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Original Article

Microbiology Section

# A Cross Sectional Study on Detection of Human Adenovirus from Clinical Samples of Conjunctivitis by Real Time PCR and Viral Cell Culture

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## ABSTRACT

**Introduction:** Human Adenoviruses (HAdV) have been implicated in a variety of infections, including conjunctivitis, respiratory tract infections, genitourinary infections and gastroenteritis. Epidemic Keratoconjunctivitis (EKC) is a severe ocular surface infection strongly associated with HAdV, known to occur in widespread outbreaks. HAdV species B, D and E are associated with ocular manifestations ranging from simple follicular conjunctivitis (types 3, 4, 7) and pharyngoconjunctival fever (types 3, 7, 14) to the more severe EKC (types 8, 19, 37, 53, 54, 56 and 64). Viral cell cultures of conjunctival specimens help confirm adenovirus infection through the characteristic Cytopathic Effect (CPE) and Polymerase Chain Reaction (PCR) is the best standard method to diagnose viral conjunctivitis due to its sensitivity, accuracy and rapidity.

**Aim:** To diagnose HAdV in clinically suspected cases of viral conjunctivitis using real-time PCR. To isolate HAdV in viral cell culture and confirm it by observing its characteristic CPE and performing adenoviral real-time PCR.

**Materials and Methods:** A cross-sectional descriptive study was conducted from July 2023 to September 2023 at the state-level Virus Research and Diagnostic Laboratory (VRDL) at Bangalore Medical College and Research Institute (BMCRI), Bengaluru, Karnataka, India. A total of 45 conjunctival swab samples from patients with suspected viral conjunctivitis, who attended the Ophthalmology Outpatient Department (OPD) or were admitted to the wards, were included in the study. Conjunctival swab samples collected in Viral Transport Medium (VTM) were centrifuged at 3000 rpm for five minutes at 4°C. Real-time PCR was performed and samples positive for HAdV by real-time PCR were taken up for viral cell culture. Confirmatory PCR was conducted for samples showing CPEs in the cell line. The data collected were analysed using descriptive statistics.

**Results:** The majority of patients in the present study were in the age group of 18-45 years, comprising 25 (56%) of the total patients. Out of 45 conjunctival samples tested, 8 samples were positive in adenovirus real-time PCR. All eight PCR-positive samples showed a CPE in viral cell culture on the A549 cell line. The study found PCR positivity in 8 samples (17.7%), while adenoviral recovery from cell culture was observed in 6 samples (13.3%).

**Conclusion:** Real-time PCR has become the standard diagnostic procedure for detecting adenovirus conjunctivitis. Rapid and accurate diagnosis is key to interrupting the contagious spread of adenoviral conjunctivitis, along with timely treatment.


**Keywords:** Adenoviral conjunctivitis, Diagnosis, Epidemic keratoconjunctivitis, Polymerase chain reaction

## INTRODUCTION

The HAdVs have been implicated in a variety of infections, including conjunctivitis, respiratory tract infections, genitourinary infections and gastroenteritis [1]. HAdV is a known cause of severe ocular surface infections, specifically EKC, which is known to occur in extensive outbreaks. Conjunctival symptoms can vary from hyper-acute, exudative conjunctivitis with the formation of conjunctival membranes to moderate follicular conjunctivitis. Adenoviral conjunctivitis can lead to subepithelial infiltrates, which may persist or recur months to years after the acute infection has resolved. This condition can also cause punctate or geographic epithelial keratitis, which may culminate in stromal keratitis [1].

The HAdVs were initially classified into 51 types based on serum neutralisation and haemagglutination inhibition. The epsilon determinant, an epitope on the hexon protein generated by two hypervariable loops programmed at the genome level by hypervariable regions 16 and 7, respectively, is responsible for serum neutralisation. Haemagglutination inhibition is a property of the fibre protein. Recently, using whole genome sequencing, HAdVs have been classified into 7 species (A-G) and 103 genotypes in GenBank [2]. The association between ocular surface infections and HAdV species B, D and E is well-established. The disease can cause a range of symptoms, from pharyngoconjunctival fever (types 3, 7, 14) and simple follicular conjunctivitis (types 3, 4, 7) to more severe EKC (types 8, 19, 37, 53, 54, 56 and 64). Worldwide, genotype 8 is more prevalent, being involved in 44% to 100% of EKC outbreaks, whereas type 4 is implicated in 7% to 11% of outbreaks [3]. Studies from South India have reported the presence of types 8 and 4 [4,5]. Previous studies from India have shown a prevalence of 13.8% to 66.6% for HAdV among patients with keratoconjunctivitis [6].

The traditional gold standard for diagnosing EKC or any adenovirus conjunctivitis has been Cell Culture in Combination with Immunofluorescence staining (CC-IFA) and characteristic CPEs [2-4]. Various cell lines are used for adenovirus cultivation, including A549, Hep-2, HeLa and 3T6 cell lines [7]. PCR, direct immunofluorescence and rapid antigen-detection immunoassays are also utilised to diagnose viral conjunctivitis. While viral cell cultures of the conjunctival specimen can help in confirming adenovirus through immunofluorescence and characteristic CPEs, they are less frequently used due to the requirement for elaborate equipment, specialised facilities, trained laboratory staff and significantly increased turnaround time [7]. PCR is considered the best standard method for diagnosing viral conjunctivitis due to its sensitivity, accuracy and rapid results, along with less technical involvement [7,8]. Most PCR assays target the

more conserved penton or hexon region sequences for detecting adenoviruses [5,6].

As mentioned earlier, EKC or adenoviral conjunctivitis is a severe ocular surface infection characterised by punctate or geographic epithelial keratitis, leading to chronic keratitis with reduced visual acuity, which can be debilitating. HAdVs cause periodic outbreaks due to their highly contagious nature in a wide range of settings, such as military recruits or hospitals. Community-based EKC outbreaks are common and are usually transmitted from person to person via respiratory or ocular secretions, by fingers, or through contaminated ophthalmologic instruments. In fact, most described epidemic outbreaks represent infections from a common source, which may include inadequately chlorinated swimming pools or contaminated ophthalmology units [1,3,4].

Laboratory confirmation of the diagnosis can guide physicians in rapidly initiating appropriate hygienic measures and determining the epidemiological significance of the infection [9]. Early rapid and accurate diagnosis helps limit the spread of the disease and accelerates recovery in patients through timely and appropriate treatment measures [5,8,10].

The current study aimed to detect adenovirus from clinical samples of conjunctivitis through real-time PCR and to isolate HAdV in viral cell culture, confirming it by its characteristic CPE and adenoviral real-time PCR. The present study updates the current state of knowledge regarding the efficacy of viral cell culture and real-time PCR in diagnosing adenoviral conjunctivitis, which is especially crucial as diagnostic methods advance and new technologies become accessible. The study's findings may have implications for public health strategies, including outbreak management and preventive measures. Rapid and accurate diagnosis can facilitate the timely implementation of isolation protocols.

## MATERIALS AND METHODS

The present cross-sectional descriptive study was conducted at the state-level VRDL laboratory at Bangalore Medical College and Research Institute, Bengaluru, from July 2023 to September 2023, after obtaining ethical clearance from the Institutional Ethical Committee (No: BMCRI/EC/11/23-24).

**Sample size calculation:** The sample size was calculated according to the study by Sharmila F et al., which reported a prevalence of adenoviral conjunctivitis of 47.8% [11].

Sample formula: $4PQ/d^2$

Prevalence (P)- 47.8% Q= 100-P= 40

Allowable error (d)= 15%

Sample Size= 44.35

Sample Size= 45

A total of 45 conjunctival samples from suspected cases of viral conjunctivitis, who attended the Ophthalmology OPD and were admitted to the wards, were included in the study.

**Inclusion criteria:**

- Patients willing to provide informed consent.
- Patients aged eight years and older.
- Patients clinically diagnosed with unilateral or bilateral acute onset of conjunctivitis, acute haemorrhagic conjunctivitis, or conjunctivitis with watery discharge.
- Patients with a recent history of unilateral or bilateral acute onset of conjunctivitis, acute haemorrhagic conjunctivitis, or conjunctivitis with watery discharge among family members.

**Exclusion criteria:** Clinically diagnosed patients with conjunctivitis associated with mucopurulent or purulent discharge, suggestive of bacterial and allergic/chemical aetiology, as well as contact lens wearers were excluded from the study.

### Study Procedure

Each specimen in the VTM was vortexed intermittently for 1 minute, then aliquoted into sterile Eppendorf tubes and centrifuged at 3000 rpm for 5 minutes at 4°C. The supernatant was used for viral culture and molecular assays.

**Viral cell culture:** A 500 µL of supernatant from each sample was inoculated into a tissue culture flask (T25) containing an appropriate, healthy A549 cell line with a confluence of ≥ 70%, which was devoid of any cytoplasmic granulation and rounded cells [12]. Fresh A549 flasks were procured from the National Centre for Cell Science (NCCS), Pune and maintained at the State Level Virus Research and Diagnostic Laboratory, BMCRI, Bengaluru. The inoculated cell lines were incubated at 37°C and observed daily for up to seven days for the characteristic CPE of adenovirus, which is characterised by grape-like clusters of cells. The CPE was confirmed by performing real-time PCR on the viral cell medium once growth was evidenced.

The QIAamp Deoxyribonucleic Acid (DNA) Mini Kit (Qiagen, Hilden, Germany) was utilised to extract viral DNA from 200 µL of the supernatant fluid, in accordance with the manufacturer's instructions. If no CPEs were observed within seven days, a blind passage was performed using a new A549 cell line flask, which was then observed for an additional seven days. Only then was it reported that the viral culture from the sample was negative for adenovirus.

**For real-time PCR:** The QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) [13] was utilised to extract viral DNA from 200 µL of VTM in accordance with the manufacturer's instructions.

The extracted DNA was subsequently processed using real-time PCR. In case of an expected delay in processing, the extracted DNA was stored at -80°C. Stored samples were thawed to room temperature before processing in real-time PCR and repeated freeze-thaw cycles were avoided.

The Helini Adenovirus Real-time PCR kit was used to perform real-time PCR according to its protocol. This kit targets the conserved region of the adenovirus genome, specifically the hexon sequence, for the direct detection of the specific amplicon in the Fam channel. An external positive control was supplied in the kit, which can be used for both qualitative and quantitative determination of viral load. The primers and probe sequences in this kit have 100% homology with a broad range of clinically relevant adenovirus groups and genotypes based on comprehensive bioinformatics analysis.

The HAdV has been categorised into seven subgenera: A-G, as per the International Committee on Taxonomy of Viruses (ICTV). A complete set of 90 genotypes has been identified phylogenetically using all of the genome sequences in the GenBank collection [14].

Widespread epidemics of keratoconjunctivitis are caused by adenoviruses 8, 19, 37, 53 and 54. The following genotypes are implicated in adenoviral conjunctivitis [15].

A: 12, 18, 31

B: 3, 7, 11, 14, 16, 21, 34, 35, 50, 55

C: 1, 2, 5, 6, 57

D: 8, 9, 10, 13, 15, 17, 19, 20, 22, 23, 24, 25, 26, 27, 28, 29, 30, 32, 33, 36, 37, 38, 39, 42, 43, 44, 45, 46, 47, 48, 49, 51, 53, 54, 56

E: 4

F: 40, 41

G: 52

Volume to be added per reaction is as per [Table/Fig-1].

A 10 µL of PCR grade water was added as Negative template control.

Thermal cycler programming was performed as per [Table/Fig-2].

Any sample showing amplification curve along with ct value of <35 was considered as positive after Internal control validation.

Qualitative Interpretation of results was according to [Table/Fig-3].

| Components | Volume per reaction |
|---|---|
| Probe PCR master mix | 10 µL |
| Adenovirus PP mix | 2.5 µL |
| IC PP Mix | 2.5 µL |
| Purified DNA | 10 µL |
| Final reaction volume | 25 µL |

**[Table/Fig-1]:** Volume to be added per reaction.

| | Step | Time | Temp |
|---|---|---|---|
| | Taq Enzyme Activation/Hold | 15 min | 95°C |
| 40 cycles | Denaturation | 20 sec | 95°C |
| | Annealing | 20 sec | 56°C |
| | Extension | 20 sec | 72°C |

**[Table/Fig-2]:** Programming of thermal cycler.

| Test sample | Negative control | Positive control | Internal control | Interpretation |
|---|---|---|---|---|
| Positive | Negative | Positive | Positive | Adenovirus specific DNA detected |
| Negative | Negative | Positive | Positive | No Adenovirus specific DNA detected. Sample does not contain detectable amounts of adenovirus specific DNA. |
| Negative | Negative | Negative | Negative | Experiment fail |
| Positive | Positive | Positive | Positive | Experiment fail |

**[Table/Fig-3]:** Qualitative Interpretation of as per kit literature [16].

## STATISTICAL ANALYSIS

The data were analysed using Statistical Package for the Social Sciences (SPSS) software version 28.0. The collected data will be statistically analysed using descriptive statistics, specifically mean, standard deviation and percentage, wherever applicable.

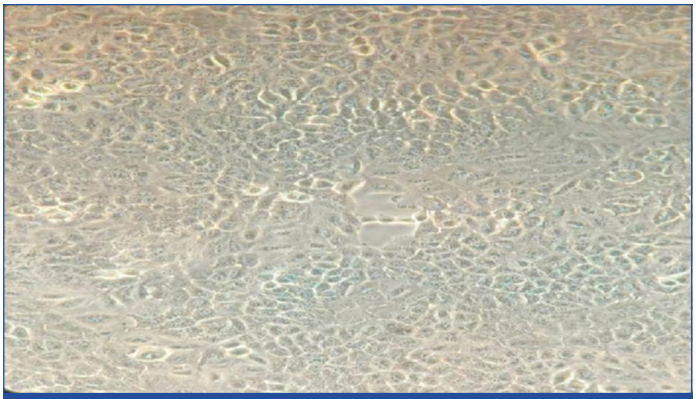
## RESULTS

The majority of patients in the present study were in the age group of 18-45 years, comprising 25 (56%) of the total patients. The age groups of <18 years and >45 years together constituted 20 (44%) of the study population. The study showed a male preponderance, with 27 (60%) male patients among the total included. All patients exhibited lacrimation as a symptom and 44 (97.7%) presented with red conjunctiva, either unilaterally 17 patients, (37.7%) or bilaterally 28 patients, (62.2%). Additionally, 19 (42.2%) patients showed signs of epibulbar or tarsal follicles, while only one patient exhibited signs of corneal involvement. Furthermore, 7 (15.5%) patients reported having similar symptoms in the past. The representation of clinical features among the patients is shown in [Table/Fig-4].

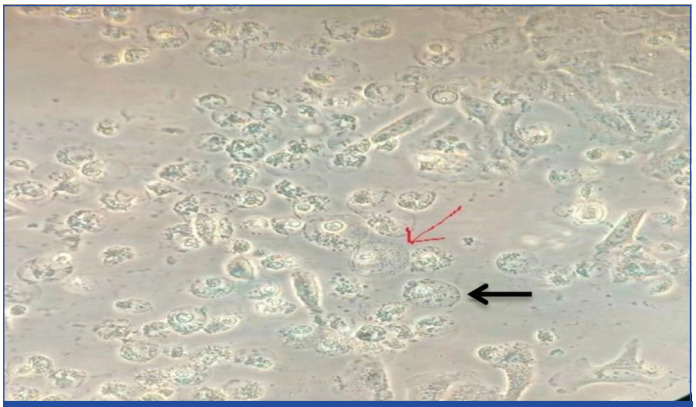
| Clinical features | No. of cases |
|---|---|
| Lacrimation | 45 (100%) |
| Red conjunctiva | 44 (97.7 %) |
| Unilateral presentation | 17 (37.7 %) |
| Bilateral presentation | 28 (62.2%) |
| Follicles | 19 (42.2%) |
| Corneal involvement | 1 (2%) |
| Previous history of similar complaints | 7 (15.5%) |

**[Table/Fig-4]:** Clinical features of study population.

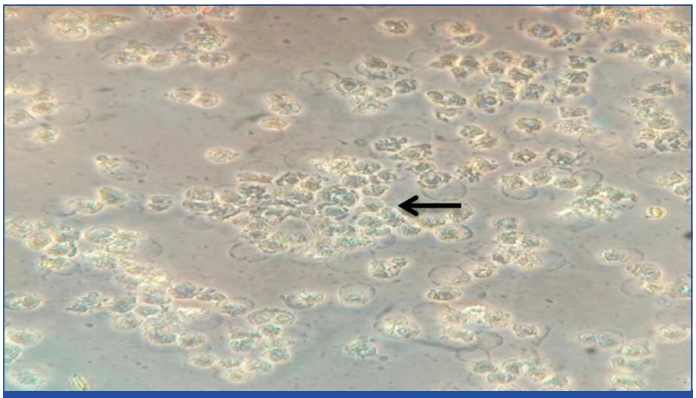
Out of 45 conjunctival samples tested, 8 (17.7%) were positive for adenovirus in real-time PCR. All eight PCR-positive samples exhibited CPEs in viral cell culture using the A549 cell line. An uninfected A549 cell line is depicted in [Table/Fig-5]. The CPE were observed in the form of cell rounding, cell detachment from the flask surface and clustering of the cells to form grape-like clusters, as shown in [Table/Fig-6,7].



**[Table/Fig-5]:** Uninfected A549 cell line (40X).



**[Table/Fig-6]:** Infected A549 cell line showing rounding of cells (40X).

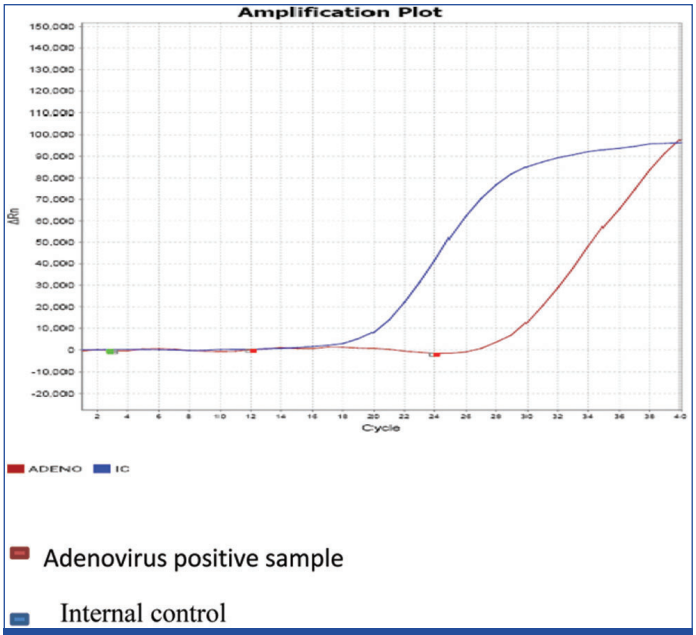


**[Table/Fig-7]:** Infected A549 cell line showing clustering of cells (Grape-like clusters) (40X).

In all cases, CPE developed within 4-5 days of inoculation. The presence of CPE was confirmed by detecting the adenovirus using the Helini Adenoviral real-time PCR kit [16] on the viral cell medium after viral growth was observed. The present study showed the presence and cell culture recovery of adenovirus in at least 6 (13.3%) of the conjunctival swab samples tested. In this study, 8 (17.7%) samples tested positive by PCR. Confirmatory PCR was positive for 6 out of the eight samples (75%) that showed CPE. The adenoviral real-time PCR amplification plot for a positive sample, along with the internal control, is shown in [Table/Fig-8].

## DISCUSSION

Adenoviral conjunctivitis is a communicable disease that can be acquired both nosocomially and in the community. Nosocomial conjunctivitis infections are particularly common, leading to severe outbreaks in ophthalmology wards. These outbreaks can force the limitation of clinical practices, including the delay of eye surgeries, early discharge of hospital inpatients and even the closure of ophthalmology wards [17]. Currently, there is no effective treatment for adenoviral conjunctivitis, making prompt detection of the aetiological agent crucial. This is important not only to initiate preventive measures due to the condition's tendency to cause outbreaks and visual impairment




**[Table/Fig-8]:** Real-time PCR Showing positive sample amplification with internal control, X-axis denotes number of PCR cycles, Y-axis denotes amount of Fluorescence.

but also because its clinical presentation can mimic other conditions that cause conjunctivitis [11]. The current study aimed to accurately diagnose Adenovirus from conjunctival swab samples collected from clinically suspected cases of viral conjunctivitis using real-time PCR and viral cell culture.

The age group distribution, gender distribution and clinical features in this present study were consistent with findings from separate studies conducted by Singh MP et al., Sundaramurthy R et al., and Gopalkrishna V et al., [5,6,12]. In the study population, the majority of cases were young adults (ages 18-45) and most cases were male 27 (60%), which can be attributed to their increased outdoor activities and, consequently, a higher risk of exposure. The symptomatic observations revealed that lacrimation (100%) and red conjunctiva (97.7%) were the most prevalent symptoms, consistent with the findings of the study conducted by Beniwal N et al., [18].

The present study found PCR positivity in 8 samples (17.7%), while adenoviral recovery via cell culture was 13.3%, similar to the study conducted by Goudarzi H et al., [19]. This study showed a 75% adenoviral cell culture recovery rate from PCR-positive samples (6 out of the eight PCR-positive samples), which is comparable to the study conducted by Yağci R et al., [10].

In the present study, it was noted that only six of the eight PCR-positive samples could be identified using viral culture. Two samples that tested positive by PCR were not detected in the viral culture, despite the fact that culture is regarded as the reference test. The confirmatory PCR from the viral culture medium for these two samples was negative. These two samples exhibited CPE, which could be due to the presence of other viruses in the samples, such as Coxsackie, Enterovirus, or Herpes. Results of other similar studies as compared to the present study is depicted in [Table/Fig-9] [5,6,10,12,19].

The diagnostic techniques for conjunctivitis include PCR, direct immunofluorescence and rapid antigen-detection immunoassays. Although viral cell cultures of conjunctival samples are less common due to the requirement for sophisticated equipment and skilled laboratory personnel and because they take longer to yield results, they can be useful in confirming the presence of adenovirus using immunofluorescence. The most accurate standard approach for diagnosing viral conjunctivitis is PCR, as many studies and research investigations have demonstrated [20].

Outbreaks of adenoviral conjunctivitis occur in healthcare facilities, educational institutions and communities. With the application of PCR testing, the adenovirus can be promptly identified as the causative agent, allowing infection control measures to be implemented to prevent the virus from spreading. By providing information on the prevalence and epidemiology of adenoviral conjunctivitis, PCR testing aids in surveillance efforts. Public health officials can use this information to monitor trends, implement preventative measures and organise responses to outbreaks.

| Study | Sample type | Place of study | PCR positivity | Cell culture positivity |
|---|---|---|---|---|
| Yağci R et al., [10] | Conjunctival swabs | Turkey | 26.5% (9/34) | 20.6% (7/34) |
| Goudarzi H et al., [19] | Conjunctival swabs | Iran | 15.7% (18/115) | 12.76% (14/115) |
| Gopalkrishna V et al., [5] | Conjunctival swabs | Pune, India | 60.9% (14/23) | - |
| Singh MP et al., [6] | Conjunctival swabs | Chandigarh, India | 65.2% (15/23) | - |
| Sundaramurthy R et al., [12] | Conjunctival swabs | Puducherry, India | 70.5% (24/34) | 67.6% (23/34) |
| Present study | Conjunctival swabs | Bengaluru, India | 17.7% (8/45) | 13.3% (6/45) |

**[Table/Fig-9]:** The table shows results of other similar studies as compared to the present study [5,6,10,12,19].

## Limitation(s)

The present study did not include the detection of other causative agents of viral conjunctivitis.

## CONCLUSION(S)

The present study suggests the presence of adenovirus causing conjunctivitis among patients attending a tertiary care centre in Bengaluru. Real-time PCR has become a standard diagnostic procedure for the detection of adenoviral conjunctivitis. With real-time PCR, adenoviral infections can be accurately detected, preventing misdiagnosis that could otherwise spread the infection. This method provides medical professionals with additional information, enabling them to utilise cutting-edge treatments and make informed clinical decisions.

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