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BRIEF REPORT

Molecular identification and phylogenetic study of coxsackievirus A24 variant isolated from an outbreak of acute hemorrhagic conjunctivitis in India in 2010

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Abstract An outbreak of acute hemorrhagic conjunctivitis (AHC) occured in India between August and October 2010. Molecular typing by RT-PCR and sequencing of a partial VP1 region identified coxsackievirus A24 variant (CV A24v) as the serotype involved in this outbreak. Phylogenetic analysis based on the VP1 and 3C genes revealed that CV A24v strains associated with the 2010 AHC outbreak in India were genetically similar to strains from Central and South America that caused outbreaks of AHC in Cuba between 2008 and 2009 and Brazil in 2009. The result shows that the Indian strain of CV A24v may be responsible for the recent AHC outbreak in Marseille, France, in 2012.

Keywords Coxsackievirus A24 · Molecular identification · VP1 · 3C · Phylogenetic analysis

Abbreviations

CV A24v	Coxsackie virus A24 variant
AHC	Acute hemorrhagic conjunctivitis
RT-PCR	Reverse transcription polymerase chain reaction
CPE	Cytopathic effect

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Human enteroviruses in the family *Picornaviridae* comprise more than 100 serotypes that are divided into four species, *Human enterovirus A, B, C,* and *D* [1]. The clinical manifestations of enteroviruses range from mild upper respiratory symptoms (common cold), hand, foot and mouth disease, myocarditis, acute flaccid paralysis, and acute hemorrhagic conjunctivitis [2]. Acute hemorrhagic conjunctivitis (AHC) is a highly contagious disease characterized by conjunctivitis, keratitis, foreign body sensation, pain, respiratory symptoms and severe neurological symptoms including acute flaccid paralysis [3]. Coxsackievirus A24 (species *Human enterovirus C*) and enterovirus 70 (species *Human enterovirus D*) are the main enterovirus serotypes associated with outbreaks of acute hemorrhagic conjunctivitis worldwide. However, some other enterovirus serotypes belonging to the species *Human enterovirus B*, including echovirus 7 and 11 and coxsackievirus B1 and B2, have been also reported in conjunctivitis cases [2, 4].

Coxsackievirus A24 variant (CV A24v), an antigenic variant of the CV A24 strain, was first isolated in Singapore from an outbreak of AHC in 1970 [5]. CV A24v has caused several epidemics and outbreaks of AHC in different countries of the world, including India, China, Nepal, Malaysia, Taiwan, Korea, Japan, Caribbean, Tunisia, Spain, Cuba, Brazil and France [6–24]. Phylogenetic analysis of the VP1 capsid and 3C protease region of the CV A24v genome has been used to determine the epidemiological relationship among strains responsible for epidemics and outbreaks [8, 9, 13–18]. Previous phylogenetic analysis of the 3C protease region of CV A24v strains described four genogroups (GI-GIV) [14]. Genogroups I and II include strains isolated from Singapore, Hong Kong and Thailand during 1970-1975. Genogroup III includes six clusters of strains isolated from Asia, Africa and Ghana

during 1985-1994. Recently, a new genogroup, genogroup IV, divided into three clusters, was identified, and it includes strains isolated from South Korea, China, India, Congo, Guiana, Morocco, Brazil and Cuba between 2000 and 2009.

Since the first epidemic in India in 1971, CV A24v has been reported in several epidemics and outbreaks of AHC in different parts of the country, including Vellore (1979), Delhi (1988), Uttar Pradesh (1994), Chennai (1999), Gujarat and Maharashtra (2003) and Mumbai (2007) [19–24]. An outbreak of AHC occurred in Northern India between August and October 2010 involving >12,000 cases. However, the actual number of cases may be much higher, since most patients did not go to hospital to seek medical assistance, and there is no specific surveillance system for AHC cases in India. CV A24v was the etiologic agent identified from this outbreak. Phylogenetic analysis based on the 3C protease region revealed that CV A24v strains associated with the 2010 AHC outbreak in India were genetically similar to strains from Central and South America associated with AHC outbreaks in Brazil and Cuba between 2008 and 2009.

A total of 66 conjunctival swab specimens were collected from patients (age range from 3 years to 65 years; mean 36.8 years) with a clinical diagnosis of AHC who attended the outdoor patient treatment at Ram Manohar Lohia Hospital and Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow, in Uttar Pradesh State in India. The conjunctival swab specimens were placed in 1 ml viral transport medium (HiMedia, Mumbai, India) and immediately transported in frozen condition to the laboratory at the Department of Microbiology, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow, and stored at −40 °C till further use.

Viral nucleic acid was extracted from conjunctival swab specimens using a QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, USA). Detection of enterovirus was performed by using Geno Sen's Enterovirus Rotor gene quantitative Real time PCR kit (Genome Diagnostics, Delhi, India) specific for the 5' non-coding region of enterovirus in a Rotor-Gene 6000 real-time instrument (Corbett Research, Victoria, Australia). Adenovirus detection was performed according to a previously described method [25].

Molecular typing of enterovirus was performed by amplification of the partial VP1 region using the AN 88 and AN 89 primers as described previously by Nix et al. [26]. The amplicons were separated by 1.8 % agarose gel electrophoresis, purified using a QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany), and sent for sequencing at Vimta Company, India. Sequencing was done on an automated sequencer (Applied Biosystem, Foster City, CA) using the AN 89 and AN 88 primers. Identification of enterovirus type was done by pairwise comparison of the VP1 amplicon sequence with a database of all enterovirus serotypes using the BLAST program (www.ncbi.nlm.gov/BLAST) from GenBank with standard criteria (≥75 % similarity at the nucleotide level for the same serotype).

Two hundred microliters of conjunctival swab specimens that were positive for CV A24v was inoculated onto HEp 2 and RD cells. Cells were incubated at 37 °C and examined daily for cytopathic effect (CPE). Two blind passages, seven days apart, were performed when no CPE was observed during the first passage. After the appearance of complete CPE, culture supernatants were collected and frozen at −40 °C until use. Viral nucleic acid was extracted from cell culture supernatant showing CPE using a QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, USA).

Amplification of the CV A24v 3C protease region was performed with primers 3C-F (5'-TACAAACTGTTTGCTGGGCA-3') and 3C-R (5'-ACTTCTTTTGATGGTCTCAT-3') according to Kishore et al. with some modifications [22, 24]. In brief, PCR reaction mixtures of 30 μL contained 2 μL cDNA, 3 μL 10X PCR buffer, 0.2 μM each primer (3C-F/3C-R), 2.5 units of Roche Taq DNA Polymerase (Roche Applied Science), and 10 μM dNTP. Reactions were incubated at 95 °C for 2 min, followed by 35 cycles of 95 °C for 30 s, 52 °C for 30 s and 72 °C for 45 s. After the last cycle, the final extension step was continued at 72 °C for 5 min. The specific product was purified using a QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany) and sequenced using primers 3C-F and 3C-R on an automated sequencer (Applied Biosystems, Foster City, CA) according to manufacturer's instructions.

Sequences (VP1 and 3C region) were aligned by the Clustal W method (www.ebi.ac.uk/clustalw). Genetic distances were calculated using the Kimura 2-parameter model, with a transition/transversion ratio of 14.0 for VP1 and 12.0 for the 3C region [27]. A phylogenetic tree was inferred by using the neighbor-joining algorithm with 1,000 bootstrap replicates in MEGA 4.0.2 software (www.megasoftware.net) [28]. Sequences obtained from CV A24v isolates in this study have been submitted to the GenBank database under accession numbers JX417146- JX417169.

Of the 66 conjunctival swab sample, 32 (48.5 %) were positive for enterovirus by real-time RT-PCR in the highly conserved 5' non-coding region. All samples were negative for adenovirus. All clinical specimens that were positive for enterovirus by real-time PCR were identified as CV A24v serotype by amplification and sequencing of a partial VP1 region. CV A24v-positive clinical specimens were inoculated onto RD and HEp 2 cells for virus isolation; CPE was observed in only 12 (37.5 %) of the inoculated HEp 2 cell cultures.

VP1 gene sequences from this study (236 nucleotides) were compared with all CV A24v isolate sequence available in the GenBank database. The nucleotide variation in

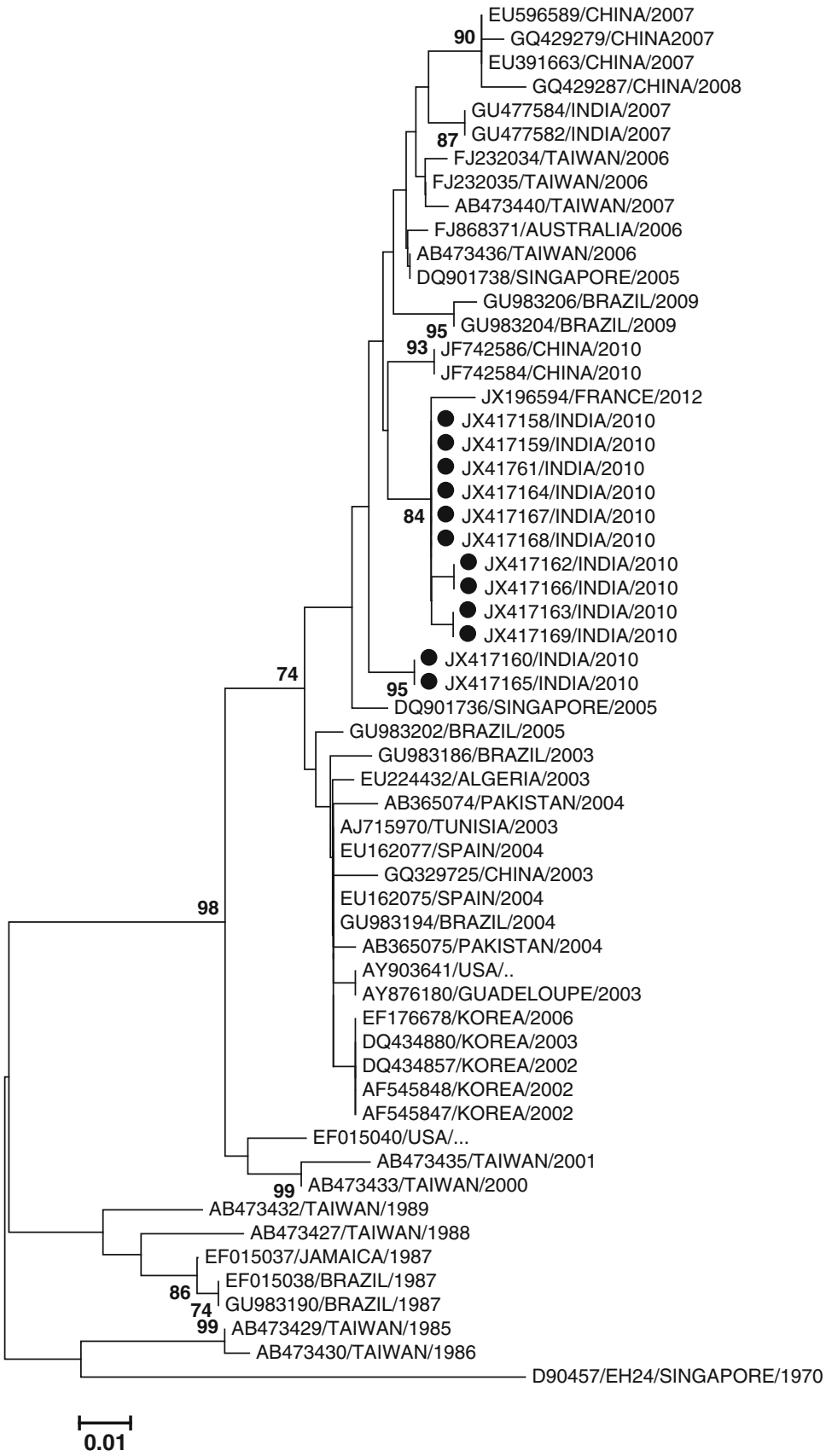

Fig. 1 Phylogenetic analysis of the partial nucleotide sequence encoding the VP1 region (236 bp) of the coxsackievirus A24 variant strain from this study (indicated by a *filled circle*) and other reference strains. The evolutionary history was inferred by the neighbor-joining method using the MEGA 4 program [28]. The scale bar indicates nucleotide substitutions per site. Coxsackievirus A24 variant isolates are indicated by GenBank accession number, country, and year of isolation. *Dotted line* no information about the year of isolation

◀**Fig. 2** Phylogenetic analysis of the partial nucleotide sequence encoding the 3C protease region (510 bp) of the coxsackievirus A24 variant strain from this study (indicated by a filled circle) and other reference strains. The evolutionary history was inferred by the neighbor-joining method using the MEGA 4 program [28]. The scale bar indicates nucleotide substitutions per site. Coxsackievirus A24 variant isolates are indicated by GenBank accession number, country, and year of isolation. *Dotted line* no information about year of isolation

the 5' VP1 region among CV A24v strains from this study was 0-2 %, and it was 14-16 % with the EH24/70 prototype strain (accession no. D90457). Phylogenetic analysis of the CV A24v isolate from this study showed that all Indian strains from the 2010 outbreak were clustered with CV A24v strains in genogroup IV associated with AHC outbreaks in Brazil in 2009 (97 % identity) [15], and China in 2010 (97-98 % identity) [16] as well as a recent outbreak in France in 2012 (97-99 %) [17] (Fig. 1).

Phylogenetic analysis of the 3C (510 nucleotide) gene sequence of CV A24v from this study was also performed. The nucleotide sequence divergence among Indian strains from the 2010 outbreak was 0-2 %, and it was 14-15 % with the EH24/70 prototype strain (accession no. D90457). Phylogenetic analysis showed that four genogroups were chronologically discerned in the tree (Fig. 2). All isolates from this outbreak were included in genogroup IV and clustered with strains from Brazil, isolated from the 2009 outbreak [15]; from Cuba, isolated between 2008 and 2009 [18]; and from China in 2010 [16].

During the past 20 years, CV A24v has been reported in the most outbreaks of AHC throughout the world. A loss of herd immunity to CV A24v has been suggested as the reason for widespread transmission, because the immunity declines considerably within seven years after infection [29]. CV A24v is a common cause of AHC in India and has been reported in several outbreaks of AHC [19–24]. A major outbreak of AHC occurred again in India between August and October 2010. To identify the causative agents of this outbreak, molecular methods were used for detection of adenovirus and enterovirus in conjunctival swab specimens collected from AHC patients. CV A24v was identified in 32 (48.5 %) samples by partial sequencing of the VP1 gene, while all specimens were negative for adenovirus. These results indicate that CV A24v was the etiologic agent of this outbreak.

VP1-gene-based sequence analysis is suitable for molecular epidemiological studies of CV A24v [13–15, 17]. To study the epidemiological link among Indian CV A24v strains from this outbreak and a previous outbreak in 2007 (unpublished data) with worldwide isolates, we performed phylogenetic analysis of a portion of the VP1 gene. All Indian isolates from the 2010 outbreak clustered with CV A24v strains associated with AHC outbreaks in China

during 2010 (97-98 % identity) [16], Brazil in 2009 (97 % identity) [15], and a recent outbreak in France in 2012 (97-99 % identity) [17]. These finding suggest that CV A24v isolates from the 2010 outbreak in India were genetically similar to isolates from Brazil, China and France.

However, most studies of AHC outbreaks caused by CV A24v have used the 3C region for molecular epidemiological analysis [9, 11, 14, 15, 18]. To perform phylogenetic analysis, we amplified and sequenced the 3C protease gene of the Indian isolates collected in this study and compared them with all available sequences. Results based on the 3C protease gene demonstrated that all Indian isolates were closely related and clustered in genogroup IV with isolates from Brazil in 2009 (96-97 % identity) [15], Cuba between 2008 and 2009 (96-97 % identity) [18], and China during 2010 (96-98 % identity) [16], forming a new clade with a bootstrap value of 86 %. Because a Chinese strain isolated from the AHC outbreak in 2010 were in the same cluster with the Indian isolates, phylogenetic analysis of the Chinese strain was also performed to study a possible epidemiological link. Interestingly, all Chinese CVA24v strains showed a high level of sequence identity to isolates from a 2009 outbreak in Brazil (96-97 % identity) and an outbreak between 2008 and 2009 in Cuba (96-97 % identity), similar to the Indian strain. These results suggest that a new lineage of CV A24v appeared in Cuba and Brazil, may have been imported into Asia, and was responsible for large outbreak of AHC in 2010.

In conclusion, an AHC outbreak occurring in India during 2010 was caused by CV A24v. Transmission of the Indian CV A24v strain in the European region may be associated with the recent AHC outbreak in Marseille, France, in 2012.

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Conflict of interest None.

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