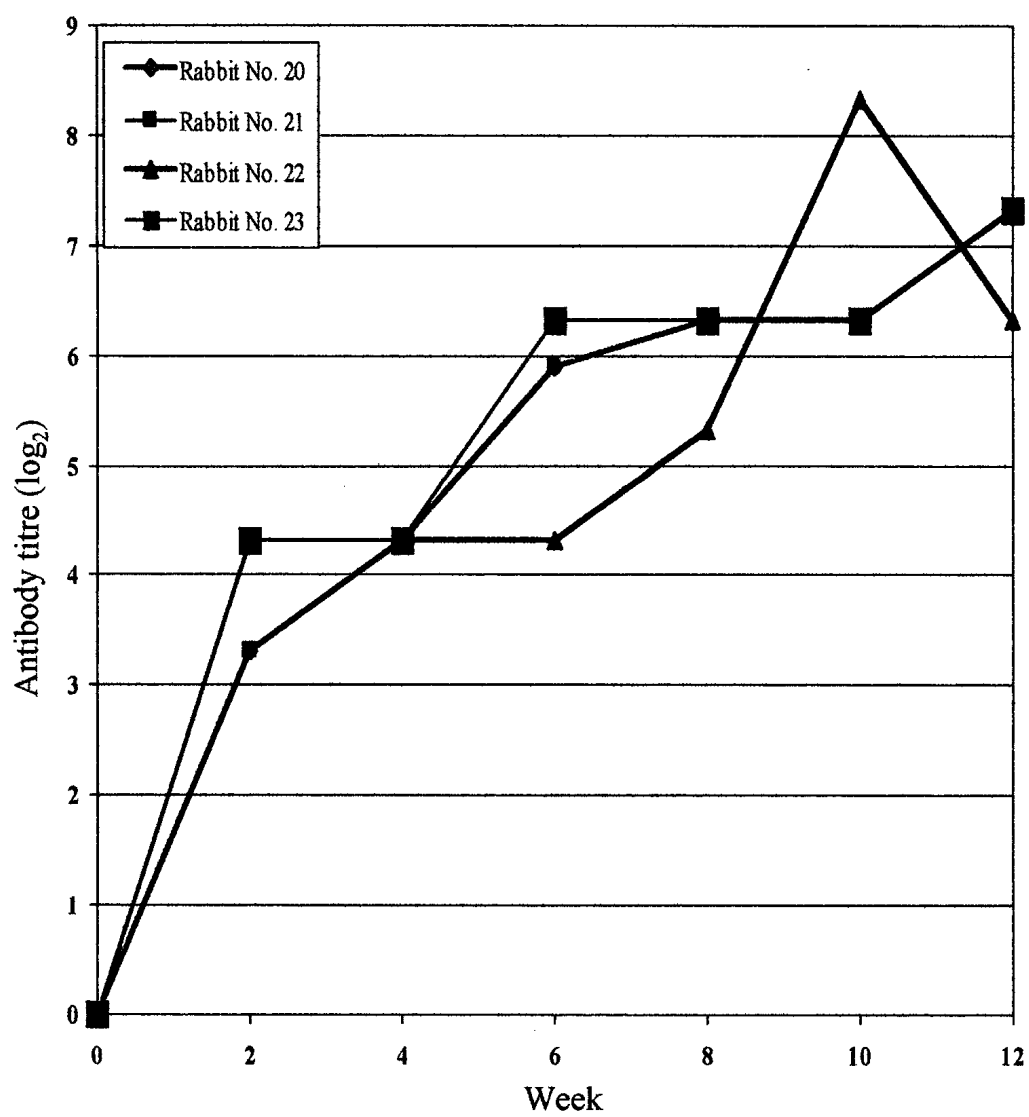
Considering the above results on the basis of virus neutralization test which was obtained from serum antibody titer of rabbits and Guinea pigs, also showed low antibody titer, therefore we arranged another set of experiment as the final effort regarding to obtain high serum antibody titer against EV 70 for diagnosis purposes. Hence, four rabbits including two males and two females, four months old were selected and as per a schedule plan experiment was carried out (Tables 12 and 13).

Followed by virus neutralization test, also it was indicated that in this group of animals, the maximum serum antibody was indicated as 1:320 in rabbit No. R-22. While the rest of rabbits i.e. R-20, R-21, and R-23 the highest antibody titer was 1:160, 1:80 and 1:80 respectively (Table 17 and Figure 12).

**Table 17: Results of neutralization test of EV 70
by the third group of Rabbits antisera**

Rabbit No.	Sex	Antibody Titer
R-20	Female	1:160
R-21	Male	1:80
R-22	Female	1:320
R-23	Male	1:80

Figure 12 : Serum antibody response in rabbits immunized with EV 70



Experiment with CA 24v:

This experiment was initiated with two type of animals i.e. a group of rabbits including three male and one female, and four Guinea pigs including four male were considered for immunization with CA 24v (the age of both group of animals were about four months old). The schedule plan for immunization as well as bleeding and serum preparation was carried out as previous experiments.

The results obtained followed by virus neutralization test in both groups of animals especially in rabbits showed high titer of immune sera against CA 24v. The peak of antisera titer in one of the animals i.e. Rabbit No. R-17 was 1:2560 in which, this titer has been achieved on week 10 post-immunization (Table 18 and Figure 13). In case of Rabbits No's R-16, R-18, and R-19 the maximum serum antibody titer is showed as 1:640, 1:1280, and 1:1280 respectively (Table 18). These titers of rabbit-anti CA 24v antibodies have been obtained during week 12, 10, and 8 in rabbits R-16, R-18, and R-19 respectively (Figure 13). It should be mentioned that in case of R-19 the peak of antibody titer has emerged for two times i.e. between week 8 and 12 post-immunization (Figure 13).

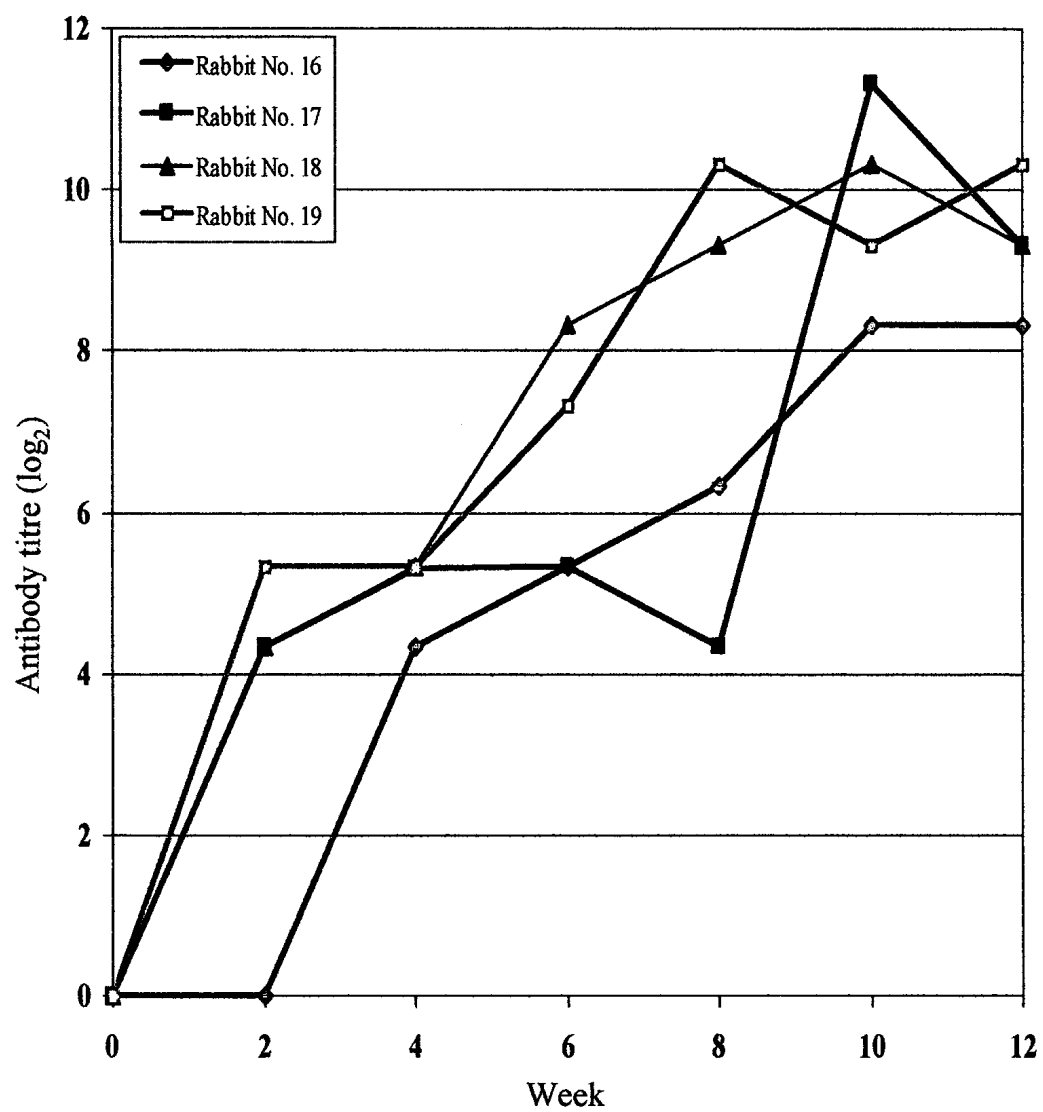
Table 18: Results of titration of CA 24v by neutralizing antibodies in immunized Rabbits and Guinea pigs sera

Rabbit/ Guinea pig	Sex	Highest antibody titer
R-16	Male	1:640
R-17	Female	1:2560
R-18	Male	1:1280
R-19	Male	1:1280
GP-6	Male	1:1280
GP-8	Male	1:40

R: Rabbit

GP: Guinea pig

Figure 13 : Antibody response in rabbits and guinea pigs immunized with cosxsackievirus CA 24v



In case of Guinea pigs, two of them i.e. GP-7 and GP-9 due to unknown reason(s) died in the period of second week post-immunization, and hence blood collection was not possible. Therefore, the serum antibody titer of these animals remained unknown. While the other two i.e. GP-6 and GP-8, their serum antibody titre was 1:1280 and 1:320 respectively. These serum antibody titers were obtained during the second week post-immunization (Table 18 and Figure 13).

For conformation of the accuracy of the immune sera obtained from rabbits and Guinea pigs immunized with CA 24v, cross-match test was carried out. For this purpose poliovirus type 1, type 2, and type 3 were used. Hep-2 cell considered as host cell for growing of polioviruses. Prior neutralization test, titration of viruses was carried out in order to find out 100 TCID₅₀ (based on previous titration test). The results of virus neutralization test using different antisera dilutions of rabbits and Guinea pigs resulted in complete cytopathic effect (CPE), that indicates absence of anti-polio-type 1, type 2, and type 3 in immune sera of rabbits and Guinea pigs, which have been immunized with CA 24 v.

Serum of rabbit No. R-17 labeled as S-5 rabbit-anti-CA 24v that showed serum antibody titer 1:2560 in virus neutralization test was used for immunoenzymatic staining.

Virus suspension of CA 24v was prepared by diluting to 10^2 (considering 100 TCID₅₀ based on the previous virus titration test), and inoculated into the complete monolayer of HeLa cell, grown on cover slips or serologic slides (with twelve flat wells) in a Petri plate. Incubation was stopped when 50% cells showed CPE, then they were kept in a fixative. The slides were air-dried after fixation.

The required amount of each dilution of rabbit serum as control i.e. pre-immunized serum of the same animal (R-17 S-0), and rabbit-anti-CA 24v antisera (R-17 S-5) was added to the cover slips i.e. 1:10 up to 1:320. Then slides reacted with goat-anti-rabbit IgG peroxidase conjugate. The, antigen-

antibody reaction was made visible by reaction with precipitating with chromogenic substrate i.e. 3, 3' diaminobenzidine (DAB). Counter staining was done using 2 % aqueous methyl green and finally mounted in DPX mounting medium. The slides were observed under light microscope.

CA 24v infected HeLa cells showed granular precipitate in the cytoplasm due to the antigen-antibody reaction followed by immunoperoxidase staining. Uninfected cells (control) treated similarly were negative. The immunoperoxidase staining was positive upto 1:80 dilution of the antisera (Figure 14 and 15).

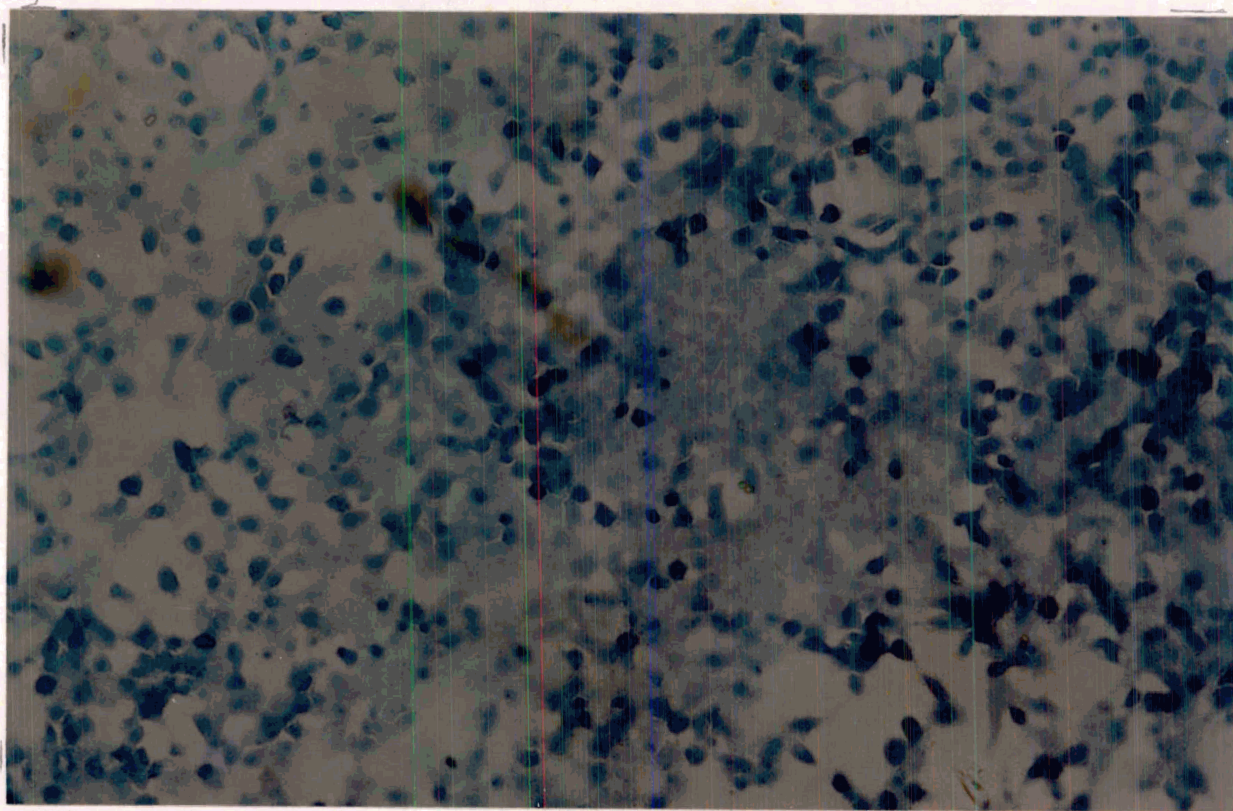
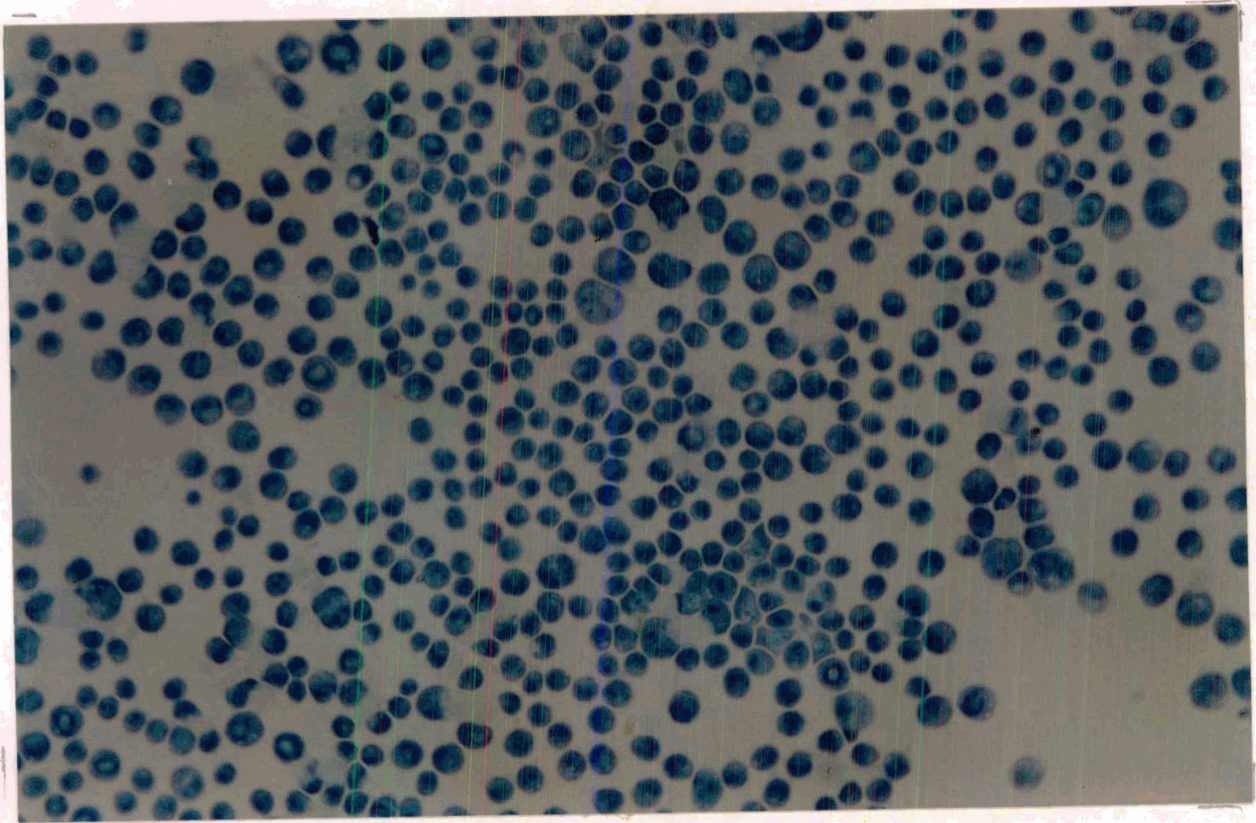


Figure 14: Immunoenzymatic staining. Infected HeLa cells with CA 24v show granules due to antigen-antibody reaction.



**Figure 15: Uninfected cells (control) treated
with immunoenzymatic staining.**

CHAPTER 6

DISCUSSION

DISCUSSION

Two enteroviruses i.e. EV70 and CA24v, the causative agents of acute hemorrhagic conjunctivitis (AHC) were inoculated in to different groups of laboratory animals i.e. rabbits, guinea pigs, and mice, in order to produce high serum antibody titer for diagnosis purpose based on antigen-antibody reaction.

Regarding EV 70 and the results obtained followed by three attempts on group of rabbits, guinea pigs, and mice followed by virus neutralization test showed poor immune response to this virus (Table 18 and Figure 13).

In the second part of the experiment i.e. inoculation of CA 24v into a group of rabbits and guinea pigs followed by the first attempt, the results obtained by neutralization test was indicated the high serum antibody titer in both group of animals. The serum antibody titer in rabbits was shown to be 1:2560, while in guinea pigs 1:1280 (Table 18).

Comparisons of results obtained by virus neutralization test in all groups of animals inoculated with EV 70 and those which were immunized with CA 24v showed different immune responses to the two enteroviruses i.e. in case of CA 24v inoculation, high titer of serum antibody, while for EV 70 poor immune response was indicated.

Considering the experimental conditions regarding maintenance of animals e.g. feeding and environmental situations and unity of different steps in laboratory investigations such as tissue culture techniques, antigen preparation, immunization and bleeding schedule, type of adjuvant, serum preparation, and virus neutralization test. All above mentioned parameters have been carried out at the same circumstances. Hence, it comes in conclusion that the results obtained from EV 70 in different groups of animals, which has shown poor immunity to this virus, could be related to antigenic properties of this virus among the other enteroviruses.

Some of properties of EV 70 on the basis of its molecular biology already have been discussed in review of literature. The other properties of this virus which make an unusual feature and also are related to present investigation are mentioned as follows:

Hierholzer and colleagues reported the results of serosurvey for "acute hemorrhagic conjunctivitis" virus (enterovirus 70) antibodies in the southeastern United States in 1975. Of 1014 residents of the eastern or southeastern United States who were screened for neutralizing antibodies to three or four strains of AHC virus (EV 70), three (0.03 %) had titers ranging from 1:10 to 1:40 (Hierholzer et al., 1975).

In one investigation using monkey inoculated with EV 70, the immunized animals were shown the serum antibody titer 1:320 as the maximum titer (Yin-Murphy, 1976)).

EV 70 is known to resist isolation in many cell lines and complete failure is not uncommon (WHO, 1981; Bernard et al., 1982; Mc Moli et al., 1984).

Results of neutralizing test using serum of patients in some investigation was indicated 1:160 as the highest serum antibody titer followed by infection (Yin-Murphy, 1985).

The present study is related for developing a reagent for diagnosis of EV 70 and CA 24v, and hence a brief history regarding methods of detection of the etiologic agents of AHC seems make a clear feature for this investigation.

Since the first outbreak of AHC due to EV 70 and CA 24v at about three decades ago, the diagnosis of causative agents mainly has been based on the following methods:

1970s: mostly virus neutralization test has played as the method of choice (Kono et al., 1974; Yin-Murphy et al., 1973; Hierholzer et al., 1975), also on report indicates use of Hemagglutination-Inhibition Test (Kono et al., 1978).

1980s: Immunofluorescence techniques for detection of virus-specific antigen in conjunctival cells (Pal et al., 1983). Electron microscopy, although this method dose not differentiate CA 24v from EV 70 morphologically, it distinguishes between picornavirus and adenovirus serotypes which are known to give rise to epidemics of acute hemorrhagic conjunctivitis clinically similar to those caused by CA 24v and EV 70 (Yin-Murphy, 1984). Application of monoclonal antibodies. Enzyme linked immunosorbent assay (ELISA), were also described (Yousef et al., 1987, Philip et al., 1985).

1990s: Electron and immunoelectron microscopy (Ramia et al., 1990); and the methods based on the molecular biology and related techniques e.g. polymerase chain reaction (PCR) and reverstranscriptase (RT-PCR) have been used for diagnosis purposes (Shulman et al., 1997).

It should be mentioned that most of the above-mentioned techniques for diagnosis of AHC viruses, virus neutralization test has been used as the collaborative method for investigation.

In spite of high specificity and sensitivity of the latest methods, to apply such techniques, at present considering some parameters, which are listed below, these methods are not considered as convenient methods for many communities specially those with low-economic status s. They are:

- (a) needs advanced scientific instruments
- (b) needs well-trained technical staff
- (c) cost

It is clear that AHC has mainly emerged in societies with low-economic situation and hygiene, and hence, to diagnose the causative agents at the first stages of its spread playing very important role, in order to suppress the spread of the disease. Regarding the above mentioned facts, therefore, a research study in order to diagnose AHC etiologic agents caused by EV 70 and CA24v was planned. Hence, convenient, applicable, easiness and rapidness of the method considering sensitivity and specificity of the test was the main scope of this study.

Therefore, inspite of failing for production of high titer of serum antibody for diagnosis of EV 70, but it has been able to develop successfully a method for diagnosis of another etiologic agent of acute hemorrhagic conjunctivitis (AHC) i.e. coxsackievirus A 24 variant (CA 24v).

Accordingly, to develop a technique independent of the above mentioned parameters, a diagnostic method on the basis of immunological techniques i.e. immunoenzymatic-staining was developed for any viral acute hemorrhagic conjunctivitis with source of coxsackievirus A24v, and helps for rapid and easy diagnosis of such viral infection.

My plans included use the reagents for diagnosis of cause of AHC as a prospective study. However, there was no outbreak of AHC during the past 2-3 years in Mumbai. Further work is necessary to it up ELISA using there antisera proposed in two specimens of animals, rabbit and guinea pigs, both the viruses.

CHAPTER 7

SUMMARY AND CONCLUSION

SUMMARY AND CONCLUSION

In order to diagnosis of acute hemorrhagic conjunctivitis (AHC), caused by enterovirus type 70 (EV 70) and coxsackievirus a 24 variant (CA 24 v), on the basis of immunological techniques i.e. immunoenzymatic- staining a reagent was developed.

The results obtained followed by virus neutralization test, was indicated low serum antibody titer i.e. poor immune response in animals which have been injected by EV 70, but high serum antibody titer was obtained in those animals that have been immunized with CA 24v.

In case of EV 70, the highest serum antibody titer was determined as 1:80, 1:160, and 1:40 in rabbits, guinea pigs and mice respectively (Table 17 and Figure 12). While in case of CA 24v, the highest serum antibody titer in rabbits and guinea pigs was indicated as 1:2560, and 1:1280 (Table 19 and Figure 13).

**Table 19: Serum antibody titer of rabbits
immunized with EV 70 and CA 24v**

Rabbit No.	S-0	S-1	S-2	S-3	S-4	S-5	S-6
R-16	NIR	NIR	1:20	1:40	1:80	1:320	1:320
R-17	NIR	1:20	1:40	1:40	1:20	1:2560	1:640
R-18	NIR	1:20	1:40	1:320	1:640	1:1280	1:640
R-19	NIR	1:40	1:40	1:160	1:1280	1:640	1:1280
R-20	NIR	1:10	1:20	1:160	1:80	1:80	1:160
R-21	NIR	1:10	1:20	1:160	1:80	1:160	—
R-22	NIR	NIR	1:20	1:20	1:40	1:320	1:80
R-23	NIR	1:20	1:20	1:80	1:80	1:80	1:160

S-0, S-1, etc indicates serum control, antisera sample No. 1, 2, and so.

NIR: No Immune Response

Considering the similarities and unity of the entire processes of the experiment i.e. type and maintenance of the animals, tissue culture techniques for antigen preparation, immunization and bleeding schedule, type of adjuvant etc. The results regarding EV 70 could be considered as antigenic properties of this "unusual" virus, in which such properties related to this present study has been discussed in review of literature.

Serum of rabbits and guinea pigs which showed the highest antibody titer against CA 24v were screened against some other enteroviruses i.e. poliovirus type 1, type 2, and type 3 to confirm the correctness of the serum antibody by virus neutralization test. The results obtained showed the presence of cytopathic effect (CPE) in all the serum samples with different dilutions, which indicates absence of the serum antibody against poliovirus type 1, type 2, and type 3.

Immunoenzymatic-staining of CA 24v was followed by virus neutralization test. The results obtained from different dilutions of serum samples (1:10 to 1:320) which showed the highest antibody titer i.e. 1:2560 of one of the rabbits (compared to preimmunized serum) was used in order to find out the cut-off at the same dilutions mentioned earlier. Cell control (untreated cells either with virus or serum antibody) was also employed. The precipitated observed due to antigen-antibody reaction up to serum dilution 1:80.

Therefore, CA 24v, we have been able to develop a rapid and easy method for diagnosis of one of this virus, by applying tissue culture and virological techniques as well as serology and immunological methods. This specific rabbit-anti-CA 24 v can be applied as diagnosis reagent for detection of any viral acute hemorrhagic conjunctivitis with source of coxsackievirus A 24v, and help rapid and easy diagnosis of such viral infection.

Utility of EV 70 antisera should be investigated further as most investigators have reported to obtain reagents of sensitive antibody titre. In my opinion the sera should be useful to identify virus isolates using this neutralization test.

CHAPTER 8

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CHAPTER 9

SYNOPSIS

A SYNOPSIS OF THE THESIS
TO BE SUBMITTED TO THE UNIVERSITY OF MUMBAI
FOR THE DEGREE OF MASTER OF SCIENCE
IN
MICROBIOLOGY

**TITLE OF THE THESIS : Development Of Reagents For Diagnosis Of
Acute Hemorrhagic Conjunctivitis Caused By
EV 70 And CA 24v Viruses.**

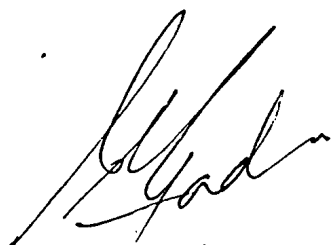
NAME OF THE CANDIDATE : Mr. Masoud M. Eslami

NAME OF THE GUIDE : Dr. S.V. Gadre

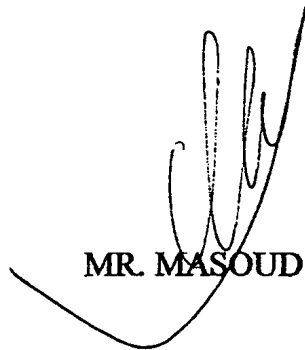
**PLACE OF RESEARCH : Enterovirus Research Center,
Haffkine Institute,
Parel, Mumbai- 400 012.**

DATE OF REGISTRATION : 11th October 1996.

REGISTRATION NUMBER : 20



DR. S.V. GADRE



MR. MASOUD M. ESLAMI

INTRODUCTION

Enteroviruses are small, non-enveloped RNA viruses belonging to the *picornaviridae*, a family currently divided into five genera: aphtho-, cardio-, entero-, hepato-, and rhinoviruses. Human enteroviruses are subgrouped into polioviruses, coxsackieviruses, echoviruses and the more recently discovered enterovirus types 68 to 71. Coxsackieviruses are assigned to two subgroups, A and B, on the basis of disease caused in newborn mice. Members of the enterovirus genus are also found in animals, e.g., bovine enterovirus (BEV) and swine vesicular disease virus (SVDV).

Human enteroviruses cause a variety of clinical diseases ranging from the common cold to severe paralysis. Some enterovirus serotypes have been associated with specific clinical patterns such as Polioviruses with poliomyelitis, Coxsackie B viruses with pleurodynia and myocarditis, Coxsackievirus A 16 with hand-foot-and-mouth disease, Enterovirus type 70 (EV70) and some Coxsackievirus A 24 strains with acute hemorrhagic conjunctivitis (AHC). However, most enteroviruses can be responsible for highly similar disease entities including meningitis and exanthema.

When the first epidemic wave of AHC hit Tokyo, Mie and Sapporo near the end of 1971, a team of Japanese investigators isolated a new picornavirus (Mirkovic *et al.* 1973) from conjunctival scrapings of patients with AHC. They reported that AHC virus, a causative agent, has the general characteristics of an enterovirus but is not neutralized by current antisera in the intersecting pools of enterovirus immune sera (Chomel *et al.*, 1973). Now it is classified as enterovirus type 70 under collaboration of WHO International Reference Center for Enteroviruses (Mirkovic *et al.* 1973).

In India, AHC was first recognized in Bombay in March 1971 (Wadia *et al.* 1973). Because the outbreak seems to have occurred earlier in the western part of the country, it is doubtful if it came in from Indonesia and is believed to have been brought into Bengal by refugees the Bangladesh war (Pramanik 1971) and according to him, it was called "Joy Bangla disease" or "Bangladesh conjunctivitis". In Calcutta alone the number of cases is reported to have reached one million (Ray *et al.*, 1972).

Coxsackie A viruses were discovered in 1948 by Dalldorf and Sickles. They were isolated from fecal specimens from children during a poliomyelitis outbreak and were recognized on the basis of their capability to cause detectable signs of disease in newborn mice. A variant of coxsackievirus A 24 (CA 24v) is one of the agents causing acute hemorrhagic conjunctivitis (AHC), which was first recognized clinically in 1969. The virus was isolated during an extensive epidemic in 1970 in Singapore (Yin-Murphy, 1972). Since CA 24v emerged as a new human pathogen from a single focus (Miyamura *et al.*, 1990) like enterovirus 70 (EV 70) (Miyamura *et al.*, 1986), the virus has been causing epidemics almost every 5 years in Southeast Asian countries (Chang *et al.*, 1977; Thongcharoen *et al.*, 1978; Christopher *et al.*, 1982; Yin-Murphy *et al.*, 1986).

Transmission of conjunctivitis can take place by direct person-to-person contact or by indirect contact with inanimate objects such as face cloth, bath towels handkerchiefs and bedding contaminated with feces, ocular and respiratory secretions. The CA 24v and EV 70 viruses can also be transmitted in respiratory droplets via aerosols, and fecal-oral route. In eye clinics, cross infection can occur by contact with contaminated fingers of ophthalmologists and nurses, ophthalmic instruments such as forceps, tonometers, slit-lamp microscopes, eyedroppers and ophthalmic solutions. Water stored in containers, public basins used for washing purposes, and spring water has been implicated (Guadin *et al.*, 1977; Arnow, 1977).

AIMS AND OBJECTIVES

Identification of a virus as the cause of a patient's illness should be accurate and cost-effective. The objective of this research project was to develop a rapid diagnostic method based on immunological techniques.

Till present day, two pandemic of viral acute hemorrhagic conjunctivitis and several major epidemics caused by enterovirus type 70 (EV 70) and several epidemics due to Coxsackievirus A 24 variant (CA 24v) as well, swept out many parts of the world and affected millions of people. The two viruses were chosen for this study.

The following experimental approach was undertaken :

1. Grow the two viruses using cell culture technique.
2. Isolation of viral antigens.
3. Immunization of laboratory animals i.e. Rabbits, Guinea pigs, and mice to develop specific high titre sera.
4. Determining antibody titres and specificity of these sera against the two viruses by virus neutralization test
5. Standardization of an immuno-enzymatic staining method for specific diagnosis.

MATERIALS AND METHODS

Cell line:

LLC-MK2 (Rhesus monkey kidney continuous cell line) and HeLa (human cervical carcinoma cell line) from Enterovirus Research Centre (ICMR) Haffkine Institute, Mumbai, were grown in MEM (Eagle with NEAA) and 10% fetal bovine serum, and cell cultures were maintained by weekly sub-culture in 80 cm² tissue culture flasks.

Virus strains :

Standard reference strains of EV 70 (J670/71), was obtained from Dr. Kono Laboratory, Tokyo, Japan, and CA 24v was obtained from Center for Disease Control (CDC) Atlanta, USA.

Stock viruses were prepared by inoculating the viruses in susceptible host cells, i.e. LLC-MK2 & HeLa, in case of EV 70 and CA 24v respectively.

Virus titration:

The virus was titrated in a 96 well microtitration plate (Nunc, Denmark) and the infectivity titre was determined by Karber method (1931).

The harvested virus fluid was diluted up to 10^{-7} . 0.05 ml of each virus dilution starting from 10^{-1} was inoculated in 10 chambers of 96 well microtitre plate. For EV 70, pre-grown LLC-MK2 was used, while for CA 24v, HeLa cells 10-15,000/well was seeded after dispensing virus dilutions. Cultures were incubated at 37°C in a CO₂ incubator and observed for 7 days, for appearance of cytopathic effect.

Antigen preparation :

In order to obtain purified viral antigen for immunization of animals, the following procedures were carried out: 0.5 ml of stock virus was inoculated in a 175 cm² tissue culture bottle containing complete monolayer cells. Incubation was carried out at 37°C in a CO₂ incubator until complete cytopathic effect was observed. Freeze thawing of cells was carried out three times to rupture the cells to increase virus yield.

After centrifugation at 10,000 rpm for 20 min at 4°C, the supernatant was collected and used for ultracentrifugation at 27000 rpm, for 3½ h, at 4°C (SW-28 rotor, Beckman L8-70M Ultracentrifuge). The pellet was collected and resuspended in 2 ml phosphate buffer saline (PBS), and kept at -40° C until further use. Virus titration was carried out at each step of purification.

Immunization :

One ml of concentrated virus mixed with equal volume of Freund's adjuvant and emulsified. Rabbits weighing 1.2-1.5 kg, were injected with 0.5 ml of the emulsion subcutaneously (sc) at four different sites on the back of the animal. For intravenous (iv) immunization, 0.8 ml of the virus was injected without adjuvant through the marginal ear vein.

Guinea pigs weighing approximately 300 g were injected subcutaneously (sc) with 0.5 ml concentrated virus along with equal volume of adjuvant, at two different sites on the back of the animal. Intraperitoneal (ip) injection was given with 0.5 ml of virus without adjuvant.

Mice weighing approximately 35 g received the virus through ip injection of 0.2 ml of purified virus mixed with equal volume of adjuvant. Immunization was repeated five to seven times at two-week intervals (booster doses).

Blood was drawn at two weeks interval following immunization. Rabbits were bled through marginal ear vein, while in case of Guinea pigs, a cardiac puncture was the method of choice, and from mice, blood was collected either by cutting the terminal tail or by cardiac puncture.

Blood was kept at room temperature for 1h for clotting. After clotting, the samples were kept at 4°C (refrigerator) overnight. The next day, serum was collected and centrifuged at 3,500 rpm at 4° C for 10 min. Serum inactivation was done by heating in a water bath at 56° C for 30 min. It was stored at -40° C until used.

Virus Neutralization test :

This test is aimed at specific inhibition of cytopathic effect in cell lines, and was used for the estimation of antibodies in serum immunized animals. In the present investigation, antibody endpoints were determined by using a constant dose of the virus and varying dilution of serum samples.

Equal volume of virus suspension containing 100 TCID₅₀ (based on the previous titration results) of virus per 0.05 ml, was added to each dilution of sample. The antigen-antibody mixture was thoroughly mixed and incubated at 37°C with 5% CO₂. The following controls were kept along with all the experiments.

- ⇒ Cell Control : Un-inoculated cultures from the same batch incubated under identical conditions served as cell controls.
- ⇒ Virus Control : or back titration. With each neutralization test, titration of virus was carried out. Four chambers (well) of the 96 well microtitre plate were inoculated with each virus dilution containing 100, 10, 1, and 0 TCID₅₀ of the virus to serve as back titration.

Plates were incubated at 37°C with 5% CO₂. Cultures were observed using an inverted TC microscope for development of cytopathic effect daily for a period of 7 days.

Presence of cytopathic effect in cultures inoculated with virus-serum mixture indicated the absence of antibodies. Conversely, absence of cytopathic effect in cells inoculated with virus-serum mixture indicated presence of the neutralizing antibody. The dose of the virus used in the test and the titre of neutralizing antibodies were calculated as per Karber (1931).

Immunoenzymatic-staining (CA 24 v):

A virus suspension was prepared by diluting to 10⁻², and inoculated in to the complete monolayer of HeLa cells grown on coverslips in a petri plate. Incubation was stopped when 50% cells showed CPE, the coverslips were kept in a 10%

formaldehyde solution in PBS for 2h for fixation of the cells. The coverslips were then air-dried.

Pre-immunized serum samples of rabbit (serum control) and rabbit-anti-CA24v (immunized rabbit) were diluted from 1:10 up to 1:320 in PBS buffer. 25 µl of each dilution was added to the coverslips, and kept in a humidified petri plate for 1 h at room temperature. Excess of antibody was removed by washing the slides three times with PBS.

The cover slips were further reacted with 1:1000 of anti-rabbit IgG horseradish peroxidase conjugate (Sigma, Cat No. P 6140, St Louis, MO, USA). The antigen-antibody-conjugate reaction was made visible by reaction with precipitating chromogenic substrate i.e. 3,3' 4,4' diaminobenzidine (DAB) (Sigma, Cat No. D-5637, St Louis, MO, USA) in 0.05 M Tris-buffer pH 7.6. Cobaltous chloride was used in order to enhance the sensitivity of the reaction.

25 µl of goat-anti-rabbit IGg-Peroxidase was added to the coverslips. The coverslips were transferred to the substrate solution and allowed to react for 5 min. They were then washed in distilled water for 5 min.

Counter staining was done with 2% aqueous methyl green for 2-5 min, rinsed in distilled water, dehydrated with a brief wash in alcohol, cleared in xylene and finally, mounted in DPX mounting medium.

RESULTS AND CONCLUSIONS

1. In case of EV70, the results indicate poor immune response to this virus i.e. in both groups of animals, a titer of 1:40 is the highest titer of antibody obtained. Hence, the experiment was repeated with another group of rabbits (two females) & group of Guinea pigs (four females). The highest antibody titers, 1:80 and less than 1:160 for rabbits and Guinea pigs respectively, shows poor immune response to EV 70. In the fourth and last attempt, four rabbits (two males and two females) were selected. The result of this experiment also has shown low titres of antibody against EV 70 i.e. 1:160.
2. In case of CA 24v, the experiment was initiated with one group of rabbit containing one female and three males, and one group of Guinea pigs containing four males. The same procedure was carried out as in case of EV 70. The result of virus neutralization test in both groups of animals showed a high titre of serum antibody. The highest antibody titer was 1:2560 for rabbit (No. 17), the rest of the rabbits and the guinea pigs (except one) showed good immune response to CA 24v.
3. Serum of rabbits and guinea pigs which showed the highest antibody titer against CA 24v were cross matched with polioviruses type 1, 2, and 3 in order to confirm the correctness of the serum antibody by virus neutralization test. The results obtained showed the presence of cytopathic effect (CPE) in all the serum samples with different dilutions, which indicates absence of the serum antibody against poliovirus types 1, 2, and 3 respectively.
4. Immuno-enzymatic staining of CA 24v was followed by virus neutralization test. The results obtained from different dilutions of serum samples (1:10 to 1:320) which showed the highest antibody titer i.e. 1:2560 for rabbit No. 17, (compared to pre-immunized serum) was used in order to find out the cut-off at the same dilutions mentioned earlier. Cell control (untreated cells either with virus or serum antibody) was also employed. We observed the precipitated due to antigen-antibody reaction up to serum dilution 1: 80.

5. Regarding the above results obtained from experiment with CA 24v in form of virus neutralization test, which shows the high immune response in both groups of Rabbits and Guinea pigs, and immuno-enzymatic staining which has been tested on one of the rabbits with high serum antibody titre i.e. Rabbit No. 17 S-5.
6. We have been able to develop a rapid and easy method for diagnosis of one of these viruses i.e. CA 24v, by applying tissue culture and virological techniques as well as serology and immunological methods. This specific serum antibody can be applied as diagnosis reagent for detection of any viral acute hemorrhagic conjunctivitis with source of coxsackievirus A 24v, and helps for rapid and easy diagnosis of such viral infection.
7. EV 70 showed poor immune response in the different groups of laboratory animals that have been used in this experiment.

The utility of antisera to EV70 and CA 24v in immuno-enzymatic tests to detect the two viruses will be discussed in detail in the thesis.

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