VIRAL INVESTIGATION OF ACUTE HEMORRHAGIC CONJUNCTIVITIS OUTBREAK (2003) IN NEPAL USING MOLECULAR METHODS

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Abstract. A nationwide outbreak of acute hemorrhagic conjunctivitis (AHC) occurred in Nepal during August to September 2003, which affected nearly half of the population. Sixty conjunctival swabs from AHC patients were collected at Tilganga Eye Center in Kathmandu. For the first time in Nepal, we demonstrated the etiologic viral agents of AHC, namely, *Coxsackievirus* A24 variant (CA24v) by reverse transcription real time polymerase chain reaction (PCR). Of the 60 samples, 19 were positive for CA24v. No difference in the two genders was observed. Conversely, *Adenovirus* (AdV) was detected in 32 samples, which suggested that the epidemic was caused by mixed infection. AdV was detected also on 10 rupee notes. Findings indicated that inadequate personal hygiene was the main cause of the spread of these highly contagious viruses in the community environment in Nepal during the summer of 2003.

INTRODUCTION

Acute hemorrhagic conjunctivitis (AHC) was first reported from Ghana in 1969 (Chatterjee *et al*, 1970) and spread across the tropical and subtropical Western and Central Africa to the Middle East, Asia including Europe. It is also called "Apollo conjunctivitis" as its emergence coincided with the landing of Apollo 11 on the moon. Following several epidemics, mostly in Asia and Africa; the second big outbreak started in India in 1981 and affected many parts of the world (Patricia, 1989; Ishiko *et al*, 1992; Wright *et al*, 1992). Recently, AHC epidemics have also been reported from Japan, Korea, Thailand, India, Singapore, Malaysia, and elsewhere (Christopher *et al*, 1977; Kosrirukvongs *et al*, 1996; Aoki *et al*, 1988; Uchio *et al*, 1999; Oh *et al*, 2003; CDC, 2004).

AHC is characterized by the sudden onset of painful, swollen, red eyes, with conjunctival hemorrhage and excessive tearing in one eye for a period of six to twelve hours (Uchida, 1989). In 80% of cases, the other eye is affected within 24 hours of onset. Usually a mononuclear cell response is elicited that is followed by a rapid and prominent hemorrhage. Pain is the initial and most notable symptom that appears after an incubation period of 24-to-28 hours. Most cases are self-limited; symptoms persist for three to five days and gradually resolve in seven to ten days (Uchida, 1989). The causative agents of this illness has been identified as *Coxsackievirus* A24 variant (CA24v) and *Enterovirus* 70 (EV70) strains belonging to the Picornaviridae.

In Nepal, AHC occurs once in every two-to-three years, during the summer season. In

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2003 (August September), nearly half of the population in the country was affected (Gurung *et al*, 2003). We detected the viral agent from the conjunctival swab taken from AHC patients by real time RT-PCR/PCR, employing the SYBR-Green method (De Medici *et al,* 2003). We presented the detailed findings of the molecular investigation of that nationwide AHC epidemic (Gurung *et al*, 2003).

MATERIALS AND METHODS

Samples collection

Two conjunctival swabs were taken from each of 60 AHC patients that attended Tilganga Eye Center in Kathmandu in September 2004 who were included in this study (Fig 1). One of the swab was subjected to bacterial and fungal study (at the National Institute of Tropical Medicine and Public Health Research, Kathmandu), while the other was frozen and sent to Japan (Kobe Institute of Health, Kobe) for virus detection. Also the six bank notes of Rs 10 (two), Rs 20 (one), Rs 50 (one) and Rs 100 (two) were tested for viral contamination. Only two of the 60 AHC cases were available for follow-up after four months (in January 2004). Seven bills of Rs 10 (three), Rs 20 (one), Rs 25 (one), Rs 50 (one), and Rs 100 (one) were also investigated.

Nucleic acid extraction and concentration

In this study, three viruses, namely, CA24v, EV70, and *Adenovirus* (AdV) were targeted.

Viral nucleic acid was extracted by the following method. Conjunctival swabs were cut into pieces, put into 1.5 ml micro tubes, followed by the addition of 200 µl of RNA grade autoclaved miliQ water (AMW). After vortexing, the cotton swab pieces were removed by centrifugation. The surface of the Nepalese currency banknotes, were cleansed using plastic cotton buds and 1 ml of AMW in a square Petri dish, and the water was collected into micro tubes.

Viral nucleic acid from the test sample (100 µl) was extracted using QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, USA) with a modification; Pellet Paint NF Co-Precipitant (Novagen, Wisconsin, USA) was used instead of the original precipitation procedure (isopropanol precipitation) as to improve the viral nucleic acid yield from the samples. After the precipitation with QIAamp Viral RNA Mini Kit, 2 µl of Pellet Paint NF Co-Precipitant and 10 µl of 3M sodium acetate, pH5.2, were added following gentle mixing after each addition. Finally, 100 µl of isopropanol was added, incubated at room temperature for three minutes, vortexed, and centrifuged at 12,000*g* for five minutes in cold. The supernatant was removed and the dark blue pellet was washed, first with 70%, and then with absolute ethanol. The pellet containing both the RNA and DNA extracted from the conjunctival swab was then dried in a vacuum centrifuge and was resuspended in 25 µl of TE buffer.

Reverse transcription

In a 200 µl capacity PCR tube, 12 µl of extract, 1.5 µl of 5X reverse transcriptase reaction buffer (enzyme component), 0.5 µl (1 unit) of cloned DNase I (TaKaRa, Otsu, Japan), and 1 µl of AMW were combined. It was then incubated at 37ºC for 30 minutes, followed by 5 minutes incubation at 75ºC, using a thermal cycler (GeneAmp 9700, ABI, California, USA) and kept in an ice bath. The high sensitivity reverse transcription (RT) was performed by adding 7.5 µl of RNA (10 nM), 0.75 µl of dNTPs (10 mM each), 0.75 µl of random 6mers primer (100 µM), 0.75 µl of DTT (100 mM), 15 U of RNase inhibitor (TaKaRa, Otsu, Japan), 2.25 µl of 5X RT reaction buffer, 150 U of Super Script II Reverse Transcriptase (Invitrogen, California, USA) and up to 15 µl of AMW into a 0.2 ml capacity PCR tube. This mixture was then incubated for 10 minutes at 25ºC, 2 hours at 42ºC, and 5 minutes at 99ºC. The cDNA thereby obtained was stored at 4ºC until tested.

Real time RT-PCR/PCR

Nucleic acid libraries of Adenovirus 3 (DL-Adv3/01), Coxsackievirus A24v (DL-CA24/04), and Enterovirus 70 (DL-EV70/05) were used for PCR control.

The primer sequences used are shown in Table 1. Real time PCR (Kurokawa *et al*, 2004) was carried out by Sequence Detection System 7900HT (ABI, California, USA).

For AdV, a nested PCR (Mitchell *et al*, 2003) was performed using a GeneAmp9700 thermal cycler (ABI, California, USA). A 25 µl reaction mixture [12.5 µl of Ampli Taq Gold Master Mix (ABI, California, USA)], 0.2 µM each of the primers (AdV-1F and AdV-1R), 2.5 µl of template, and up to 25 µl of autoclaved MiliQ grade water) in a 96-well micro reaction-plate was subjected to thermal cycling (10 minutes at 95ºC, followed by 40 cycles of PCR; 30 seconds at 95ºC, 60 seconds at 55ºC, and 60 seconds at 72ºC). The second PCR (real time PCR) mixture contained 2X SYBR Green PCR Master Mix (ABI, California, USA) 12.5 µl, primers (0.1 µM) (AdV-2F and AdV-2R), 1:10 diluted first PCR product (2.5 µl) and AMW (up to 25 µl). In a thermal cycler, the mixture was heated at 50ºC for 10 minutes and then at 95ºC for 10 minutes, followed by 45 cycles of heating at 95ºC for 30 seconds, at 55ºC for 60 seconds and 72ºC for 60 seconds. The intensity of the fluorescence released due to the amplification of target sequence in each cycle was collected automatically and was analyzed using SDS 2.0 version software.

Enteroviruses (CA24v and EV70) were detected using primers shown in Table 1. PCR was performed in 25 µl reaction mixtures consisting of 2X SYBR Green PCR Master Mix (ABI, California, USA) 12.5 µl, 0.1 µM primers, cDNA template 2.5 µl and AMW up to 25 µl. Thermal cycling consisted of heating at 50ºC for 10 minutes, 95ºC for 10 minutes, following 45 cycles of 95ºC for 30 seconds, 55ºC for 60 seconds, 72ºC for 60 seconds. Results were confirmed by melting curve analysis on SYBR Green PCR. Positive samples were also carried out the PCR using another set of primers (S3F and S3R for CA24v and S4F and S4R for EV70) identified by product size, and representative PCR products were examined by sequencing (Shulman *et al*, 1997).

Table 1
Sequences of primers.

Name	Target	Region	Sequences (5′-3′)	Length (bp)	References
Adv-1F	Adenovirus	hexon	GCC-GCA-GTG-GTC-TTA-CAT-GCA-CAT-C	301	Mitchell *et al,*
Adv-1R			CAG-CAC-GCC-GCG-GAT-GTC-AAA-GT		2003
Adv-2F	Adenovirus	hexon	GAC-GCC-TCG-GAG-TAC-CTS-WSY-CC	185	Mitchell *et al,*
Adv-2R			TAC-GAG-TAC-GTG-GTG-TCC-TCK-CGR-TC		2003
CA24v-VP31F	CA 24v	VP-3 to VP-1	GCC-TGC-CCA-GAT-TTC-AGT-GT	384	Kishore *et al,*
CA24v-VP31R			GTC-AGC-TTC-GGT-CGT-GGC-GT		2002
EV70-VP1F	EV 70	VP-1	CTT-AGG-CAG-ATC-TGC-ACT-TG	372	Yamazaki *et al,*
EV70-VP1R			AAT-GGA-ATT-GTT-AGA-CGC-GC		1995
S3F	CA 24v	VP-1	GCA-CAA-GGC-ATT-GAG-GAG-ACC-ATT-G	171	Shulman *et al,*
S3R			TGC-CTG-GCC-IGA-TAC-ICC-AGT-CTC		1997
S4F	EV70	VP-1	AAT-TGG-AGA-AAT-AGT-GAA-AAC-TGT-GGC	113	Shulman *et al,*
S4R			CTG-TGT-TGG-ATG-TAG-CIC-CTG-TCT-C		1997

Naming of incomplete nucleic acid sequences followed by NC-IUB recommendation 1984 (IUB, 1986).

RESULTS

Of the total 60 episodes carried out, 19 (31.7%) episodes were positive for CA24v, whereas 32 (53.3%) episodes were positive for AdV. In 7 (11.6%) episodes, both CA24v and AdV were positives. None of the episodes was positive for Enterovirus 70 (Table 2, Fig 2). Bacterial and fungal cultures were sterile except the growth of normal bacterial flora (*Staphylococcus epidermidis* and diphtheroid bacilli) in 6 (10.0%) of the cases studied (Table 2). Conversely, one of five notes (10 Rupee) was positive for AdV (Table 3). Most of the CA24v and AdV positive cases fell in the age groups of teens to 30s and teens to 40s, respectively. No differences in the detection rate of CA24v and AdV were seen between two genders (Table 2). The follow-up of some of the positive cases (after three months of the epidemic) showed no virus in both conjunctival swabs as well as Nepalese Rupee notes. When two nucleic acid precipitation methods, Pellet Paint NF Co-Precipitant(R) and isopro-

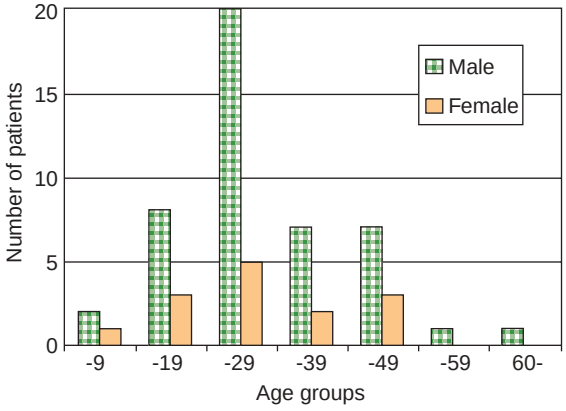
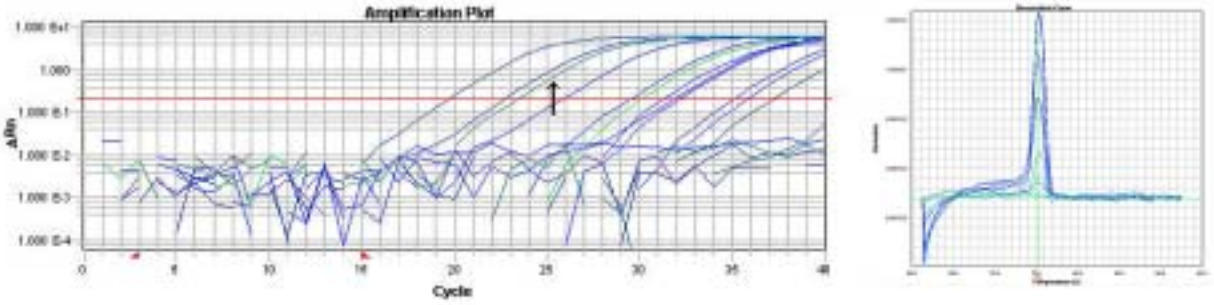


Fig 1–Prevalence of acute hemorrhagic conjunctivitis with age.

Adenovirus



CA24v

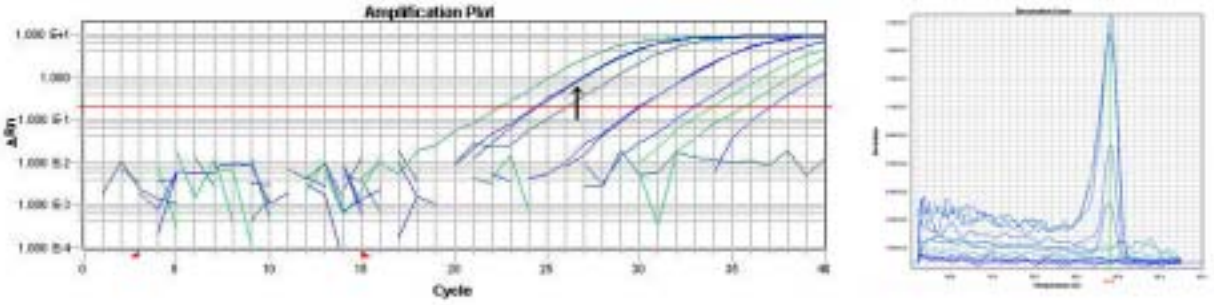


Fig 2–Progress of a PCR reaction is shown by the curve showing the increase in fluorescence (Rn). Up-pointing arrow in same windows indicate amplification curve from positive control. In right window, the sharp curve shows melting temperature of PCR product (Tm) for confirmation of PCR specificity. Temperatures shown in the graph peak in right-side windows as Tm of AdV (above) and CA24v (below) were 75.2ºC and 83.9ºC, respectively.

Table 2
Number of pathogen positives from conjunctival swab.

	Number	Adenovirus	CA24v	Bacteria
Sex				
Male	46	24 (52.2%)	14 (30.4%)	3 (6.5%)
Female	14	8 (57.1%)	5 (35.7%)	3 (21.4%)
Age groups				
-9	3	1	2	1
-19	11	4	4	1
-29	25	13	6	1
-39	9	5	4	1
-49	10	7	2	2
-59	1	1	0	0
60-	1	1	1	0
Total	60	32 (53.3%)	19 (31.7%)	6 (10.0%)

Table 3
Comparison of nucleic acid precipitation method for concentration of RNA/DNA.

		Adenovirus		CA24v	
	Number	With carrier[a]	No carrier[b]	With carrier	No carrier
Conjunctival swab	60	32 (53.3%)	27 (45.0%)	19 (31.7%)	13 (21.6%)
Bills	5	1 (20.0%)	1 (20.0%)	0	0

[a]Pellet Paint NF Co-Precipitant; [b]Isopropanol precipitation

panol were compared, the first method showed better results (46.2%; 19 versus 13 for CA24v and 18.5%; 32 versus 27 AdV (Table 3).

DISCUSSION

Several large outbreaks of AHC have occurred in Nepal (Gurung *et al*, 2003). However, the causative viral agents were not established due to the lack of detection facilities. To the best our knowledge, we have been able to identify for the first time AdV and CA24v viruses associated with nationwide AHC in Nepal (2003) using the real time RT-PCR technique.

The concentration of the target nucleic acid is very important when using the PCR technique. Viral RNA is very small compared with a DNA molecule; therefore, enough RNA cannot be extracted using only the alcohol precipitation method. The addition of a carrier, such as glycogen, tRNA, or polyacryl, into the solution enhances the precipitation of RNA/DNA (Gallagher *et al*, 1987), and thus increases the sensitivity. Significantly better results were obtained with real time PCR by detecting fluorescence in the computer as compared with the conventional PCR method. This technique, coupled with carrier precipitation methods (Pellet Paint NF Co-Precipitant), is also effective for the direct detection of target nucleic acid from clinical sample, especially RNA virus like CA24v (CA24v mutates easily and numerous mutants are being kept in the BLAST database at the National Center for Biotechnology Information, Mary-

land, USA). To identify the enteroviruses by molecular technique, common primers are usually used for detection and product sequencing for viral identification. With the combined use of specific primers, we could detect the viruses associated with the AHC epidemic of 2003 in Nepal.

Detection of AdV on the 10 Rupees note indicated that the viruses were widely spread in the environment (community), and the infection was transmitted person-to-person through contaminated hands and/or contaminated items, including bank notes. It appeared that the notes played a significant role in the transmission of the nationwide AHC epidemic because the notes circulated widely. In addition, other factors also might have played an important role in the spread of infection. However, we did not have the means to investigate them. No viral pathogens were detected from the follow-up conjunctival swabs (three months after the epidemic) or from the bank notes, which indicated that the AHC causing viruses disappeared from the environment soon after the epidemic.

No growth of pathogenic bacteria or fungi indicated that the nationwide epidemic of 2003 in Nepal supported the viral findings. Most of the CA24v and AdV positive cases fell into the physically and socially active age group, and this may have been associated with their active day-to-day lifestyle. No differences in the detection rate of these viruses among two genders suggested the viruses were widely spread in the communities (Gurung *et al*, 2003).

AHC epidemics are mostly caused by EV70, CA24v, and, less commonly, by AdV 11 (Wright *et al*, 1992). AHC-causing CA24v was first detected during an outbreak in Singapore in 1970 (Lim and Yin-Murphy, 1971; Mirkovic *et al*, 1974), and it was not detected outside of Southeast and South Asia until 1986 when an outbreak was reported in American Samoa that subsequently spread to the Caribbean Islands and other parts of America (Ghazali *et al*, 2003). Many AHC outbreaks due to CA24v/EV70 have been reported from India during the years 1977 to 2002 (Christopher *et al*, 1977; Bhatia and Swami, 1999; Maitreyi *et al*, 1999; Wairagkar *et al*, 1999; Madhavan *et al*, 2000; Kishore and Isomura, 2002). However, the entry route of these viruses into the country was not clear because there were AHC cases in both neighboring countries, India and China (Tibet Autonomous Region) during 2003 summer. This, therefore, remains for further investigation.

Because of its characteristic epidemic nature and the morbidity caused by the AHC, education about AHC is strongly advocated. This will benefit, not only the ophthalmic workers to recognize and treat this illness (Wright *et al*, 1992), but also make the people in general aware of this illness that will become useful in combating such an outbreak in the future.

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