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CHARACTERIZATION OF A VIRUS ASSOCIATED WITH EPIDEMIC CONJUNCTIVITIS IN THAILAND

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SUMMARY A strain of virus, named the A-E strain, was isolated from a patient suffering from acute haemorrhagic conjunctivitis during an epidemic of the disease in Bangkok in 1972. The virus had the characteristics of an enterovirus but was not neutralized by any known enterovirus antiserum. Cross neutralization tests indicated that the isolate was closely related to the J670–71 virus isolated in Japan. The virus produced conjunctivitis in rabbits and monkeys following conjunctival inoculation.

INTRODUCTION

In June and November 1971, there was an outbreak of acute conjunctivitis among school children in Bangkok Municipal Area, and the eastern, and the western provinces of Thailand (Dumvibhat et al., 1973). The disease was first recognized in Cholburi province, about 93 km to the east of Bangkok. It was characterized by the acute onset of marked conjunctival injection with certain degrees of chemosis, various degrees of subconjunctival haemorrhage and watery discharge. The lesions involved both eyes, but corneal involvement was rare. Preauricular lymph glands in some cases were enlarged and tender. Patients had a low grade fever, commonly with cold-like symptoms. The disease was self remitting within a week and complete recovery ensued within 14 days.

It seemed to be highly contagious and spread very rapidly among school children, involving mainly children of 10–15 years of age. In 1971 there were over 5,000 cases. The epidemic occurred again in the following year when there were more than a thousand cases.

This report describes virological and serological studies of specimens obtained from patients during an epidemic of this disease in a school in Bangkok Municipal Area in 1972.

MATERIALS AND METHODS

1. *Clinical specimens*

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Eye and throat swabs were randomly collected from 16 of 132 patients in the acute phase of illness. Each swab was dipped in 2 ml of phosphate buffered saline (PBS) containing 0.25% gelatin, and kept in an ice bath until used. The specimens were used for viral isolation within 12 hr after collection after filtration through a Millipore filter, type HA, of 0.45 μm pore size.

2. *Cell culture*

A primary monkey kidney (MK) cell culture and four cell lines, i.e. KB, HeLa, Hep-2, and human embryonic lung fibroblasts (HELF), were used simultaneously for virus isolation. For cultivation of cells Earle's balanced salt solution (BSS) supplemented with 0.5% lactalbumin hydrolysate and 10% fetal bovine serum was used for KB, Hep-2, and HeLa cells and medium 199 with 10% fetal bovine serum was used for HELF and MK cells.

These media were routinely supplemented with 100 μg/ml of streptomycin, 100 units/ml of penicillin, 100 μg/ml of Fungizone and 100 μg/ml of Kanamycin.

The maintenance medium consisted of 0.5% lactalbumin in BSS supplemented with 2% fetal bovine serum.

3. *Virus isolation and infectivity assay*

Virus isolation was performed in duplicate on each cell culture. Each culture tube was inoculated with 0.2 ml of filtered sample, and after adsorption for 1 hr at 36.5 C, was maintained in 1.8 ml of maintenance medium. The cells were incubated at 36.5 C and observed daily for cytopathic effects (CPE).

Culture fluids from infected cells were harvested and pooled between the 3rd and the 4th day after inoculation. Further passages were performed by inoculating cell cultures with pooled samples of the respective cell lines. Passages were repeated successively for five generations.

Culture fluids from each cell culture showing definite CPE from each sample were pooled after the 5th passage. Infectivity titration was performed on various cell cultures, using the tube method to determine the number of $TCID_{50}$ per 0.1 ml (Dulbecco and Vogt, 1954).

4. *Virus identification*

1) Biological properties

The following properties of the isolates were characterized by standard methods (Melnick and Wenner, 1969; Kapikian, 1969).

a) Ether sensitivity: An isolate that had been titrated for infectivity was treated with 1/5 volume of ethyl ether for 6–24 hr at 4 C and its residual infectivity was compared with that of the untreated specimen in KB cells.

b) Type of viral nucleic acid: The amount of virus was measured quantitatively in KB cells in the presence of 40 μg/ml of 5-iododeoxyuridine. Herpes simplex, type 1, virus was used as a positive control.

c) Thermostability: The isolate was stored at various temperatures, i.e. 4 C, 25 C, 50 C and —70 C, in the presence and absence of 1 M $MgCl_2$. The residual virus titer was titrated daily in KB cells except in samples stored at 50 C, in which the titers was only titrated once after one hour.

d) pH-Sensitivity: The pH-sensitivity of the virus was tested by incubation at pH 3.0 and 7.2 at room temerature for 3 hr, following the method of Tyrrell and Chanock (1963). Poliovirus, type 1, was used as an acid stable control virus.

e) Haemagglutinating activity: On the third day after infection cell sheets were washed with Hanks' balanced salt solution (HBS) and incubated with 0.2 ml of 0.5% chick erythrocytes or 0.5% human " O " erythrocytes in normal saline solution (NSS) at 4 C, 20 C, or 37 C for 30 min. Then the cell sheets were washed twice with HBS and observed for haemadsorption to detect haemagglutinating activity. As controls 4 tubes of cells were also tested to rule out non-specific haemadsorption (Dowdle and Robinson, 1966).

f) Animal susceptibility: The virus was inoculated into suckling mice intracerebrally or intraperitoneally and into adult mice intranasally.

Conjunctival inoculation was performed in rabbits and cynomolgus monkeys by applying 2 drops of virus suspension containing about 10^6 $TCID_{50}$ to each eye. The animals were observed for symptoms and signs of illness and their body temperature was recorded daily for 4 weeks.

2) Virus concentration and electron microscopy

KB monolayers in 32 ounce Roux bottles were infected with isolated virus at an input multiplicity of 10 $TCID_{50}$/cell. After adsorption at 36.5 C for 30 min, maintenance medium was added without washing the cell layers and the infected cell cultures were incubated at 36.5 C until 4+ CPE appeared. Cultures were then harvested, frozen and thawed three times, and clarified by centrifugation at 3,000 $\times g$ for 30 min. The virus was precipitated by cen-

trifugation at 40,000 rpm for 4 hr at 4 C in a Hitachi model 55P-2 ultracentrifuge using an RP-55 rotor. The supernatant fluid was discarded and the pellet was resuspended in a small amount of PBS, stained with 2% phosphotungstic acid at pH 6.7 and examined in a Hitachi HU-11C electron microscope at 75 KV.

5. Neutralization test and antisera used

Virus was grown in KB cells and partially purified as described above. Then virus suspension containing about 10^7 $TCID_{50}$/ml was emulsified with an equal amount of complete Freund's adjuvant and 1 ml of the mixture was injected subcutaneously into rabbits and monkeys at weekly intervals for 4 weeks.

Monkey and rabbit antisera against J670–71 virus were kindly supplied by Dr. R. Kono from the National Institute of Health, Tokyo, Japan.

An intersecting pool of antisera to enteroviruses was prepared in this Institute by the method of Melnick and Wenner (1969). The antisera pool consisted of type-specific antisera against polioviruses 1–3, coxsackie viruses A7 and A9, coxsackie viruses B1–B6, echoviruses 1–9, 11–27, and 29–33, and reoviruses 1–3.

The method used for the tube neutralization test was based on the method described by Melnick and Wenner (1969) using KB cell cultures. Sera were inactivated by heating at 56 C for 30 min, diluted with maintenance medium without fetal calf serum and allowed to react with an equal volume of virus suspension at room temperature for 1 hr.

In the neutralization test against the intersecting pool of enterovirus antisera, 20 units of antisera were used.

Neutralization was performed by testing both the homologous system and cross testing with the J670–71 virus and anti-J670–71 sera.

RESULTS

Virus was only isolated from one specimen of an eye swab and this virus was characterized as follows:

1. *Growth characteristics in cell cultures*

The isolate produced CPE on the first passage in KB, and HELF cells, but no definite CPE in $LLCMK_2$, primary monkey kidney, HeLa or Hep-2 cell cultures. After the 5th passage the isolate produced distinct CPE within 48 hr in KB and HELF. Therefore, the KB cell culture was used as the host system for subsequent characterization of the virus. The CPE produced by the isolate had the same characters as that produced by enterovirus.

2. *Growth characteristics in animals*

The isolate, when injected intracerebrally or intraperitoneally into suckling mice failed to produce any sign or symptom in the animals. Adult mice inoculated intranasally with the isolate also showed no sign of infection.

However, when inoculated onto the conjunctiva of rabbits, the virus produced signs and symptoms, as shown in Fig. 1. The body temperature began to rise on the first day after inoculation, reached a maximum on the 4th and 5th days and then gradually decreased within two weeks.

On the 2nd day after infection, the animals developed conjunctivitis of both eyes. The lesions were moderate with watery discharge.

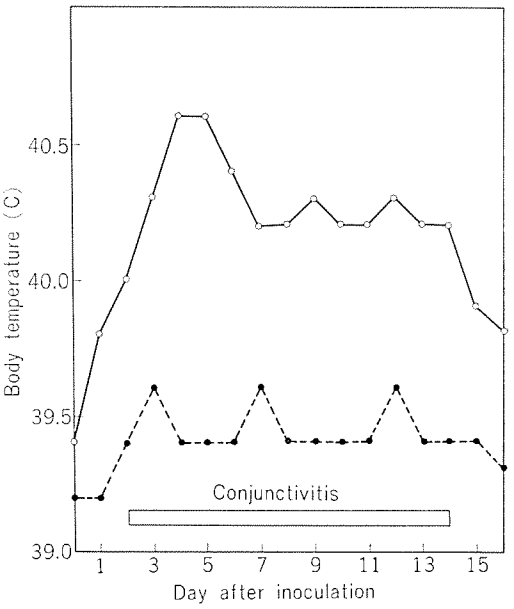

FIGURE 1. *Experiment in rabbits. Two drops of 10^6 $TCID_{50}$/ml virus suspension were inoculated in each eye.* ○, *infected rabbit;* ●, *control rabbit.*

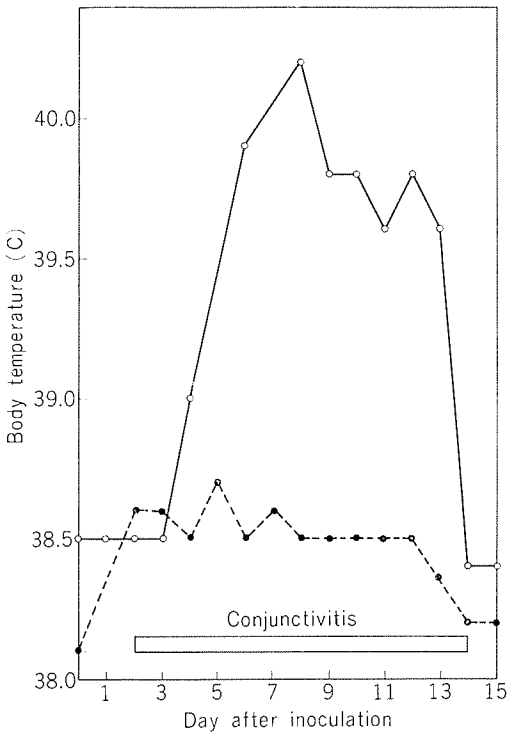

FIGURE 2. *Experiment in monkeys. Two drops of 10^6 $TCID_{50}$/ml virus suspension were inoculated in each eye.* ○, *infected monkey;* ●, *control monkey.*

Neither subconjunctival haemorrhage nor corneal involvement was observed. The lesions persisted for 10 days and subsided completely within 14 days. Control animals inoculated with culture fluid from uninfected KB cell cultures showed no abnormal signs or symtoms.

The virus produced similar symptoms and signs in monkeys to those in rabbits (Fig. 2). Fever developed on the 3rd day after inoculation and lasted for 10 days. Conjunctivitis developed on the 2nd day of infection and lasted for 12 days. Lesions developed in both eyes with watery discharge. No subconjunctival haemorrhage or corneal involvement was observed.

One of three monkeys infected was weak after the conjunctivitis had subsided and developed muscular weakness in the third week. The general condition of the animal remained grave and it died on the 28th day after inoculation.

3. *Biological properties*

As shown in Table 1 diethyl ether failed to inactivate the virus, even on treatment for 24 hr at 4 C.

The virus could replicate without apparent decrease in titer in the presence of 40 μg/ml of 5-iododeoxyuridine while herpes simplex virus used as a control showed definite decrease in titer (Table 2).

The virus was inactivated completely within one hour on heating at 50 C at pH 7.2 (Fig. 3). This heat inactivation was completely inhibited by the presence of 1 M $MgCl_2$. After heating at 36.5 C for 4 days, only 10^{-4} of the viral infectivity survived. At 25 C and 4 C the virus was stable for 3 days and then its infectivity gradually decreased. After 7 days at —70 C the infectivity of the virus had decreased about two powers and then it remained steady for several months.

TABLE 1. *Effect of ether on the A-E virus*

Time of ether treatment (hr)	Virus titer ($TCID_{50}$/ml)[a]	
	Before treatment	After treatment
6	5×10^6	5×10^6
12	5×10^6	5×10^6
20	5×10^6	5×10^6
24	5×10^6	5×10^6

[a] Virus titration was performed on KB cell culture.

TABLE 2. *Effect of IUDR on the A-E virus*

Virus	Virus titer ($TCID_{50}$/ml)[a]	
	Treated[b]	Untreated
A-E #5KB	5×10^5	5×10^5
HSV #10KB	0	5×10^5

[a] Viruses were titrated on KB cells.

[b] 5-iododeoxyuridine was used at a concentration of 40 μg/ml.

As shown in Table 3, the virus was not affected by incubation at pH 3.0 for 3 hr.

In the haemadsorption test with either chick or human "O" erythrocytes no heamagglutinin was demonstrated in infected cells.

4. *Morphology of the virions*

Electron microscopy showed that the virus particles were spherical and about 29 nm in diameter, suggesting cubic symmetry of the capsid (Fig. 4).

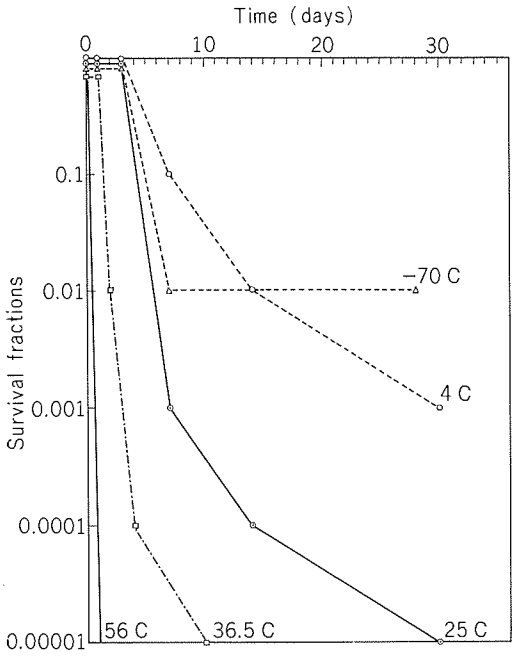

FIGURE 3. *Thermostability of A-E virus at pH 7.2.*

TABLE 3. *Effect of pH on A-E virus at room temperature*[a]

Virus	pH of treatment	Virus titer ($TCID_{50}$/ml)[b]	
		Treated	Untreated
A-E #5KB	3.0	5×10^6	5×10^6
	7.2	5×10^6	5×10^6
Polio, type 1	3.0	5×10^6	5×10^6
	7.2	5×10^6	5×10^6

[a] Treatment was performed at room temperature for 3 hr.

[b] A-E virus was titrated on KB cells and poliovirus was titrated on primary monkey kidney cell culture.

5. *Neutralization tests*

Neutralization tests of the isolated virus against intersecting pools of enteroviruses gave negative results. None of the antisera tested neutralized the virus although they neutralized control virus.

Table 4 shows the results of cross neutralization tests between the isolate and the J670–71 virus from Japan, demonstrating that the two viruses are related.

DISCUSSION

The results described in this paper indicate that the isolated A-E strain of the virus is an RNA virus, having a particle size of about 29 nm ϕ with cubic capsid symmetry and contained no envelope. The virus was resistant to acid under the conventional conditions used for testing rhinoviruses. It was inactivated by heating at 50 C for 30 min and this effect of heat inactivation was prevented by 1 M $MgCl_2$. These results indicate that the virus should be classified as an enterovirus.

The absence of pathogenicity in mice excludes the possibility that it is a coxackie virus, and its failure to produce CPE in monkey kidney cells rules out its classification as a polio-/or echo-virus.

Neutralization tests with intersecting pools of enteroviruses antisera support the above findings that the virus is not identical with any known enterovirus. This result is similar to those of others (Kono, et al., 1972; Mirkovic, et al., 1973) showing that the enterovirus associated with acute haemorrhagic conjunctivitis is a new type of enterovirus, namely enterovirus 70.

The results of cross neutralization tests indicated that the A-E strain is closely related to J670–71 virus, a prototype of enterovirus 70 isolated in Japan, though the neutralization end points were somewhat different with homolog-

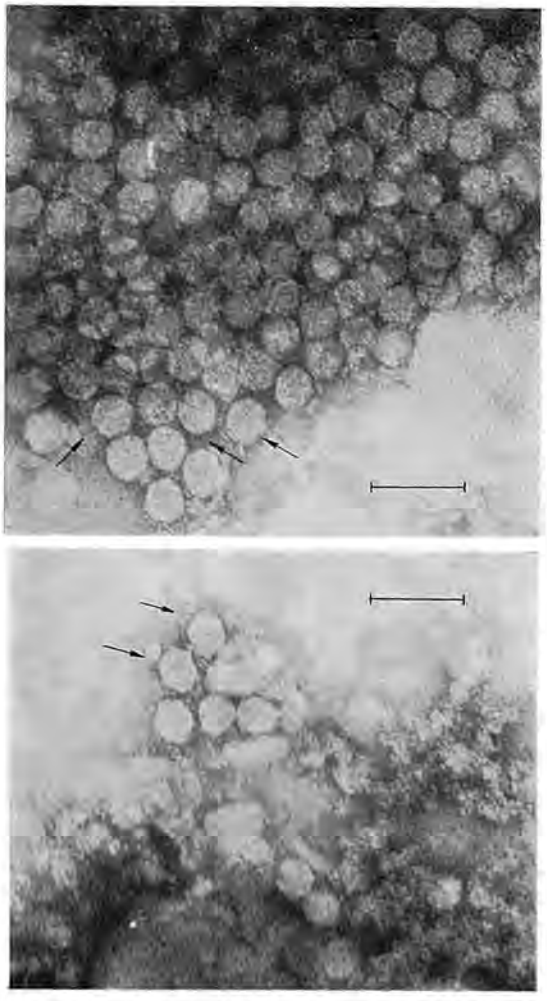

FIGURE 4. *Virions of A-E strain virus. Negatively stained with phosphotungstic acid. Scale: 100 nm. Particles marked by arrows suggest the cubic symmetry of the capsid.*

ous and heterologous systems.

The results of experiments in rabbits and monkeys provide additional evidence that the organism could be the causative agent of the disease, though Koch's postulate has not yet been satisfied. The eye lesions and general symptoms observed in animals were similar to those observed in human cases although no subconjunctival haemorrhage developed in animals.

The neurological symptoms observed in one monkey following fever and conjunctivitis are of great interest. Bharucha et al. (1972) and Wadia et al. (1972, 1973) reported patients who developed neurological complications following an epidemic of acute haemorrhagic conjunctivitis in India. This was serologically confirmed later by Kono et al. (1974). These findings suggest that this type of enterovirus may have neurovirulence. Further studies on this possibility are in progress in this laboratory.

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TABLE 4. *Results of cross neutralization test between A-E and J670–71 viruses*

Antiserum against	Prepared in	Serum dilution end point with:	
		Japan 670–71 virus	A-E virus
Japan 670–71 virus	Monkey #11845	810	203
	Rabbit #109	505	160
A-E #7KB	Monkey[a]	81	64
	Rabbit #10	64	127
$TCID_{50}$ in test		32	320

[a] This monkey serum was prepared in Bangkok.

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