Etiology of Acute Conjunctivitis Due to Coxsackievirus A24 Variant, Human Adenovirus, Herpes Simplex Virus, and Chlamydia in Beijing, China

Jie Li¹, Yongsheng Yang², Changying Lin¹, Weihong Li¹, Yang Yang¹, Yong Zhang³, Lei Jia¹, Xitai Li¹, Lijuan Chen¹, and Quanyi Wang¹*

¹Institute for Infectious Disease and Endemic Disease Control, Beijing Center for Disease Prevention and Control, Beijing, 100013; ²Optic Nerve Department, Eye Hospital of China Academy of Chinese Medical Sciences, Beijing, 100040; and ³Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing, 102206, People’s Republic of China.

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SUMMARY: Acute conjunctivitis is a common disease associated with high morbidity and economic burden. To clarify the etiological characteristics of acute conjunctivitis in Beijing, surveillance of acute conjunctivitis was conducted from July to October during 2007–2012 by collecting eye swabs from patients treated at surveillance hospitals affiliated with a surveillance program of 18 districts Center for Disease Prevention and Control in Beijing. Coxsackievirus A24 variant (CA24v), enterovirus 70 (EV70), human adenovirus (HAdV), herpes simplex virus (HSV), and chlamydia were identified by PCR. Phylogenetic analysis of the VP1 region of CA24v was conducted. Comparisons of proportions and statistical significance were performed using the chi-square test. HAdV was found to be the most prevalent pathogen, followed by CA24v, chlamydia, and HSV. Significant differences in the symptoms of ocular pain, photophobia, and epiphora were identified among the 4 agents. The prevalence of HAdV- and CA24v-mediated conjunctivitis peaked in July or August and September or October, respectively. Nucleotide sequences of the VP1 regions among the isolated CA24v strains shared 92.8–100% homology. In conclusion, HAdV followed by CA24v, chlamydia, and HSV were the most common causative agents of acute conjunctivitis in Beijing. Comprehensive, continuous surveillance and advanced laboratory techniques are needed for further studies.

INTRODUCTION

Conjunctivitis is described as any inflammation of the conjunctiva, generally characterized by irritation, itching, foreign body sensation, and watering or discharge. It commonly occurs worldwide due to infection by viruses, bacteria, or, in some cases, chlamydia. This infection is usually mild and resolves spontaneously within 2–5 days in most cases without treatment. However, complications that threaten sight can arise and can be associated with significant morbidity and economic burden.

Previous reports have shown that cases of conjunctivitis comprise approximately 2% of cases treated by general practitioners (1,2). Approximately 20%–70% (3–5) of infectious conjunctivitis is thought to be of viral etiology, which has been reported to account for 8%–75% of acute cases (6–8). Several previous studies have reported that approximately 65%–90% of viral conjunctivitis cases in Japan and Pakistan in the 19th and 20th centuries were caused by human adenovirus (HAdV) (8–10). Herpes simplex virus (HSV) is regarded as the leading infectious cause of corneal blindness among developed nations (11). A previous study estimated the incidence rates of HSV eye disease ranging from approximately 4 to 13 new cases per 100,000 persons (12). For more than 40 years, coxsackievirus A24 variant (CA24v) has cocirculated with enterovirus type 70 (EV70) worldwide as the causative agents of acute hemorrhagic conjunctivitis (AHC), a highly contagious eye disease. As a major pathogen, CA24v was first isolated from an outbreak of 60,000 reported cases of AHC in Singapore that occurred in 1970. CA24v epidemics were initially limited to India and Southeast Asian before 1985. Since then, CA24v outbreaks have spread worldwide (13–16). Chlamydia is also a causative agent of conjunctivitis that can cause focal corneal scarring, neovascularization, and chronic inclusion conjunctivitis, which may lead to otitis media and pneumonitis in neonates. Previous data have shown an increase in the annual incidence of adult inclusion (chlamydial) conjunctivitis. Moreover, chlamydia has become the most frequent identifiable cause of neonatal conjunctivitis in many countries and the incidence has continued to rise annually (17,18).

Previous reports have revealed that the prevalence of conjunctivitis varies by season, with age, and among geographical areas. However, few studies have been conducted on this subject and most were limited to only one agent. Thus, there is relatively little information available on this disease. The aim of this study was to
identify the etiological and epidemiological characteristics of conjunctivitis caused by CA24v, EV70, HAdV, HSV, and chlamydia to obtain further information about this disease in order to propose effective preventive measures to control the disease in the future.

METHODS

Clinical definition: Conjunctivitis, which is also referred to as red eye, is any inflammation of the conjunctiva, generally characterized by conjunctival congestion, irritation, itching, foreign body sensation, and watering or discharge.

Sample collection: A total of 28 surveillance hospitals of 18 districts in Beijing, China, participated in this surveillance program from July to October each year during 2007–2012. The surveillance system was designed and managed by Beijing Center for Disease Prevention and Control (CDC). The surveillance included patients with acute conjunctivitis diagnosed by ophthalmologists in the surveillance hospitals. From each hospital, 1–5 outpatients with acute conjunctivitis were randomly selected to participate in this study. At each clinic, trained personnel obtained eye swab samples from these patients with prior written informed consent. Obtained samples were stored in minimum essential media (MEM) in plastic containers at −70°C until analyzed.

Sample processing, nucleotide extraction, and virus identification: The specimens stored in MEM were vortexed violently and centrifuged at 4,000 g for 10 min prior to nucleotide extraction. Total nucleotide extraction was performed using a Roche MagNA Pure LC 2.0 nucleic extraction system (Roche Diagnostics Ltd., Rotkreuz, Switzerland) with the MagNA Pure LC Total Nucleic Acid Isolation Kit–Large Volume (Roche Diagnostics), according to the manufacturer’s instructions.

HAdV, HSV, and chlamydia were identified using a PCR kit (Qiagen, GmbH, Hilden, Germany) according to a previously described method using 3 pairs of primers (19). CA24v and EV70 were identified using a real-time one-step RT-PCR kit (Qiagen) using 2 pairs of previously described primers (20). Complete nucleotide sequences of VP1 genes of CA24v-positive specimens were amplified using a one-step RT-PCR kit (Qiagen) with specific primers as described previously (14,21). PCR products of 915 bp were purified and sequenced using an ABI PRISM310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Phylogenetic analysis: Sequence analysis of 915 bp of the complete gene of the VP1 region was performed using Molecular Evolutionary Genetics Analysis software (version 5.0; MEGA 5). Phylogenetic trees were constructed using the neighbor-joining method with bootstraps of 1,000 replicates. Sequences of CA24v strains isolated in Beijing were aligned and compared with the corresponding regions of the 35 worldwide CA24v strains available in the GenBank database.

Statistical methods: Statistical analysis was performed using SPSS 11.5 software (IBM SPSS Inc., Chicago, IL, USA). Comparisons of proportions and statistical significance were performed using the chi-square test. A P value of <0.05 was considered significant.

Ethics statement: This study was conducted in compliance with the Declaration of Helsinki, and the protocol was approved by the Human Research Ethics Committee of Beijing CDC. Patients or their guardians, as appropriate, submitted written informed consent before sample collection.

RESULTS

Distribution of different pathogens linked to conjunctivitis: A total of 962 conjunctivitis cases, which included 552 males and 410 females (male:female ratio = 1.35:1) aged 5 months to 106 years were enrolled in this study from July to October each year during 2007–2012. Among the 962 specimens, 49 (5.09%) eye swabs were positive for CA24v. HAdV, HSV, and chlamydia were not identified among the specimens collected in 2008 and 2009 for unknown reasons. Among the 888 specimens collected in 2007 and 2010–2012, excluding those from 2008 and 2009, single infections were observed in 49.89% (443/888) of cases with a male:female ratio of 1.46:1 and mixed infections were detected in 2.36% (21/888) with a male:female ratio of 0.61:1. The results of the remaining 47.75% (424/888) of cases were negative for infection. The results showed that 42.23% (375/888) of eye swabs were identified as HAdV-positive, 4.95% (44/888) as CA24v-positive, 3.38% (30/888) as HSV-positive, and 4.05% (36/888) chlamydia-positive (Table 1).

Mixed infections: As shown in Table 2 of the 888 specimens collected in 2007 and 2010–2012, 21(2.36%) were positive for mixed infections. Five different combinations of infectious pathogens were observed, of which the most common was HAdV and chlamydia (n = 8), followed by HAdV and HSV (n = 5), CA24v and HSV (n = 4), CA24v and HAdV (n = 3), and CA24v and chlamydia (n = 1). As shown by the data presented in Table 3, the patients aged 20–39 years comprised the highest proportion (47.62%) of cases among all age groups. With regard to gender distribution of mixed infections, according to the manufacturer’s instructions.
infectious cases, the male:female ratio was 0.61:1. A significant difference in gender distribution was identified between the single and mixed infection groups \((P = 0.047)\). A symptom of conjunctival congestion comprised the highest proportion (100.00\%) of mixed infection cases followed by the symptom of eye discharge (88.23\%). No significant differences in clinical manifestation were identified between the single and mixed infection groups.

**Clinical features of admitted patients infected with different pathogens:** Symptoms, such as eyelid swelling, ocular pain, conjunctival congestion, eye discharge, cauma, photophobia, and epiphora, were evenly distributed among infections with the above mentioned agents. As shown in Table 4, symptoms of conjunctival congestion (91.12\%) and eye discharge (81.00\%) were the most common, followed by ocular pain (63.13\%), photophobia (60.75\%), epiphora (59.88\%), foreign body sensation (25.87\%), and itching (13.50\%). Significant differences were observed in the prevalence of symptoms of ocular pain \((P = 0.042)\), photophobia \((P = 0.002)\), and epiphora \((P = 0.002)\) among the 4 infectious agents. Acute conjunctivitis associated with HSV infection showed the lowest rate of ocular pain (46\%), photophobia (42.31\%), and epiphora (42.31\%), whereas the highest rates (80\%, 73.33\%, and 73.33\%, respectively) were associated with chlamydial infection. Significant differences were observed in the prevalence of symptoms of ocular pain \((P = 0.008)\), photophobia \((P = 0.019)\), and epiphora \((P = 0.019)\) between patients with HSV and chlamydial infections, but no differences were observed in the prevalence of symptoms of foreign body sensation \((P = 0.150)\), conjunctival congestion \((P = 1.000)\), eye discharge \((P = 0.200)\), or itching \((P = 0.087)\).

**Trends of pathogenic agents:** The temporal distributions of infectious agents are presented in Fig. 1. Regarding CA24v, the positive rate increased in July (1/281, 0.36\%) and August (6/268, 2.24\%), peaked in September (27/224, 12.5\%), and then decreased in October (9/108, 8.33\%). The highest positive rate of HAdV was observed in July (133/281, 47.33\%) and then declined from August (124/268, 46.27\%) to September (77/224, 34.38\%) and attained the lowest point in October (35/108, 32.41\%). The positive rate of HSV infection initially increased from July (8/281, 2.85\%) to August (11/268, 4.10\%), decreased in September (5/224, 2.23\%), and then peaked in October (6/108, 5.6%). The highest positive rate of chlamydial infection was observed in July (13/281, 4.63\%), decreased in August (9/268, 3.36\%) to September (6/224, 2.68\%), and then increased in October (4/108, 3.70%).

**Sequence comparison and phylogenetic analysis of the CA24v VP1 region:** The phylogenetic relationships of the VP1 regions among the CA24 isolates worldwide were analyzed, resulting in 4 major clusters (lineages 1–4). Lineage 1 contained a CA24v sequence isolated in 1970, lineage 2 contained isolates from 1987, lineage 3 contained isolates from 1993 to 1998, and lineage 4 contained isolates from 2002 to 2012 (Fig. 2). The CA24v isolates in this study were all located in lineage 4. Our result showed that VP1 sequences obtained in this study shared 82.9%–85.3% nucleotide identity and 94.6%–95.3% amino acid identity with EH24/70 strains, and 86.8%–88.2% nucleotide identity and 95.6%–98.3% amino acid identity with strains isolated in Brazil and Jamaica in 1987. Comparisons with strains isolated in the USA in 1993 and Dominican Republic in 1998 revealed a nucleotide identity of 91.1%–92.1%, corresponding to an amino acid identity of 98%–98.7%. Isolates in this study shared 93.7%–99.9% nucleotide identity and 97.7%–100% amino acid identity with strains isolated in other countries and regions from 2002.
Infectious conjunctivitis is a very common disease among all age groups and is characterized by many clinical presentations depending on whether the infection is due to viruses, bacteria, or parasites. The most important outcome of this study was the description of the 5 etiological agents (CA24v, HAdV, EV70, HSV, and chlamydia) associated with acute conjunctivitis in outpatients of all ages in Beijing. HAdV is the most common pathological agent of conjunctivitis, followed by CA24v, chlamydia, and HSV. No EV70-mediated conjunctivitis was identified from 2007 to 2012 in Beijing. Under the auspices of the surveillance system in Beijing, the study period covered 6 years and 962 outpatients to determine the prevalent agents of acute conjunctivitis in Beijing. Using a combination of real-time RT-PCR and PCR, 54.62% of specimens demonstrated an infection with at least one agent among CA24v, HAdV, HSV, and chlamydia. The positivity rate implied that other agents may also be responsible for the cause of this disease. It is well known that bacteria, including Staphylococcus aureus and Streptococcus pneumoniae, as well as many allergens can also cause acute conjunctivitis. When CA24v as a single causative agent of AHC was taken into account, the positive rate in this study was 3%. The prevalence of CA24v in Beijing has been associated with low morbidity since 2007. As the third most common causative agent of acute conjunctivitis, the positive rate of HSV was 3.38%, which was in accordance with the rate of 4.3% in a previous report from Japan (32). Young et al. (12) reported that the likelihood of developing ocular HSV in the state of Minnesota (USA) from 1976 to 2007 was 0.23% by the age of 30 years, 0.49% by 50 years, and 0.94% by 80 years. However, this phenomenon was not observed in this study. Other than the 3 main pathogenic viruses, chlamydia was also detected using collected eye swabs. The prevalence of chlamydia in this study (4.05%) was similar to that in a previous study (2.5%) conducted in Japan from 1990 to 1994 (9). However, there was much higher prevalence in other countries, such as India, in Korea, and affects approximately 1 million people in Japan per year (9,23). In this study, the positive rate of HAdV was 42.23%, which is consistent with the prevalence of 49% throughout East Asia in the 1980s (24). However, the prevalence of HAdV in this study was lower than that reported in Japan during 2005–2006 (82%) (25) and Pakistan (75%) (26), whereas there was a much lower prevalence in other countries, such as the USA (17.16%) (27) and Mexico (24.39%) (28). It is well known that CA24v is highly contagious, especially due to the incredible mobility of populations worldwide (29). Several recent CA24v outbreaks have been reported in south China (Guangdong province in 2010 and Yunnan province in 2007) (30,31) and throughout the world (Egypt in 2010; Brasil in 2003, 2004, and 2005; Africa in 2003 and 2004; and Spain in 2004) (13–16,29).

HAdV is an important causative agent of acute conjunctivitis in many countries and the primary cause of viral conjunctivitis in East Asia, including Japan and
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which the antigen positivity of chlamydia varied between 22\% and 28\% of patients attending an eye care hospital (33).

Co-infection is a common phenomenon among many infectious diseases. Different modes of co-infection in conjunctivitis, such as mixed viral infections and mixed infections of bacteria and virus, have been reported previously (34,35). In this study, a total of 21 co-infections were observed. HAdV was the most frequently identified agent of conjunctivitis and the most common agent in combination with chlamydia and HSV, which may be due to the high positive rate of HAdV infections in Beijing. Although the positive rate of acute conjunctivitis caused by chlamydia or HSV in this study was not very high, the positive rates of chronic conjunctivitis caused by HSV and chlamydia in Beijing were reportedly 30.9\% and 23.4\%, respectively (34,36). This situation may contribute to the high incidence of co-infection with HAdV and HSV or chlamydia. Among case with mixed infections, patients aged 20–39 years, as with single infection cases, comprised the highest proportion among all age groups, which may be due to the increased exposure time and frequency of contact with the source of infection. A significant difference in gender distribution was identified between cases with single and mixed infections ($P = 0.047$). The low positive rate of
single infections among females compared with males may have been due to better hygiene habits. However, females had a higher positive rate of mixed infections, which may be related to differences in immunocompetence. No significant difference was observed in clinical manifestations between single and mixed infections.

In this study, the prevalence of CA24v, HAdV, HSV, and chlamydia was based on specimens obtained over 4 months each year in a 6-year period. From the limited data in this study, we deduced that different agents were associated with different regularities of epidemic status. Epidemics of HAdV and CA24v-mediated acute conjunctivitis was distinct, with peaks in July and September, respectively, which was in agreement with the results of previous studies (37). The high temperature and suitable humidity during the summer months may play important roles in the transmission of CA24v and HAdV. However, because of the limited number of positive cases and limited surveillance period, we were unable to accurately define trends for HSV and chlamydial infection.

In this study, some symptoms were significantly associated with specific agents ($P < 0.05$). For example, ocular pain, photophobia, and epiphora were more common in chlamydial infection. However, there were no significant correlations among other symptoms, such as foreign body sensation, conjunctiva congestion, and eye discharge with CA24v, HAdV, HSV, and chlamydia conjunctivitis. The result of this study revealed that although there were differences in clinical presentation, there were also some overlaps. Marango et al. (37) found that the clinical and laboratory correlation in ocular viral disease was $< 90\%$. Because of the considerable overlap in presentation, infectious conjunctivitis is difficult to distinguish clinically. The results of the present study were in agreement with those of previous reports (38).

Comparisons of VP1 sequences have been widely used in epidemiological studies of enteroviruses. A previous study identified 4 CA24v genotypes by phylogenetic analysis of VP1 regions of the genome (39). In this study, each CA24v strain was isolated from local residents of Beijing and analyzed on the basis of VP1 sequences. VP1 phylogenetic trees were divided into 4 lineages chronologically. CV24v isolates obtained each year from 2007 to 2011 were all located within one cluster, and the CA24v isolates 206/Beijing/China/2012 and 295/Beijing/China/2012 were clustered with the strains isolated in 2008 and 2007, respectively. In general, CA24v isolates of each year from 2007 to 2011 were clustered chronologically in one lineage of the tree. Isolated CV24v strains from 2007 to 2012 in this study were all gathered in one lineage. A nucleotide divergence of $0\%$–$4.5\%$ of the VP1 region was found among the isolated CA24v strains, suggesting that CA24v-associated acute conjunctivitis in Beijing has a common source.

In conclusion, infectious conjunctivitis is a very common disease in Beijing, and HAdV, followed by CA24v, HSV, and chlamydia were identified as the most common agents of acute cases. A limitation of this study is that data of acute conjunctivitis due to HAdV, CA24v, EV70, HSV, and chlamydia were collected from July to October inclusively, as the surveillance period did not span the entire year. Because of the insufficient data in this study, we were only able to draw limited conclusions. Hence, more data are needed regarding the prevalence of these agents over the entire year to better describe risk factors of infection, conjunctivitis due to other agents, and serotypes of known agents to implement efficient control programs and reduce the negative impact on the individual and the high cost of health care to society.

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Conflict of interests None of declare.

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