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Original Article

Investigational approach to adenoviral conjunctivitis: comparison of three diagnostic tests using a Bayesian latent class model

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Abstract

Introduction: Highly contagious adenoviral conjunctivitis represents 15-70% of all conjunctivitis worldwide. Human adenovirus (hAdV) serotypes 3,4,7,8,19 and 37 contributes to 89% of all adenoviral conjunctivitis. Accurate and rapid diagnosis of adenoviral infections at serotype level could prevent misdiagnosis, spread of disease, unnecessary antibiotic use and increased treatment costs.

Methodology: Sixty-two suspected viral conjunctivitis cases were recruited from November2013-January2015. Swabs collected from inferior palpebral conjunctiva and processed for viral culture (Hep2 cell line), immunofluorescence assay (IFA) and polymerase chain reaction (PCR) (targeting hexon gene). Serotype 3,4,7,8,19 and 37 identification was carried out with an optimized multiplex-PCR (based on hypervariable region of hexon gene) and confirmed by sequence analysis. Bayesian Latent Class Model (LCM) analysis was used to compare sensitivity and specificity of three tests.

Results: Adenovirus was detected in 54.8% (34/62) of cases by combination of all three methods. Culture was positive in 23/34 cases (67.6%). PCR and IFA detected adenovirus in 24 (70.5%) and 21 (61.7%) cases respectively. LCM analysis revealed, sensitivity and specificity of PCR, Culture and IFA was 77.8% and 92.4%; 72.2% and 90.8%; 67.6% and 92.9% respectively. Serotyping by multiplex-PCR showed, two cases each were hAdV3 and hAdV4, 18 hAdV8 and two remained unidentified. Results of Multiplex-PCR and sequence analysis showed 100% concordance

Conclusion: LCM analysis revealed, PCR is the most appropriate method for identification. Multiplex-PCR is a simple and rapid method (serotypes identification within two days); owing its short turnaround time and accuracy, it can be used as a diagnostic tool for surveillance of adenoviral keratoconjunctivitis.

Key words: adenovirus; keratoconjunctivitis; mPCR; immunofluorescence assay; viral culture; Bayesian latent class model.

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Introduction

Viral conjunctivitis is one of the most commonly encountered ocular infections. Human adenoviral conjunctivitis represents 15-70% of all conjunctivitis cases worldwide [1]. Human adenoviruses (hAdVs) belong to the family of *Adenoviridiae* under the genus of *Mastadenovirus,* which contains seven known hAdV subgroups (A, B, C, D, E, F and G) based on their ability to agglutinate with various red blood cells. Among the 68 known types of adenoviruses, serotypes 3, 4, 7, 8, 19 and 37 account for up to 89% of all adenoviral conjunctivitis cases [2]. Ocular adenoviral infection may present itself as pharyngoconjunctival fever (serotypes 3 and 7), epidemic keratoconjunctivitis (serotypes 4, 8, 9, 19 and 37) or acute hemorrhagic conjunctivitis (serotypes 3, 7, 11 and 25) [3]. Conjunctivitis caused by serotypes 8, 19 and 37 is more severe and can spread faster than the one caused by other serotypes [4–7]. The propensity of adenoviral conjunctivitis to cause outbreaks and visual impairment makes identification of adenovirus essential so that preventive measures are initiated as early as possible [8]. Serotype identification of adenovirus is critical for epidemiological surveillance, detection of new strains and understanding the pathogenicity of the virus [5-8].

Traditionally, viral culture has been considered as the gold standard for the identification of adenovirus in any clinical sample, and the diagnostic accuracy of all other methods is evaluated considering the viral culture sensitivity and specificity as 100%. Even though adenoviruses are stable, the detection rate of adenovirus by culture can be affected by various factors like pH

alterations, temperature changes and concentration of viral particles in the sample, as well as transport medium properties and viability of the virus during transport. Additionally, some adenoviruses are fastidious in nature and will not grow in culture [9]. Since the sensitivity and specificity of cell culture has been assumed to be perfect (100% sensitivity and specificity), which may not be true, the accuracy of the different diagnostic tests which have been evaluated against the culture may has not been accurately estimated. Studies have demonstrated the utility of Bayesian latent class models (LCM) in estimating the true sensitivity and specificity of alternative tests when the reference test is imperfect [10-12].

This study was carried out (i) to compare the results of immunofluorescence assay (IFA), PCR and viral culture using the Bayesian LCM model of analysis to detect adenovirus in conjunctival swabs and (ii) to optimize multiplex-PCR so as to identify serotypes 3, 4, 7, 8, 19 and 37 and confirm the results with sequence analysis.

Methodology

Study design

A total of 62 suspected viral conjunctivitis cases (sample size based on our previously registered data) from the ophthalmology outpatient department of Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER) during the period November 2013 to January 2015 were included in the study. Patients with clinical diagnosis of herpetic keratitis, bacterial or fungal corneal ulcer and viral conjunctivitis superimposed with bacterial infections were excluded. Institutional ethical committee clearance was obtained (JIP/IEC/SC/2O13/5/458 dated 13/02/14) before sample collection.

Specimen collection and transport

Conjunctival swabs were collected from the infected patient's inferior palpebral conjunctiva using a commercially available, sterile, dacron swab without applying any local anaesthetics. Swabs were dipped in a commercially available viral transport medium (Hi-Media, Mumbai, India) validated for both cell culture and PCR and transported in an ice box. An informed written consent was obtained from each patient prior to sample collection. Collected samples were immediately transported to the laboratory and further processed for viral culture and IFA analysis. Upon arrival, each specimen in the viral transport medium was vortexed intermittently for 1 minute, then aliquoted in two sterile eppendorf tubes and centrifuged at 10000 rpm for 30 minutes at 4°C. The supernatant was used for viral culture and molecular assays and the sediment (pellet) was used for indirect immunofluorescence staining. Furthermore, samples were stored at -70°C for PCR analysis.

Viral culture

Five hundred µL of supernatant from each sample were inoculated into a tissue culture flask with an appropriate, healthy, Hep-2 cell line which had a confluence of ≥ 70% and was devoid of any cytoplasmic granulation and rounded cells (Figure 1a). Inoculated cell lines were incubated in a CO_2 incubator at 37°C for 30 minutes for enhanced adsorption. Then, 5 mL of maintenance medium were added and the mixture was incubated in a CO_2 incubator at 37°C and observed daily for up to 7 days for the characteristic

Figure 1.a) Hep-2 cell line used for adenovirus isolation; **b)** Hep-2 cell line inoculated with sample and showing cytopathic effects (grape like cluster) suggestive of adenovirus infection.

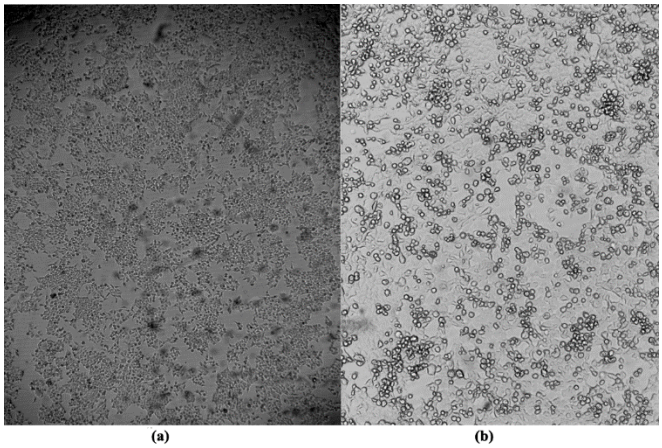
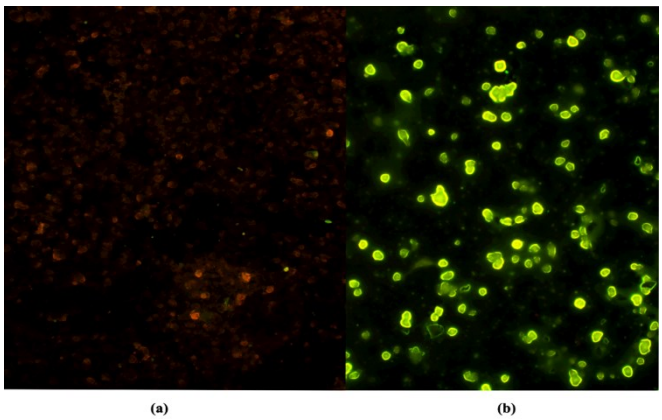


Figure 2. IFA from viral culture fluid for detecting adenoviral infection -demonstrating **a)** negative IFA results (reddish hue); **b)** Positive IFA results (green fluorescence) from provisionally detected viral culture fluid.

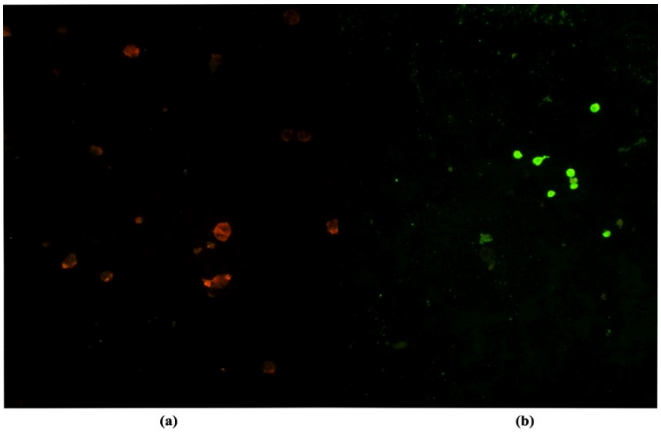


cytopathic effect (grape-like cluster of cells) of adenovirus (Figure 1b). Provisional detection of adenovirus in the viral culture was confirmed by IFA using primary pan adenoviral antibodies and secondary FITC labeled antibodies (Figure 2). If no cytopathic effects were observed within 7 days, a blind passage was performed in a new Hep-2 cell line and the inoculate was observed for seven more days. Only then it was reported that the viral culture from the sample was negative for adenovirus [11]. Even though the virus can disseminate and patients remain infectious for about 2 weeks, the virus can be detected by conventional culture usually in the first 7 days and then the detection rate drops down to 25% by day 10, and at 5-10% by day 14 [13].

Indirect immunofluorescence staining

Antigen detection is one of the most frequently used methods for the rapid identification of adenoviral infections. The commercially available test Rapid Pathogen Screening (RPS) Adeno Detector (Rapid Pathogen Screening Inc., South Williamsport, USA), which is based on the conserved hexon region, has been used in earlier studies [23,25] however we were not able to use it due to local non-availability of the kit. Instead, we performed an alternative antigen detection method i.e an indirect immunofluorescence assay (IFA) which can be more specific than direct IFA. For adenoviral antigen detection by indirect IFA we used a pan adenoviral primary antibody against the hexon region (mouse anti-adenovirus monoclonal antibody), in 1:50 dilution and a secondary goat anti-human antibody labeled with fluoresce in isothiocyanate (FITC) in 1:50 dilution with Evans blue counterstain. Both antibodies were purchased from Chemicon International Millipore Corporation (Temecula, California, USA). Cells from the pellet of the centrifuged sample were placed on the designated spot, left to air dry, and subsequently fixed with 100% cold acetone (HiMedia Laboratories Pvt. Ltd, Mumbai, India). After the addition of 1% BSA (HiMedia Laboratories Pvt. Ltd, Mumbai, India) as the blocking agent, cells were washed with PBS and 10 µL of primary antibody were added, followed by an incubation at 37˚C for 1 hour in a moist chamber. After washing the slide three times, for 5 minutes, with PBS,10 µL of the secondary FITC-labeled antibody was added in the presence of Evans blue counterstain (HiMedia Laboratories Pvt. Ltd, Mumbai, India) followed by an incubation at 37˚C in a moist chamber for 30 minutes. After a final wash with PBS, cells were observed under the fluorescence microscope [14]**.** Cells showing green fluorescence either in the cytoplasm or in the nucleus were considered as positive (Figure 3a), while cells showing reddish hue due to the Evans blue counterstain used for the FITC-conjugated antibody dilution were considered as negative for the presence of adenoviral antigen (Figure 3b).

Figure 3. a) Indirect IFA from direct sample (pellet) showing negative IFA results (reddish hue); **b)** Positive IFA results for adenoviral infection(green fluorescence in cytoplasm and nucleus).



PCR procedure

Viral DNA was extracted from 200 µL of the supernatant fluid using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) as per the manufacturer's instructions. The semi-nested PCR was performed in order to simultaneously have two amplification products in order to increase the sensitivity of the detection. A reverse and a forward genus-specific universal semi-nested outer primer (able to identify all the adenovirus subgroups, A-G) along with one semi-nested forward primer, were used in the reaction. The outer forward and outer reverse primers will amplify 150 bp long products from the target and at the same time, the semi-nested forward primer and the outer reverse primer will amplify 100 bp long products. PCR for adenovirus was performed using primers targeting the conserved hexon region (Outer F-5'CTGTGGTCGACT TGCAAGAC3' Outer R-5'ACCGCAGAGTTCCACATACT3' and semi-nested F 5'-ACATGCACATCGCCCGTGAC-3'), provided by HELINI Biomolecules, (Chennai, India) as per the manufacturer's instructions. The cycling conditions were: 95°C for 5 minutes for initial denaturation followed by 30 cycles of denaturation at 95°C for 30 seconds; annealing at 56°C for 30 seconds; extension at 72 °C for 45 seconds and a final extension step at 72°C for 10 minutes. Amplified fragments of 105-130 bp along with an internal control fragment of 400 bp were indicative for the presence of adenovirus (Figure 4).

Samples that were tested positive by the semi-nested PCR were further processed for identification of serotype by an in-house multiplex-PCR. Serotypes 3, 4, 7, 8, 19 and 37 were detected with primer design based on the hypervariable region of the hexon gene (Table 1). The reaction mixture contained 0.3 pmole of each primer, 2.5mM concentration of $MgCl_2$,1.5 Units per reaction of Taq polymerase and 50ng to 250ng in 10uL volume of DNA . The reaction was carried out in an Eppendorf Master Cycler Gradient thermocycler (Wesseling-Berzdorf, Germany) with a final reaction mixture volume of 25µL. Cycling conditions were 95°C for 5 minutes for initial denaturation followed by 38 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 45 seconds and a final extension step at 72°C for 10 minutes. Amplicons (serotype 3, 150 bp; serotype 4, 230 bp; serotype 7, 250 bp; serotype 8, 550 bp; serotype 19, 103 bp and serotype 37, 322 bp) were detected by agarose gel (2.8%) electrophoresis in the presence of ethidium bromide (Figure 5). Serotypes identified by multiplex-PCR were outsourced for sequencing (Xcelris Genomics, Ahmedabad, India).

Statistical analysis - Bayesian latent class model

The absolute accuracy of each diagnostic test was evaluated using Bayesian LCMs [12,15]. In brief, Bayesian LCMs assess the accuracies of various diagnostic tests based on the actual disease status. Bayesian LCMs do not assume any diagnostic test as a perfect test. LCM hypothesizes the existence of one or more unobserved categorical latent variable (multiple positive diagnoses) to explain the relationships between a set of observed categorical variables (test's results-positive and negative). In the LCM model, a posterior probability of the infections of interest was estimated for each possible test combination and if the posterior probability was > 0.50, subjects were classified as infected [16].

Three-tests-in-one-population –Walter and Irwing simplified interfaces web-based model (web server R, and WinBUGS programs- http://mice.tropmedres.ac),

Figure 4. Universal semi-nested PCR for identifying adenovirus showing amplified fragments in the 130 bp area (sample 6-10) and no amplification product for negative samples (samples 1-5), the internal control (IC) band at 400 bp and the appropriate positive (PC) (130 bp) and negative (NC) (no band) controls.

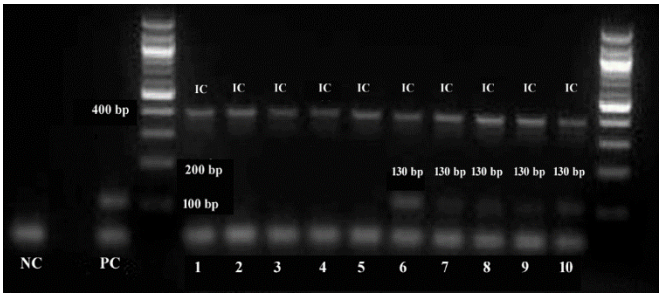


Figure 5. Multiplex PCR image for identifying adenovirus showing amplified fragments for serotype 8 (550 bp) (samples 4,5,10,11,12), serotype 3 (150 bp) (sample 9), serotype 4 (230 bp) (sample 7) and the corresponding positive (PC) and negative controls (NC).

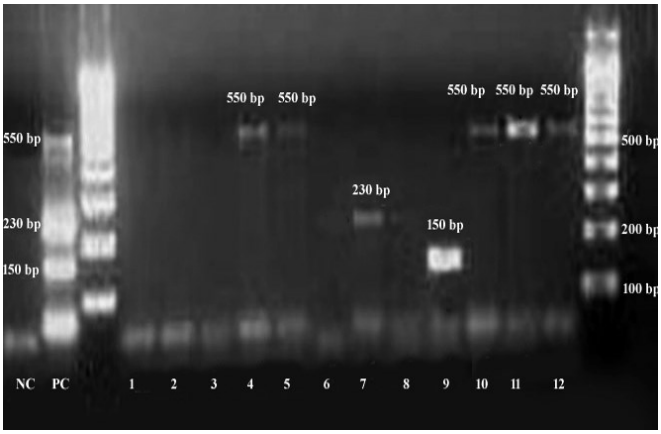


Table 1. Multiplex-PCR primers used for the identification of the most common adenoviral serotypes with their product size in base pairs (bp).

Serotype	Primers	Product Size (bp)
Serotype 3	F- TGCACCTACTATGAGACAAGGG R-GACATGAAGTTGCTGGAGAAGG	150bp
Serotype 4	F-GGTGGTGGACGAGGTTAACTA R-GACATGAAGTTGCTGGAGAAGG	230bp
Serotype 7	F-ACATTACTGCAGACAACAAGCCC R-CTCCTCAGCTTCAACATCTCCTTC	250bp
Serotype 8	F-TTTGTTTACTCGGGCACCATC R-GACATGAAGTTGCTGGAGAAGG	550bp
Serotype 19	F-CTCTGGTACCAATGCTGCCTA R-GTTACGATCTGCGACTTTGGTATC	103bp
Serotype 37	F-AGGAACTGGAGCAGAAAAAGATGTTAC R- GTATTGAGGATCGGTACCATTGGG	322bp

have been used in various studies to analyze the accurate sensitivity and specificity of various tests when a reference test is imperfect [10,11,15-17]. We have also used the Walter and Irwing-three tests in one population model to analyze our results from viral culture, IFA and universal semi-nested PCR.

Results

Data Summary

We have examined 62 suspected viral conjunctivitis cases; the majority of the patients were males (35/62, 56.4%) with a mean age of 38 ± 19 years. Most cases (57/62, 93.5%) presented within four days of onset of symptoms with, predominantly, single eye involvement (92%). Chemosis was the most common clinical sign of presentation (51.6%) followed by pseudomembrane (50%) and hyperaemia (48.4%). The cornea was involved in one-third of the patients. Similarly, one-fourth of the patients had ipsilateral preauricular lymphadenopathy.

Overall, adenovirus was detected in 54.8% (34/62) of the total suspected cases. Viral culture was positive in 23 patients (67.6 %); Figure 1a represents the viral culture showing normal, healthy Hep-2 cells and Figure 1b shows the Hep-2 cell line inoculated with clinical material showing positive cytopathic effects (grape like clusters). All positive for adenovirus viral culture results were further confirmed by IFA using primary pan adenoviral antibodies and secondary FITC labeled antibodies. Figure 2 represents IFA from provisionally detected adenovirus culture fluid; Figure 2a represents IFA results from adenovitus-negative culture fluid (red hue due to Evans blue) and Figure 2b represents IFA from adenovirus-positive culture fluid (green fluorescence of both cytoplasm and nucleus due to FITC).

Indirect IFA detected adenoviruses in 21 (61.7 %) cases. Figure 3a represents the indirect IFA from direct sample (pellet) showing negative IFA (red hue due to Evans blue) and Figure 3b represents IFA positive (green fluorescence of both cytoplasm and nucleus due to FITC) from direct sample.

PCR detected adenoviruses in 24 (70.5 %) cases. Figure 4 represents the semi-nested PCR in identifying adenovirus showing amplification fragment in the 130 bp area (sample 1-5) and no amplification for negative samples (samples 6-10) with internal control at 400 bp and satisfactory positive (130 bp) as well as negative control (no band). Detection of adenovirus from the sample by various combinational tests (Table 2).

Table 2. Number of samples positive for adenovirus by various combinational modalities.

Various methods	No. of positive samples
All three methods (PCR, culture, IFA)	11
PCR & culture	5
PCR & IFA	4
Culture & IFA	3
PCR alone	4
Culture alone	4
IFA alone	3
Total positive	**34**

Bayesian latent class model analysis

The absolute accuracy of each diagnostic test was evaluated using Bayesian LCMs using three-tests-in-one-population model (Walter and Irwig model) simplified interfaces (Table 3).

Serotype distribution

Of the 24 semi-nested PCR positive samples, 8 were positive by PCR but negative by culture; serotyping by multiplex-PCR showed that 18 were hadV-8 (75%), two were hadV-3 (8.33%), another two were hadV-4 (8.33%), while the two remaining strains could not be identified by serotype-specific multiplex-PCR. Figure 5 represents the multiplex-PCR image in identifying adenovirus showing amplification fragments for serotype 8 (550 bp), serotype 3 (150 bp), serotype 4 (230 bp) with their corresponding satisfactory positive and negative controls.

Table 3. Diagnostic accuracy of each test by Bayesian Latent Class Model analysis with their confidence intervals.

Parameters	Bayesian latent class model*(CI)**
Prevalence	44.2 (24.2 - 63.2)
Viral culture	
Sensitivity	72.2 (50.2 - 95.3)
Specificity	90.8 (73.1 - 99.9)
PPV	86.3 (53.0 - 99.9)
NPV	80.2 (57.8 - 97.6)
Universal semi-nested PCR	
Sensitivity	77.8 (56.0 - 98.6)
Specificity	92.4 (73.5 - 100)
PPV	89.2 (54.9 - 100)
NPV	84.0 (60.4 - 99.3)
IFA	
Sensitivity	67.6 (46.7 - 93.3)
Specificity	92.9 (76.0 - 100)
PPV	88.2 (55.5 - 99.9)
NPV	78.4 (55.4 - 97.2)

* Bayesian latent class model assumed that all tests evaluated are imperfect; **Values shown in brackets are estimated median with 95% confidence interval; PPV: Positive predictive value; NPV: Negative predictive value.

Table 4. Time to positivity by viral culture for serotypes 3, 4, and 8.

	Serotype 8		Serotype 3	Serotype 4
Time to positivity (in days)	5-7	10	9-10	9-10
Number of Culture isolation Positive During this time period	12	2	All (2)	All (2)

Serotype identification by multiplex-PCR was confirmed by sequence analysis. Sequences were submitted to GenBank (GenBank accession no. KR150657-67 and KR084326 and 27**)** and were published online.

Time to positivity by viral culture

Twenty-threesamples were positive by viral culture. Time to positivity varied from 5 to 10 days (Table 4).

Discussion

Adenoviruses, one of the leading causes of infectious conjunctivitis, are stable against most of the physical and chemical agents of disinfection and survive for long periods in the environment [18]. Although the most frequent route of transmission is through patient's contaminated hands, healthcare-associated outbreaks through tonometry contact, eye drops and hands of healthcare workers have been also reported [19,20]. Clinical presentation of adenoviral keratoconjunctivitis may mimic conjunctivitis caused by other agents like HSV, enterovirus E70, coxsackie virus A24 and *Chlamydia trachomatis* [21]. Adenoviral conjunctivitis may lead to permanent visual impairment due to corneal involvement [8]. The propensity of adenoviral conjunctivitis to cause outbreaks as well as visual impairment while its clinical presentation mimics other conjunctivitis causing conditions makes early identification of the etiological agent essential, so that preventive measures are initiated since there is no effective treatment available.

In the study population, the majority of the cases were either young adults (18-35 years) or middle-aged individuals (36-55 years) with a mean age of 38 ± 19 years. The majority of the cases were males (56.4%) which can be attributed to their increased outdoor activities and hence increased risk of exposure.

In the present study, adenovirus was detected in 54.8% of the total suspected viral conjunctivitis cases. This is similar to the results of a study by Percivalle *et al.* [5], which reported an hAdV detection rate of 48.8%, while studies by Loseva *et al.* [22] and Maysaa El-Sayed Zaki *et al.* [23] have reported a higher adenoviral detection rate of 95% and 72.2% respectively. In contrast, lower viral detection rates were reported by Madhavan *et al*. (13.8%), Torres *et al.* (20%), Stevens *et al.* (26 %) and Nashwa Al-Kasaby *et al.* (29.2%) [2, 24–26]. The variation in the reported detection rate among different studies can be attributed to the time of sample collection, quality of samples, transport and storage conditions and diagnostic modalities used for viral detection. Comparatively, the higher detection rate observed in our study could be attributed to the fact that samples were collected early in the course of the disease as most of the patients presented within four days of the onset of symptoms and samples were processed immediately with minimal storage. Also, our study involved all three modalities for viral detection i.e. viral culture, antigen detection by immunofluorescence technique and PCR.

Of the 34 positive samples, 7 were positive by indirect IFA but negative by culture. Immunofluorescence-positive and culture-negative results can be attributed to the presence of non-viable viral particles and free viral antigen. Loseva *et al.* [22] have demonstrated the persistence of viral antigens over a period of 1-3 months in 22-30% of the cases even after clinical recovery. Also, inflammatory substances produced in response to the infection like interleukin 1, interferon γ, and tumor necrosis factor are known to interfere with viral culture growth [23]. Also, certain serotypes of adenoviruses like hAdV-8, exhibit poor culture recovery compared to other serotypes [27]. Similarly, 8 cases were detected by PCR but were negative by culture. Failure to detect adenovirus by culture, in PCR-positive cases, could be attributed to the higher sensitivity of PCR and that the method can detect even few copies of viral DNA and non-viable virus particles [3].

Accuracy of Viral culture

Traditionally, the diagnosis of most of the viral diseases has relied on the isolation of the virus from cell culture. Even though the cell culture approach is slow and requires considerable technical expertise it has been regarded as the gold standard for virus detection. Due to the development of rapid tests for the detection of viral antigens or viral nucleic acids, the usefulness of the viral culture has been questioned. The isolation of viruses by cell culture depends on various factors such

as selection of the appropriate cell line, proper collection of samples to contain the highest possible viral titer, and viral infectivity preservation until cell culture inoculation by transporting the samples at 2 to 8°C or on wet ice. All these factors can directly impact the viral recovery rate and thus affect the sensitivity of the viral culture that has been traditionally considered to be the gold standard or reference test [9]. When the reference test is imperfect, the sensitivity and the specificity of an alternative test can be determined by Bayesian LCM statistical analysis which does not assume any test as perfect [13,14]. In this study, the actual sensitivity and specificity of the viral culture, as determined by Bayesian LCM statistical analysis, was 72.2% and 90.8% respectively, although they are traditionally considered to be 100%. Our analysis is comparable to that of Sobotzki *et al*., who applied LCM to analyze the true sensitivity of a combined set of tests for pertussis, adenovirus, and influenza A and B in a large population of adult patients with respiratory disease and cough and reported that LCM is a well-documented and statistically valid tool in the absence of a gold standard test [12].

Accurate sensitivity and specificity of IFA and PCR based on LCM

Antigen detection by immunofluorescence is one of the most extensively used rapid methods for the identification adenoviral keratoconjunctivitis with sensitivity ranging from 60% to 90% and specificity from 75% to 100% [5,21,25,28-30]. In our study, the sensitivity and specificity of IFA by LCM was 67.6% and 92.9% respectively. Our results are in concordance with the findings of Aoki and Tagawa *et al*. [30] who reported the sensitivity and specificity of IFA to be 67% and 78.6% respectively.

Molecular methods have emerged as an important tool in the laboratory diagnosis of viral infections. In our study, we used a PCR based on the conserved hexon region and designed to identify all adenoviral serotypes except 40 and 41. Our study revealed that of detection of adenovirus from conjunctival samples by PCR had 77.8% sensitivity and 92.4% specificity. Both the sensitivity (true positive) and the specificity (true negative) of the semi-nested PCR was higher than that of the viral culture in identifying adenovirus from the conjunctival swab. This is in concordance with the findings of Kinchington *et al.* [31], who reported sensitivity and specificity of PCR in detecting hAdV from the conjunctival sample as 79% and 97% respectively.

We observed that out of the 24 PCR-positive samples, only 16 were detected by viral culture. Even though viral culture is considered to be the reference test, eight samples that were positive by PCR were not identified by culture. The PCR products of these 8 samples were sequenced and their analysis revealed they all belonged to adenovirus.

Serotype distribution

The identification of adenovirus serotype is critical for epidemiological surveillance, detection of new strains and understanding pathogenesis. Up till now, 68 hAdV types [32] have been identified. Among these, serotypes 3, 4, 7, 8, 19 and 37 are more frequently associated with conjunctivitis, accounting to approximately 90% of all adenoviral conjunctivitis cases [1]. Many serotype identification techniques are available like neutralization technique, multiplex-PCR, sequence analysis and PCR-RFLP [33]. We have optimized multiplex-PCR to identify the most common serotypes associated with keratoconjunctivitis (serotypes 3, 4, 7, 8, 19 and 37) based on the hypervariable region of the hexon gene of adenovirus. In our study, among the 24 semi-nested PCR-positive samples, 8 isolates were from the pre-epidemic period, and the remaining 16 were isolated during the epidemic. Among the strains isolated during the pre-epidemic period; two were serotype 3, two were serotype 4, two were serotype 8 and the remaining two strains could not be identified by serotype-specific multiplex-PCR. All the 16 strains isolated during the epidemic period were identified as serotype 8 by serotype-specific multiplex-PCR.

The specificity of multiplex-PCR was verified by sequence analysis of the amplified by semi-nested PCR products. The sequence analysis results were in 100 % concordance with the serotype identification results of multiplex-PCR. The method is rapid as the serotype could be identified within two days and simple since no post-PCR processing is required. However, our multiplex-PCR did not identify two isolates that were detected by semi-nested PCR. This may be due to the fact that the isolates were not among the six serotypes (3, 4, 7,8, 19 and 37) identified by our multiplex-PCR. These two strains were also detected by IFA using adenoviral pan antibody; however, the viral cultures for these samples were negative. This could be attributed to non-viability of the strains in the sample which however gave a positive result with PCR and IFA.

When comparing culture results across the 6 investigated serotypes, it was observed that all serotype 3 and 4 casesgave positive viral culture results, whereas

out of the 19 serotype 8 cases ,5 were negative by viral culture. This could be due to the fastidious nature of adenovirus serotype 8 well known for its poor culture recovery [27].

Conclusion

In our study we have compared the sensitivity and specificity of three diagnostic tests used for the identification of the etiologic agent in cases of keratoconjunctivitis. Understanding the limitations of viral culture as the gold standard for adenoviral detection, we have analyzed the results of the three different diagnostic tests by Bayesian LCM statistical analysis which does not presume that any test is perfect. Analysis by LCM revealed that PCR is the most appropriate method for identifying adenovirus. The most common causes of adenoviral keratoconjunctivitis in our population were hAdV-8, hAdV-3 and hAdV-4. Serotype identification by multiplex-PCR was in 100% concordance with sequence analysis results and was simple and rapid as serotype could be identified within two days. Accurate and rapid diagnosis of adenoviral infections with serotype specificity will prevent the misdiagnosis that leads to the spread of disease, unnecessary antibiotic use and increased health care costs and allows clinicians to be more informed regarding treatment decisions and use of novel therapeutics.

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Author's Contribution

RD, SK, BNH has given the concepts and intellectual content; RS designed the study; RS, RD carried out the literature search; RS, SK have collected and analysed the data; RS, RD, SK, BNH carried out the statistical analysis; RS done the manuscript preparation; RD, SK have done the manuscript editing; RD, SK, BNH carried out the manuscript review.

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