Egypt. J. Microbiol. 37, No. 3, pp . 311 - 322 (2002)

Viral Etiology of Hemorrhagic Conjunctivitis

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FIFTY cases of acute hemorrhagic keratoconjunctivitis of different ages were subjected to full ophthalmic examinations. The cases were presented with unilateral (32 eyes) or bilateral (18 eyes) follicular conjunctivitis bulbar and tarsal conjunctival haemorrhages, chemosis, eye lid oedema, profuse watery or mucopuralent discharges, preauricular adenopathy and fine punctate epithelial keratitis. Although follicular reaction was the earliest sign, yet subconjunctival haemorrhages were the most constant findings in all the cases. Conjunctival swabs were collected from all patients and inoculated in three types of cell lines to increase the chance of virus isolation. Enterovirus common primers and also adenovirus group specific primers were used. Enterovirus 70 was isolated in 3 cases by RT-PCR for cell culture lysates at the third cell culture passage. No coxsackie and adenoviruses were detected.

Keywords: Hemorrhagic conjunctivitis, enterovirus type 70, Coxsackieviruses, adenoviruses, PCR.

Epidemics of acute hemorrhagic conjunctivitis (AHC) had been occurred at different countries in the world. It is caused by a virus transmitted mainly through contaminated water or foods. The main etiologic agent is enterovirus type 70 (EV70), which is responsible for explosive epidemics of AHC (Uchio *et al.*, 1999). Coxsackievirus type A4 (CA24) and adenovirus type 8, 19 and 37 were found as competent agents as EV70 for AHC (Guyer *et al.*, 1975;Sprague

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et al.,1973). An outbreak of AHC occurred in Delhi, India, during August and September 1996. The etiologic agent was confirmed as EV 70. After nearly a decade, this virus is reemerging as a cause of AHC in India (Maitreyi, 1999).

AHC due to enterovirus 70 has caused extensive outbreaks in tropical areas since 1969. Between December, 1990, and 1991, an outbreak of AHC due to EV70 occurred in American Samoa, where an outbreak due to the same agent had occurred in 1981. A survey of 5% of the households (134 households, 1,095 individuals) was conducted throughout the island of Tutuila. The outbreak affected 58% of the population, with age-specific attack rates greater than 50% for all age groups except children younger than 2 years. Attack rates were significantly higher for children 2-10 years old (65%) than in the remainder of the population. Women aged 21-40 years had higher rates than did men the same age (66 vs. 49%), possibly because of the close association of women and young children. At higher preepidemic titers, there was evidence of protection from clinical disease among males but not among females. EV70 can cause large outbreaks even in a population already exposed in a previous large outbreak; protection due to previous infection is only partial (Bern *et al.*, 1992).

The immune response to EV70 infection had been studied by Aoki *et al.* (1992). In August 1984, an outbreak of AHC occurred at a nursing home in Sapporo, Japan. During the subsequent 7 years, they have monitored the neutralization antibody of EV70 in 13 patients with acute hemorrhagic conjunctivitis and 15 persons with subclinical EV70 infection among the 108 persons in the nursing home. During the first one to two years, the neutralization antibody in 6 of the 28 subjects (21.4%) decreased to less than 1:8. Seven years later, the neutralization antibody had decreased to less than 1:8 in 92% of the subjects. There was an individual difference in the level of neutralization antibody titer against EV70. However, overall the antibody levels markedly decreased every year. It is important to remember that the neutralization antibody titer of EV70 decreases with the passage of time. Consequently, most of the patients who had AHC will not have resistance against reinfection 7 years after the initial infection.

The reverse transcription-polymerase chain reaction test provides a reliable diagnostic method that readily allows specific detection of enterovirus 70 in patients with AHC in whom low enterovirus 70 recovery rates have been obtained recently by cell culture (Uchio *et al.*, 1996).

Reverse transcription-polymerase chain reaction (RT-PCR) and stringent

microplate hybridization (SMH) methods were assessed for the detection of EV70 in conjunctiva swabs. Furthermore, with the use of the SMH method, the genetic homogeneity of EV70 can be compared. (Yoshino *et al.*, 1998).

Subconjunctival hemorrhage (SCH) can be a predominant presentation of adenoviruses 19 and 37 keratoconjunctivitis and may herald a new stage in the evolution of adenoviruses. PCR and PCR-RFLP are rapid and reliable methods for adenoviruses detection and typing (Chang *et al.*, 2001). Although EV70 has been identified as a major etiological agent of acute hemorrhagic conjunctivitis in some surrounding countries, no EV70 strain has been identified in Egypt since 1989. Our aim was to virus isolation and a molecular approach to

Material and Methods

Patients and specimens

Patients presented with acute onset of severe unilateral (32 eyes) or bilateral (18 eyes) follicular conjunctivitis, bulbar and tarsal conjunctival haemorrhages, chemosis, eye lid oedema, profuse watery or mucopuralent discharges, preauricular adenopathy and fine punctate epithelial keratitis.

Conjunctival follicles were masked in 15 eyes by the severity of chemosis and pseudomembrane. The peak intensity of conjunctivitis occurred 5-6 days after the onset of symptoms. Upper respiratory tract infection was observed in three cases with mild fever. The course of the disease lasted between 8-11 days in 42 eyes and only 7 days in 8 eyes. Ten samples without manifestation (normal eyes) were collected as control.

Conjunctival swabs were collected from all patients (50 swabs). Samples were kept in 3ml Hanks' Balanced salt solution (HBSS) and 50 µl of antibiotic-antimycotic mixture (10,000 U penicillin G sodium, 10,000 µg streptomycin sulfate and 25µg amphotericine B, GIBCO BRL) were added for 30 min at 37°C. Samples were aliquoted and stored at -20°C. The first aliquots were inoculated in African green monkey kidney cells (Vero), Buffalo green monkey kidney (BGM) cells and human embryonal rabdomyosarcoma (RD) cells with daily observation for cytopathic effect. The second aliquots were used in PCR for detection of EV70, EV and Adenoviruses.

Primers

Oligonucleotide primers E1 and E2 were derived from conserved sequences in the nontranslated region of the enterovirus genome (Chapman *et.al.*, 1990).

The sense primer is E2 (5'- TCC GCC CCC TGA ATG-3') and antisense E1 primer (5'- CAC CGG ATG GCC AAT CCA-3'). For EV70, sense S4 (5'-AAT TGG AGA AAT AGT GAA AAC TGT GGC-3') and antisense AS4 (5'-CTG TGT TGG ATG TAG CIC CTG TCT C-3') were derived from VP1 region (Shulman *et al.*, 1997). The amplified product segments were 196 bp length for enteroviruses, and 114 bp length for EV 70. For adenoviruses, sense primer (5'-GCC GCA GTG GTC TTA CAT GCA CAT C-3') and antisense (5'-CAG CAC GCC GCG GAT GTC AAA GT-3') were designed to amplify a 308-bp sequence of the hexon region of all adenovirus serotypes (Allard *et al.*, 1990).

Extraction of Virus RNA and cDNA synthesis

RNAs were extracted directly from100µl of each cell culture lysate by guanidinium thiocyanate/ phenol / chloroform followed by chloroform/isoamyl alcohol and precipitated by ethanol as described by Chomczynski and Sacchi (1987). The extracted RNA was dissolved in 20µl of DEPC-treated water and mixed with 40 U RNase inhibitor (Promega, Madison, Wis.). The mixture was heated at 95°C for 5 minutes, and then chilled on ice. Reverse transcription was done in 20µl reaction mixture containing, 4µl extracted RNA, 0.3µl (200ng) antisense primer E1 for EV and AS4 for EV70, 0.3µl of 10mM dNTPs mixture (Pharmacia Biotechnology), 4µl of 5X RT buffer (50mM Tris-HCl, pH 8.3, 75mM KCl, 3mM $MgCl_2$ and 10mM Dithiothreitol [DDT]), 3µl (30U) avian myeloblastosis reverse transcriptase (AMV-RT, Promega, Madison, Wis.) and DEPC-treated water up to 20µl. The reaction mixture was overlaid with 50µl mineral oil and incubated at 37U°C for 1 hr, heated to 96°C for 10 min, and then cooled to 4°C for at least 2 min (Martin *et al.*1994).

PCR

Four microliters of the first strand cDNA was combined with 25 pmol/L (4µl) of each sense and antisense primers, 5µl 10X PCR buffer (10mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, and 0.1% Triton X-100), 2µl of 50mM $MgCl_2$ and 1µl dNTPs in a 50 µl reaction volume. Taq DNA polymerase, 5 U (1µl) (Promega, Madison, Wis.) was added after initial 5 min incubation at 95°C. Thirty-Five rounds of amplification were performed under the following conditions: 95°C for 30 seconds, 57°C for 30 seconds and 72°C for 1 min for EV and 24 cycles of DNA amplification (93°C for 1 min and 30 s, 56°C for 45s and 73°C for 45s) for EV70 and the reaction was terminated with 10 min elongation at 73°C using a Perkin-Elmer 9600 thermocycler.

For adenoviruses, 3µl of extracted total DNA was combined with 25

pmol/L of each primer, 5µl 10X PCR buffer (Promega, Madison, Wis.), and 5µl of 2.5mol/L dNTPs in a 50 µl reaction mixture. Taq DNA polymerase (5U) was added after an initial 5 min incubation at 95°C. Forty rounds of amplification were performed at the following temperatures: 95°C for 30s, 57°C for30s, and 72°C for 1min.

PCR products were analyzed by electrophoresis in 2% agarose gel containing ethidium bromide .DNA bands were visualized under UV light and compared at 196bp, 114bp and 308bp length for enteroviruses, EV70, and adenoviruses, respectively, to DNA marker (100 bp ladder, Pharmacia Biotechnology). Control viruses were coxsackievirus type 4,adenovirus type 8 and EV70, which was isolated during an earlier epidemic at 1989 (Bahgat *et al.*,1989 and Mostafa *et al.*,1990).

Results

Clinical data

The 50 hemorrhagic kerato-conjunctivitis cases were at different age groups (Table. 1). Clinical manifestations had been divided into 3 groups as shown in Table-2.

Isolation of cytopathogenic virus

All the collected samples were inoculated in three cell culture types to isolate EV,EV70 or non enteric adenoviruses. The results of the three successive passages in cell cultures revealed that only three samples showed CPE in both RD and BGM but two of them were cytopathic for Vero cells (Table. 3).

Identification by RT-PCR

With the specific enterovirus primers, RT-PCR identified the CPE-induced samples as enterovirus. Accordingly, other EV70 specific primers were used which in turn identified EV70 in the three samples. This also meant that there was no coxsackieviruses in the samples. Adenovirus-specific primers were also used for all cell culture lysates at the third passage but no adenovirus was detected. It was noticed that all the three EV70 -carrying samples were in 5-50 years age group. The three EV70 positive patients were aged 15-25 years belong to one family and live in a small village at Daqahlya Governorat . Since, the socioeconomic and health care are slightly poor which may explain the transmission of the virus between the members of this family.

TABLE 1 . Hemorrhagic kerato-conjunctivitis clinical data.

		No. of cases	%
Age group	3- 5 years	10	20
	6-50 years	30	60
	> 50 years	10	20
Sex	Male	15	30
	Female	35	70
Other family members			
	Positive	36	72
	Negative	14	28
Bilateral		18	36
Unilateral		32	64
Urban		26	52
Rural		24	48
Symptoms:			
- Red irritable eye		50	100
- Discharge			
	- Watery	45	90
	- Yellowish	5	10
- Sore throat		3	6
- Headache		7	14
- Fever		4	8

TABLE 2 . Clinical findings in acute hemorrhagic conjunctivitis

Clinical finding	No. of cases	%
Subconjunctival hemorrhage	50	100
Superficial punctate keratitis	45	90
Preauricular lymphadenopathy	43	86
Type of discharge		
- Watery	45	90
- Mucopurulent	5	10
Resolution:		
- Within 8-10 dayes	42	84
- Within 7 dayes	8	16

TABLE 3 . Isolation of cytopathogenic virus by cell culture inoculation with acute hemorrhagic conjunctivitis samples .

No. of A.H.C. cases	CPE-producing samples in cell culture types		
	Vero	RD	BGM
50	2	3	3

TABLE 4 . Results of PCR for acute hemorrhagic conjunctivitis samples at different age groups.

Age group	No. of samples	RT-PCR		PCR
		EV	EV70	Adenoviruses
3-5 years	10	0	0	0
6-50 years	30	3	3	0
> 50 years	10	0	0	0

EV =Enteroviruses EV70 = Enterovirus 70

Discussion

An outbreak of AHC was noticed in 1999 in different locations in Egypt. The epidemiological features were more or less similar to those in other countries; involvement of adults and young age mainly those living in crowded communities and irrelevance of the socioeconomic levels. The exact means of spread are not known. Hands and fomites contaminated by virus are the most likely vehicles. Weather conditions of heat and high humidity may play a role.

Many viral etiological causes may be involved. Enterovirus 70 may be associated with adenoviruses. It may be difficult to evaluate the role of the two groups in the determination of outbreak. The detection of the residual antibodies is not yet possible to ascertain whether those antibodies are specific or reflects an antigenic crossing between EV70 and one or several enteroviruses.

The results of the present study showed that the wide age group affected those from 5 to 50 years with a higher incidence in females. This is in agreement with Bern *et al* (1992).

The conjuctival hyperemia was present in all patients. This is in agreement with Uchio *et al* (1999) albeit the presence of subconjunctival hemorrhage, superficial punctate keratitis and lymphadenopathy were (24.0%, 11.7% and 9.3%) compared to our results (100%,90% and 86%).

Changes in clinical features might be due to biological transformation of EV70. AHC caused by EV70 and a variant of coxasakie virus A 24 is characterized by the rapid onset of severely painful red eye with watery discharge and bilateral inyolvement. The results of this study agree with Wright *et al* (1992).

Our study did not detect neurological symptoms. Waldman *et al* (1990) recorded nine cases presented with acute cranial nerve paralysis. The severity of subconjunctival haemorrhage and prevalence of upper respiratory and systemic symptoms are greatly variable from an EV70 outbreak to another. Upper respiratory and systemic symptoms were less common in our studied group while they were more common in the study of Sawyer *et al* (1989).The results of this study show no disease difference according to residence in urban and rural areas. This disagrees with the results recorded by Reeves *et al* (1986) whereas poor sectors of the city had a high attack rate. Household crowding and communal bathroom were the most important risk factors. In spite of the bulbar conjunctival haemorrhages, follicular conjunctival reaction and the frequent superficial punctate keratitis, the infection was short self limited and free of significant ocular sequelae. This is in agreement with Sklar *et al* (1983).

Acute keratoconjunctivitis with prominent subconjunctival haemorrhage is usually perceived by a clinician as acute hemorrhagic conjunctivitis associated with enteroviruses. However, subconjunctival haemorrhage can also be an adenoviruses infection. A rapid and sensitive laboratory diagnoses is helpful for differential diagnosis and epidemiological purposes. Therefore the sensitivity and applicability of cell culture and PCR and RTPCR diagnoses were evaluated for AHC associated with viral infection.

Results of different cell culture inoculation showed that the cytopathic effect was positive in 4%,6% and 6%of the AHC studied cases on Vero, RD and BGM cell types. Results of RT-PCR showed 6% of EV and 6% of EV70 but no adenoviruses DNA had been detected. Chang *et al* (2001) did not detect Enterovirus type 70 but they detect adenovirus in 39.9% by PCR and 37.1% by culture isolation. The difference in the results may be multifactorial: the site of the work done, the time of viral outbreak from one out break to another. This can supported by the previous studies that proved reappearance of EV70 as a viral aetiology of AHC every 7-10 years epidemic interval (Aoki *et al.*,1992; Doraisingham *et al.*, 1987).

Moreover the variability of severity of symptoms from one virus genotype to another was by Chang *et al* (2001). The genome of EV70 tends to be conserved during natural infection with a possible consequence on the transient

nature of the disease (Kew *et al* 1983). Virus isolation upon cell culture in this study detected 6% positive isolations compared to 17.2% given by Ramia and Arif (1990). From 29 conjunctival scrapings from patients with AHC in Saudi Arabia.This difference can be explained by the fact they had used two human diploid cell lines: human skin fibroblast (HSF) and human embryonic lung fibroblast (MRC-5) which are quite sensitive for the isolation of this virus. Another attributal factor, may be the timing of collection of specimens and the fact that conjuncitival cell scraping contained more virus particles than did eye swabs.

In conclusion EV70 was prominent in AHC in Egypt and that RTPCR is a rapid sensitive method of diagnosis of such clinically confusing cases with acute keratoconjunctivitis with prominent subconjunctival hemorrhage perceived by clinician as acute hemorrhagic conjunctivitis associated with enteroviruses and adenoviruses.

References

Bahget, M., Mostafa, O., Ali,M.A., and Abdel -Saed,S. (1990) Rapid Diagnosis, clinical features treatment of epidemic conjunctivitis in Egypt, 1989. *Bull Ophthalmol. Soc. Egypt*, **83**, 453.

Mostafa,O., Bahgat,M. and Ali,M.A. (1990) Epidemiology and Diagnosis of an outbreak of conjunctivitis in Egypt, 1989. *Bull. Egypt. Soc. Physiol Sci.*, **10** (3), 97.

Uchio, E., Yamazaki , K., Ishikawa, H., Matsunaga, I., Asato, Y., Aoki, K., Ohno, S. (1999) An epidemic of acute hemorrhagic conjunctivitis caused by enterovirus 70 in India Okinawa, Japan, in 1994. *Graefes Arch Clin Exp Ophthalmol* , **237**(7), 568.

Maitreyi, RS., Dar, L., Muthukumar, A., Vajpayee, M., Xess, I., Vajpayee, RB., Seth, P., Broor, S., (1999) Acute hemorrhagic conjunctivitis due to enterovirus 70 in India. *Emerg Infect Dis* , **5**(2), 267.

Yoshino, S., Yamamoto, S., Kawabata, N., Itokazu, K. (1998) Detection of enterovirus 70 in acute hemorrhagic conjunctivitis by PCR-stringent microplate hybridization method. *Kansenshogaku Zasshi* ,**72** (2), 136 .

Uchio, E., Yamazaki, K., Aoki, K., Ohno, S. (1998) Detection of enterovirus 70 by polymerase chain reaction in acute hemorrhagic conjunctivitis. *Am J Ophthalmol,* **122** (2), 273.

Chomczynski,P. and Sacchi, N. (1987) Simple-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem*., **162**,156.

Martin, AB., Webber, S Fricker., FJ, Jaffe, R . Demmler,G. Kearney, D. Zhang, YH. Bodurtha, J. Gelb B . and Ni J . (1994) Acute myocarditis rapid diagnosis by PCR in children. *Circulation* , **90** (1), 330.

Allard, A.,Girones, R., Juto, P.,Wadell , G. (1990) Polymerase chain reaction for detection of adenoviruses in stool samples. *J.Clin.Microbiol.*, **28**, 2659 .

Guyer,B., O'Day, D.M. Heirholzer, J.C. and Schnaffner W. (1975) Epidemic Keratoconjunctivitis: a community outbreak of mixed adenovirus type 8 and 9 infection. *J.Infect.Dis.*, **132**, 142.

Sprague, J.B., Hierholzer, J.C. Currier., R.W.Hallwick, M.A.W and Smith D. (1973) Epidemic Keratoconjunctivitis: a severe industrial outbreak due to adenovirus type 8. N.Engl.*J.Med.* **289**, 1341.

Bern, C. Pallansch, M.A., Gary, H.E. Jr., Alexander, J.P., Torok, T.J., Glass, R.I., Anderson, LJ. (1992) Acute hemorrhagic conjunctivitis due to enterovirus 70 in American Samoa: serum-neutralizing antibodies and sex-specific protection. *Am J Epidemiol* ,**15**, 136 (12), 1502.

Doraisingham, S., Goh, K.T., Ling, A.E., Ramia, S., Arif, M. (1987) Epidemiology of viral infections in Singapore. *Ann Acad Med Singapore* , **16**(2) 243.

Ramia, S., Arif, M. (1990) Isolation of enterovirus 70 (EV70) from patients with acute hemorrhagic conjunctivitis in two areas of Saudi Arabia. *Trans R Soc Trop Med Hyg* , **84** (1),139.

Waldman, E.A., Takimoto, S., Ishida, M.A., Kitamura, C., Mendonca, L.I. (1990) Enterovirus 70 in the metropolitan region of Sao Paulo, Brazil, from 1984 to 1987: infection aspects in endemic and epidemic periods. Rev I*nst Med Trop Sao Paulo* , **32**(3), 221.

Aoki, K., Kawana, R., Matsumoto, I., Maekawa, H., Sawada, H., Sakurada, N., Nobrega, M.J., Del Rey Filho, M., Belfort, R Jr. (1987) An epidemic of acute hemorrhagic conjunctivitis in the city of Sao Paulo. *Jpn J ophthalmol* , **31**(4), 532.

Wright, P.W., Strauss, G.H., Langford, M.P. (1992) Acute hemorrhagic conjunctivitis. *Am Fam Physician Jan;* **45**(1), 173.

Sawyer, L.A., Hershow, R.C., Pallansch, M.A., Fishbein, D.B., Pinsky, P.F., Broerman, S.F., Grimm, B.B., Anderson, L.J., Hall, D.B., Schonberger, L.B. (1989) An epidemic of acute hemorrhagic conjunctivitis in American Samoa caused by coxsackievirus A24 variant. *Am J Epidemiol Dec,* **130**(6), 1187.

Reeves, W.C., Brenes, M.M., Quiroz, E., Palacios , J., Campos, G., Centeno, R. (1986) Acute hemorrhagic conjunctivitis epidemic in Colon, Republic of Panama. *Am J Epidemiol* , **123**(2), 325.

Kew, O.M., Nottay, B.K., Hatch, M.H., Hierholzer, J.C., Obijeski, J.F. (1983) Oligonucleotide fingerprint analysis of enterovirus 70 isolates from the 1980 to 1981 pandemic of acute hemorrhagic conjunctivitis: evidence for a close genetic relationship among Asian and American strains. *Infect Immun,* **41**(2), 631-5.

Sklar, V.E., Patriarca, P.A., Onorato, I.M., Langford, M.P., Clark, S.W., ulbertson, W.W., Forster, R.K. (1983) Clinical findings and results of treatment in an outbreak of acute hemorrhagic conjunctivitis in southern Florida. *Am J Ophthalmol* , **95** (1),45.

Chang, C.H., Sheu, M.M., Lin, K.H., Chen, C.W. (2001) Hemorrhagic viral keratoconjunctivitis in Taiwan caused by adenovirus types 19 and 37: applicability of polymerase chain reaction-restriction fragment length polymorphism in detecting adenovirus genotypes. *Cornea* , **20**(3),295 .

(*Received* 8 / 7 2002 ;
accepted 9 /10/2002)

السبب الفيروسى للالتهاب الدموى للملتحمة

مها رشدي عبد الواحد ، *محمد احمد احمد على ** ، نانا عبد الرحمن محمد*** و مصطفى عوض هيكل ****

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اشتملت هذه الدراسة على خمسين حالة مصابة بنزيف حاد بالقرنية والملتحمة لأعمار مختلفة . وقد تم فحصهم فحصا رمديا كاملا . وقد كانت الإصابة فى عين واحدة فى عدد ٣٢ حالة وفى العينين فى ١٨ حالة . حيث أن الأعراض المرضية كانت ممثلة فى التهاب بثرى ونزيف فى الجزء الحجاجى والجسمى من نسيج الملتحمة مع ارتشاحات في الملتحمة والجفون وإفرازات كثيرة من النوع المائى وقليل منه كان مخلوط بالنوع المخاطى الصديدى مع تورم مصحوب بألم فى الغدد الليمفاوية قبل الاذنية مع وجود تقرحات نقطية بالنسيج الطلائى للقرنية وكان النزيف تحت الملتحمة هو السائد فى معظم الحالات . وقد جمعت مسحات من الملتحمة من الملتحمة من كل المرض وبعد معالجتها تم حقنها فى ثلاث أنواع من الخلايا الحية لاعطاء فرصة كبيرة لعزل الفيروسات منها . واستخدمت طريقة التفاعل النووي المتسلسل العكسى للتعرف على الفيروسات المعوية وكذلك الادينو . وقد أظهرت النتائج وجود الفيروس رقم ٧٠ فى ثلاثة حالات فقط بعد زراعتها فى مزارع الأنسجة ثلاث مرات متتالية ولم يتم التعرف على فيروسات الكوكساكى أو الادينو بالعينات مما يدل على أن الفيروس المعوى رقم ٧٠ هو المسبب لهذا المرض فى العينات الإيجابية .