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ORIGINAL ARTICLE

Outbreak of acute respiratory disease caused by human adenovirus type 7 in a military training camp in Shaanxi, China

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ABSTRACT

Outbreaks of ARD associated with HAdV have been reported in military populations in many countries. Here, we report an ARD outbreak caused by HAdV-7 in a military training camp in Shaanxi Province, China, from February to March of 2012. Epidemic data and samples from the patients were collected, and viral nucleotides from samples and viral isolations were detected and sequenced. IgG and IgA antibodies against HAdV, and the neutralization antibodies against the viral strain isolated in this outbreak, were detected. Epidemiological study showed that all personnel affected were males with an average age of 19.1 years. Two peaks appeared on the epicurve and there was an 8-day interval between peaks. Laboratory results of viral nucleotide detection carried out with clinical specimens were positive for HAdV (83.33%, 15/18). Further study through serum antibody assay, virus isolation and phylogenetic analysis showed that HAdV-7 was the etiological agent responsible for the outbreak. IgA antibody began to appear on the 4th day after the onset and showed 100% positivity on the 8th day. The virus strain in the present outbreak was highly similar to the virus isolated in Hanzhong Shaanxi in 2009. We conclude that HAdV-7 was the pathogen corresponding to the outbreak, and this is the first report of an ARD outbreak caused by HAdV-7 in military persons in China. Vaccine development, as well as enhanced epidemiological and virological surveillance of HAdV infections in China should be emphasized.

Key words China, human adenovirus type 7 (HAdV-7), military training camp, outbreak.

Human adenoviruses (HAdV), belonging to the family Adenoviridae and the *Mastadenovirus* genus, are associated with a broad spectrum of clinical diseases, including uncomplicated acute respiratory and gastrointestinal infections in humans, especially in children and immunodeficient individuals (1). HAdV are non-enveloped particles with linear double-stranded DNA. On the basis of biological properties and DNA sequence homology, more than 65 types of HAdV have been characterized and classified into seven species (A–G) (2, 3). HAdV species B

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List of Abbreviations: ARD, acute respiratory disease; CCID50, 50% cell culture infective dose; CPE, cytopathic effect; HAdV, human adenovirus; IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M; MTC, military training camp; SXCDC, Center for Disease Control and Prevention of Shaanxi Province.

is classified into two subspecies B1 (types 3, 7, 16, 21, and 50) and B2 (types 11, 14, 34, and 35). Subspecies B1 cause respiratory infections, whereas subspecies B2 is usually associated with kidney and urinary tract infections but sometimes also associated with respiratory diseases (4, 5).

Shaanxi province, located in northwestern China, has had a few epidemic outbreaks of HAdV in recent years. HAdV-11a (HAdV-55) was reported in high school students in 2006, while HAdV-7 was reported in infants in 2009 (6, 7). In present study, we report a HAdV-7 outbreak in a military training camp that affected about 200 soldiers. This is the first report about a HAdV outbreak in military recruits in China.

MATERIALS AND METHODS

Outbreak description

Since 21 November 2011, military training was started in a MTC in Shaanxi Province, China. There were a total of 625 personnel in the MTC including 549 recruits (509 male and 40 female) in 46 squads of five companies, and 76 support personnel in a tutor company. On 13 February 2012, three recruits from the same squad were reported ill with a body temperature of approximately 38.5°C. Eleven days later, this outbreak was reported to the SXCDC. Definition of an ARD case was given as an individual with a body temperature over 37.8°C and with at least a respiratory symptom such as cough or sore throat. Recruits who were randomly distributed in the MTC, but did not have any symptoms were chosen as healthy controls. Until 8 March, 2–20 ARD cases per day were reported over an almost 4-week period, and 176 patients were confirmed as having ARD. On 25 February, an epidemical survey for every patient was carried out and precautionary measures including separation, environmental disinfection and morning checks were implemented, and no new case was reported since 8 March.

Specimen collection

On 25 and 26 February, 18 new cases were discovered and in order to investigate the causative pathogen, swab specimens from these 18 new cases were collected aseptically in 3-mL viral transport medium and transported to SXCDC. From these, eight and 10 specimens were collected on 25 and 26 February, respectively. Eighty serum samples from available persons who had been reported as having ARD since 13 February were also collected on 26 February. On 7 March, 30 paired serum samples were collected again. Throat swabs and serum samples were collected from 12 healthy controls. Sera and throat swab samples were stored at -20 and −80°C, respectively, until further analysis.

Extraction and detection of viral nucleic acids from throat swabs

Viral nucleic acids were directly extracted from throat swab specimens using a QIAamp MiniElute Virus Spin Kit (Qiagen, Valencia, CA, USA). Viral RNA was reverse transcribed by a first-strand cDNA synthese kit (Fermentas, Vilnius, Lithuania). PCR was carried out using the Seeplex RV 15 ACE Dection system (Seegene, Seoul, Korea). This system can detect 14 types of RNA virus (including influenza A and B virus, human respiratory syncytial virus A and B, human metapneumovirus, human parainfluenzavirus 1, 2, 3, 4, human rhinovirus A, B, C, and human coronavirus 229E/NL63 and OC43, human bocavirus1/2/3/4, and human enterovirus) and a DNA virus (HAdV), responsible for most respiratory diseases.

HAdV typing from samples

DNA from the clinical specimens were subjected to HAdV-specific PCR on a BioRad thermal cycler (C1000; BioRad, Singapore) by using primer pairs ADSD/AD52 as described by Zhen, and the amplicon was targeted on the *hexon* gene of HAdV (7). The amplicons were detected by 2% agarose gel. After purification, PCR products were sequenced directly by the dye terminator method (BigDye Terminator, version 3.1, cycle sequencing kit; Applied Biosystems, Austin, TX, USA) using the same primers on an ABI 3500XL genetic analyzer (Applied Biosystems). Nucleotide sequence homology was inferred from the identity scores obtained using the BLASTn program (National Center for Biotechnology Information, Bethesda, MD, USA).

Cell culture and virus isolation

Throat swabs of patients positive for HAdV-DNA by PCR were separately inoculated into HEp-2 cells and cultured in a maintenance medium (minimal essential medium containing 2% FCS, 100 U/mL penicillin G, 100 μg/mL streptomycin) at 37°C in a closed system in a 5% CO_2 incubator. Cultures exhibiting an adenovirus-like CPE were passaged again to confirm the presence of the virus. Primary identification of positive isolates was done by PCR with adenovirus-specific primers.

Neutralization test

A HAdV strain (HAdV SXWN1205) was used as the neutralization virus, and CCID50/50 μL was calculated as described previously (8). Stored serum samples were inactivated at 56°C for 30 min, filtered through a

0.22-μm filter membrane, and diluted with the maintenance medium. A total of 50 μL of several dilutions (1:2–1:256) of serum samples and 50 μL of the viral antigen diluted in CCID50 of 100 were added to the wells of a 96-well microtiter plate. The contents were mixed well, and the plates were incubated for 2 hr in an open system in the presence of 5% CO_2. The HEp-2 cell suspension was then added to each well. Positive and negative controls were carried out. The plate was incubated again in the open system in the presence of 5% CO_2, and the CPE was observed daily.

Sequencing of *hexon* gene of the virus isolates and molecular analysis

To sequence the *hexon* gene of viral isolates, DNA was extracted from cell culture supernatant using the QIAamp Viral DNA minikit (Qiagen, Hilden, Germany) and the entire *hexon* gene was amplified by specific primers with three overlapping regions (Table 1). The amplified products were sequenced in both directions, directly or after cloning into a pGEM-T Easy vector (TA Cloning Kit; Invitrogen, Leek, The Netherlands). Analysis of nucleotides and predicted amino acid sequence was carried out by using the BioEdit program (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) (9). Nucleotide and deduced amino acid identities were calculated by using the DNA Star program (DNASTAR, Madison, WI, USA). The reference sequences were retrieved from GenBank (www.ncbi.nlm.nih.gov/Genbank).

Antibody against HAdV in serum tested using ELISA

HAdV IgA was detected from 80 patient sera collected from the acute phase and 12 healthy control serum samples by using an ELISA Classic adenovirus IgA kit (Institute Virion/Serion GmbH, Würzburg, Germany). HAdV IgG was also detected from 30 paired sera of IgA-positive samples in the acute period and 12 healthy control serum samples. All these kits enabled the detection of serum antibodies against all types of HAdV that are human pathogens.

Ethics

Written informed consent from all patients was obtained and this research was permitted by the ethics committee of the School of Medicine, Xi'an Jiaotong University (reference number 2012–0222).

Statistical analysis

All statistical analyses were carried out using SPSS 13.0 (SPSS, Chicago, IL, USA). Categorical variables were compared by χ^2-test or Fisher's exact test. Non-categorical variables were compared by the Mann–Whitney *U*-test. A *P*-value <0.05 was considered significant. All statistical operations were two-tailed.

RESULTS

Outbreak description and clinical features of the patients

In this ARD outbreak, the index cases were three men in the same squad with a body temperature of over 38.5°C accompanied by a sore throat. A total of 176 patients were involved and all the patients were males aged 16–34 years; no females were infected. The outbreak lasted for 24 days and the distribution of daily cases is shown in Figure 1. Patients were reported in all six companies. The company with index cases had the most reported cases (24.43%, 43/176) and the tutor company had seven reported cases (3.98%). The case distribution in dormitory rooms showed clustering characteristics; the number of dormitories with one patient in each room was 36; and those with two, three and four patients in each room were 34, 12 and nine, respectively.

Clinical features of the patients are summarized in Table 2. All infected individuals had fever, and most of them had sore throat or tonsillitis. Eleven were sent to hospital and others were observed and treated on the spot. Common symptoms for inpatients were cough and sore throat, and most of them had dyspnea (63.64%). In non-hospitalized cases, conjunctivitis (1.71%), abdominal pain (1.14%), diarrhea (0.57%), and vomiting (0.57%) were also observed.

Table 1. Primers used for hexon sequencing in the present study

Primer	Sequence (5′–3′)	Position	Amplicon size (bp)
ADV-7-hexon-1F	GCGCCGTCGCTGCTATTAATTAAAT	18,186–18,210	1265
ADV-7-hexon-1R	GTCCACAGCCTGATTCCACATGC	19,450–19,428	
ADV-7-hexon-2F	GTGGTTGACTTGCAGGACAGA	19,347–19,367	1194
ADV-7-hexon-2R	GCATTGGGCCACATTGTATCC	20,540–20,520	
ADV-7-hexon-3F	AGTCAGCTGGCCTGGCAATG	20,466–20,487	902
ADV-7-hexon-3R	AAAGCCAGCCAGTGCTCTCC	21,368–21,349	

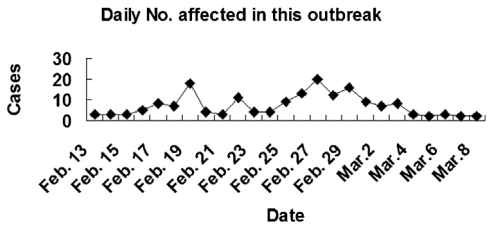

Fig. 1. Case distribution in a military training camp during an outbreak of HAdV-7.

Serological assays

HAdV IgA antibody in the serum samples from 80 affected soldiers in the acute phase and from 12 healthy individuals were examined by ELISA Classic adenovirus IgA kit (Institute Virion/Serion GmbH). A total of 40 (50.00%) samples from patients and two samples (16.67%) from the control group were positive for IgA. IgA antibody increased as the disease progressed and, in the first 3 days of infection, IgA antibody was negative but on the 9th day all cases became positive (Table 3). The proportion of samples positive for HAdV IgA was significantly higher for the patients than for the controls ($P < 0.05$). Thirty serum samples from patients positive for IgA were further determined during convalescence 11 days after onset. From paired serum samples ($n = 30$), 29 were positive for IgG in the acute phase, whereas 30 were positive in the convalescence period. From control samples, two were positive in both acute and convalescence periods. The neutralization antibody against HAdV SXWN1205 was detected from 30 paired sera, in which 11 samples showed more than a fourfold increase, 11 showed a twofold increase, five had no change, and three decreased. The neutralization antibody of two IgA-positive samples from the control group showed more than a fourfold increase (Fig. 2).

Screening of viral nucleic acids from throat swabs

From eight throat swab samples collected on 25 February, six samples were positive for HAdV-DNA and two were positive for parainfluenza virus RNA. From 10 swab samples collected on the following day, nine were positive for HAdV-DNA. Sequence analysis revealed that all the 15 amplicons positive for HAdV-DNA had 99.9% similarity with HAdV-7 strain (GU230898), which was isolated in 2009 from Hanzhong, Shaanxi.

Molecular analysis of the HAdV strain

Throat swab samples positive for HAdV nucleic acid by PCR ($n = 15$) were separately inoculated into Hep-2 cells, and a characteristic adenovirus-like CPE was observed for 12 samples. These isolated viral strains were further confirmed by specific PCR. The entire *hexon* gene (3116 bp) of three strains (Genbank accession number KC689913–KC689915) was highly identical with each other (99.9–100%).

Phylogenetic analysis based on the entire *hexon* gene of isolated strains in this study as well as 17 viral strains representing the seven HAdV species and 29 strains belonging to HAdV B showed that isolates from this study belonged to HAdV-7 and had high homology with the HAdV HZ/SHX/CHN/2009 (GU230898) strain (Fig. 3) (6).

Table 2. Characteristics, clinical signs, and symptoms of 176 patients in the present study outbreak

		Unhospitalized	Hospitalized	
Characteristics	All (176)	$n = 165$	$n = 11$	*P*-value
Age (mean, years)	19.11	19.10	19.22	0.860
Median duration (days)	3 (1–13)	3 (1–9)	7 (4–13)	<0.001
Signs and symptoms				
Fever				
Duration, days (range)	3 (0–9)	2 (0–6)	5 (3–9)	<0.001
Median peak temperature, °C (range)	39.0 (37.8–41.2)	38.9 (37.8–41.2)	39.4 (38.7–40.2)	0.036
Sore throat	150 (85.22)	139 (84.24)	11 (100.0)	<0.001
Exudative tonsillitis	132 (75.00)	123 (74.50)	9 (81.80)	0.012
Cough	22 (12.50)	11 (6.67)	11 (100.0)	<0.001
Coryza	12 (6.82)	9 (5.50)	3 (27.30)	<0.001
Dyspnea	7 (3.98)	0 (0)	7 (63.64)	<0.001
Conjunctivitis	3 (1.71)	3 (1.82)	0 (0)	0.820
Abdominal pain	2 (1.14)	2 (1.21)	0 (0)	0.880
Diarrhea	1 (0.57)	1 (0.61)	0 (0)	0.940
Vomiting	1 (0.57)	1 (0.61)	0 (0)	0.940

Values are no. (%) patients, except as indicated.

Table 3. IgA antibody assay progress

Days after onset	No. cases	Adenovirus ELISA-IgA	Positive rate (%)
0	5	0	0
1	11	0	0
2	4	0	0
3	3	0	0
4	8	2	25.00
5	3	1	33.33
6	2	1	50.00
7	17	11	64.70
8	6	5	83.33
9	8	8	100.0
10	4	4	100.0
11	3	3	100.0
12	2	2	100.0
13	2	2	100.0

DISCUSSION

The present study describes an outbreak of febrile respiratory illness (176 affected) in a military training unit in Shaanxi, China, in early spring of 2012. From the epidemical survey and laboratory examination, the etiological pathogen of the reported outbreak was confirmed as HAdV-7.

HAdV are classified into more than 60 types belonging to seven species. HAdV subspecies B1 (types 7, 3, 21) and species E (type 4) cause outbreaks in military and civilian communities (10). Stress, fatigue, and crowding are important factors facilitating the transmission, and increase the susceptibility of different diseases in a military training unit (11, 12). Many outbreaks have been reported in military units in the USA, the Netherlands and Portugal (13–16). This is the first report of an outbreak with a definite pathogen from a Chinese military training camp and indicates the need for close surveillance in the future. Adenoviral respiratory disease has been recognized as a frequent cause of illness in US active duty military populations, particularly in basic training installations, for more than several decades (17, 18). In 1971, the US army started a vaccination program against HAdV types 4 and 7 (17, 19) that was ceased in 1996–1999 (20). In the vaccination period, there were only five reported adenovirus (HAdV-4 and -7)-associated deaths. In the post-vaccine period (1999–2010), eight HAdV-associated deaths have been reported (21). After a 12-year absence, the HADV vaccination program has been resumed for military recruits since October 2011 (22). Vaccination programs for Chinese military recruits include vaccination against hepatitis B, measles, tetanus and *Neisseria meningitides*. HAdV vaccines are unavailable in China now, and indigenous HAdV vaccines should be developed from the lessons of this outbreak.

A series of HAdV outbreaks have been reported from Shaanxi province recently (6, 7). In March 2006, the causative pathogen of an acute respiratory outbreak in Qishan county, Baoji prefecture, was found to be HAdV-11A, later renamed HAdV-55 (3). During this infection, 254 people including 247 senior high school students were infected, which resulted in the death of one patient who had bone marrow megaloblastic anemia (7). In

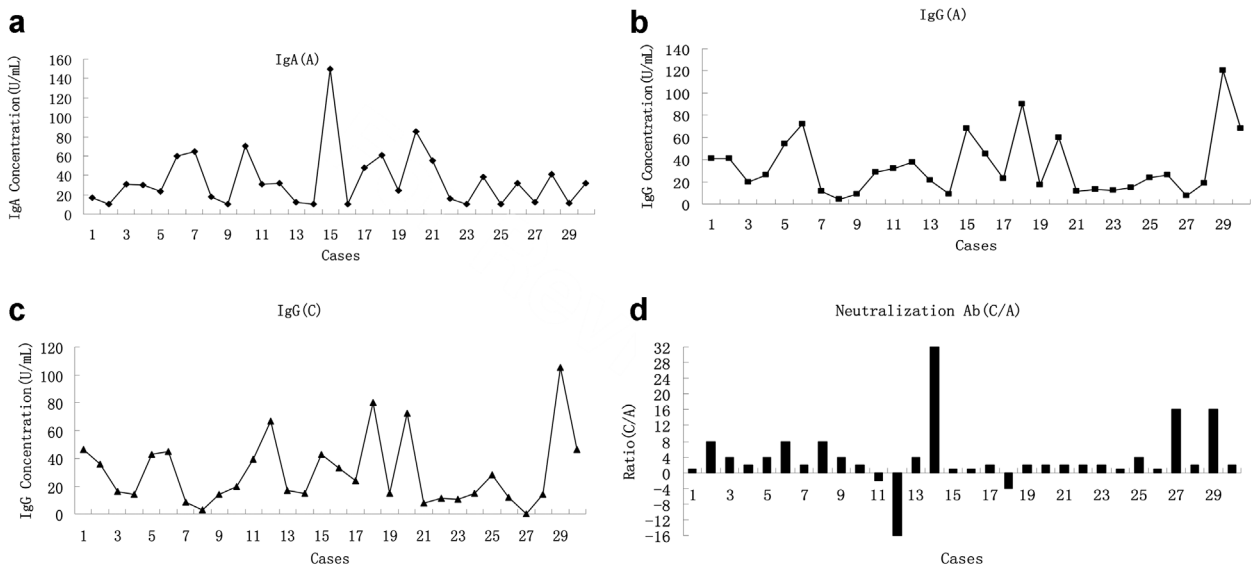

Fig. 2. Antibody concentration against HAdV in 30 paired sera. (a) IgA in the acute phase; (b) IgG in the acute phase; (c) IgG in the convalescence phase; and (d) neutralization antibody titers in the convalescence phase *vs* the acute phase.

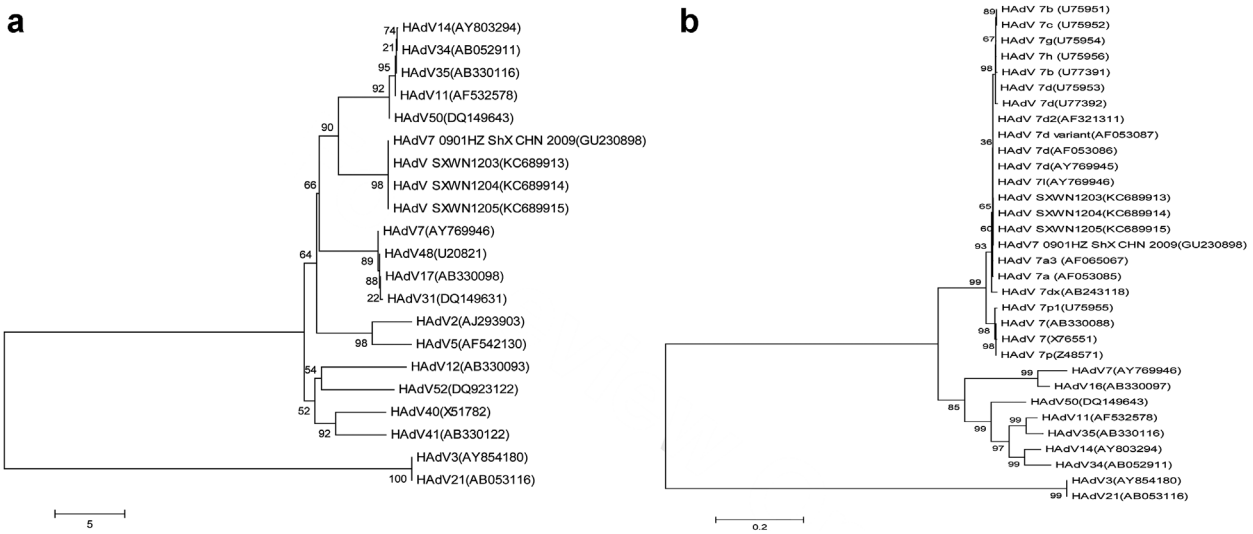

Fig. 3. Phylogenetic analysis of the entire *hexon* gene for strains described in the present study and other reference strains of HAdV. Mega 5.1 was used to construct the phylogenetic trees by using the neighbor-joining method with 1000 bootstrap replicates. Numbers in parentheses are GenBank accession numbers. (a) Strains in the present study with the strains in the A–G groups of adenovirus and (b) the strains in the present study with other reference strains belonging to HAdV B.

February 2009, an outbreak of severe lower respiratory tract disease in infants in Xixiang county, Hanzhong prefecture, located in the southwest part of Shaanxi province, was due to HAdV-7 (6). In July and August 2009, an outbreak of HAdV-3 infection caused pharyngo-conjunctival fever in 67 pupils while another outbreak of HAdV-4 infection caused acute upper respiratory tract disease in 11 students (15–18 years old) in Xi'an, located in central Shaanxi (data not published). The isolated strains of HAdV-7 in Shaanxi ($n = 4$, three from this outbreak and one from Hanzhong in 2009) showed high similarity in sequences (99.8–100%) and indicated the possibility of the same ancestor. It is interesting that several types of HAdV are circulating in Shaanxi province which indicates the need for a surveillance network for adenovirus infections.

Different HAdV-7 genome types have predominated in different areas and 13 (7p, 7a–7l) genome types have been found (23). The prototype strain Gomen of HAdV-7p was isolated in an outbreak of ARD among new military recruits in California in 1955, while 7a was isolated in 1958, 7b from Paris in 1956, 7c from South Africa in 1958, 7d from China in 1980, 7e from Brazil, 7f from the former Soviet Union, 7g from China, 7h from South Africa, 7i from Korea in 1999, 7k from Israel in 1968, 7l from Korea in 1996, and 7m has recently been mentioned (24–33). Both epidemiological and molecular evidence strongly suggest that unique patterns of genome type shifts are restricted to geographic areas. Therefore, surveillance for HAdV genome types in China should be strengthened.

The IgA antibody plays an important role in the diagonosis of HAdV infection (6, 7). As we know that IgM antibody predominates in early immune responses, this is always used as an early diagnosis for infection. However, Kornadt *et al.* found that in adenoviruses infections, fewer antibody rises and weak reactions were observed in the IgM test than in the IgA test. A test for IgM antibodies was found to be unrewarding and IgM antibodies were present in individuals without fresh HAdV infection. Therefore, the IgM antibodies test was not suitable for the early diagnosis of HAdV infection (34). Previous reports from the 2006 and 2009 outbreaks have shown 83.3% and 35% IgA positivity rates, respectively. Similarly, serum samples from the acute phase in this study showed 50% IgA positivity. From 30 IgA-positive samples, 29 were also positive for IgG both in the acute phase and in the convalescence phase. IgG in the acute phase and in the convalescence phase showed no obvious change, whereas the neutralization antibody was significantly different in various phases. It was also noted that IgA against HAdV was negative in all samples in the first 3 days of disease onset, but all were positive on the 8th day. From 15 samples that were HAdV positive by PCR, IgA antibody was not present. It was noted that PCR could detect HAdV within 5 days from onset. Two individuals from the control group (apparently healthy) were also positive for IgA, indicating a possible non-apparent HAdV infection.

To confirm HAdV infection, the 30 paired sera titers of neutralization antibody against the viral strain isolated in this outbreak were detected. Although most (22/30) titers

of sera increased, in our study, three samples were found to be decreased. A decrease in neutralization antibody has been reported previously, but the reason is unknown (6). We assume that personal differences may play a role in this finding, but this needs to be studied in future work.

From the present study, it is concluded that the etiological agent of an outbreak in military recruits in Shaanxi province in the spring of 2012 was HAdV-7. The nucleotide sequence, virus isolation, IgA antibody, and neutralization antibody were all attributed to the HAdV infection. IgA antibody began to appear on the 4th day after onset and showed 100% positivity on the 8th day. The virus strain in this outbreak was highly similar to the virus isolated in Hanzhong prefecture, Shaanxi in 2009. Recent outbreaks of HAdV in Shaanxi province indicate that vaccine development, and enhanced epidemiological and virological surveillance of HAdV infections are necessary in Shaanxi as well as in China.

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DISCLOSURE

Authors declare that they do not have a commercial interest with any company or other association that might pose a conflict of interest regarding this work.

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