Original Article

Detection and Serotyping of Human Adenoviruses from Patients with Influenza-Like Illness in Mongolia

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SUMMARY: Human adenoviruses (HAdVs) are responsible for approximately 5%–10% of acute respiratory infections. The serotypes of commonly detected respiratory HAdV in Asian countries are diverse. However, there are no well-documented reports of circulating HAdV serotypes in Mongolia. Between January 2010 and May 2011, 1,950 influenza-negative samples from patients with influenza-like illness, including eye swabs from patients with eye symptoms, were screened for HAdVs, and 40 samples (2.1%) were positive for HAdVs. Among these 40 samples, 31 samples were positive for the hexon gene used in phylogenetic analysis, as determined by PCR. We identified 7 different serotypes. We constructed the phylogenetic trees of HAdV-B7 and HAdV-B3, the 2 most commonly detected serotypes in this study. All detected HAdV-B7 and -B3 Mongolian strains had identical sequences. HAdV-D8, known to be associated with epidemic keratoconjunctivitis (EKC), was detected from nasopharyngeal and eye swabs. There was no difference between the amino acid sequences of the hexon and fiber genes that may affect tissue tropism in Mongolian strains and those in EKC-causing strains.

INTRODUCTION

Human adenoviruses (HAdVs) are non-enveloped, double-stranded DNA viruses belonging to the genus Mastadenovirus and the family Adenoviridae (1). There are 52 serotypes that have been classified into 7 species (A–G) (2,3). Recently, full genome sequencing of HAdVs was performed, and some novel HAdVs (HAdV-D53, -D54, -B55, -D56, -C57, and -D58) were discovered through phylogenetic analysis (4–9). HAdVs cause variable serotype-specific diseases, such as acute respiratory infection, conjunctivitis, and gastroenteritis, and are responsible for approximately 5%–10% of acute respiratory infections (1). HAdV-C1, -C2, -B3, -E4, -C5, -C6, and -C7 are the most common serotypes that cause respiratory infections while HAdV-D8 is the one of the major causes of epidemic keratoconjunctivitis (EKC) (1).

The circulating respiratory HAdV serotypes vary both geographically and temporally among Asian countries. For example, HAdV-B3 and -B7 are the most commonly encountered serotypes in surveillance conducted from 1991 to 2007 in Korea (10), and HAdV-C2 and -B3 were common in Japan between 2000 and 2007 (11). HAdV-C, especially HAdV-C1 and -C2, were common in Malaysia between 1999 and 2005 (12), whereas HAdV-B3 was common in Taiwan in 2004 and 2005 (13). However, there are no available data on currently circulating HAdV serotypes in Mongolia. Between January 2010 and May 2011, HAdVs were detected in patients with influenza-like illness (ILI), and phylogenetic analysis of the hexon gene from these HAdVs was performed to determine which serotypes were circulating in Mongolia. To our knowledge, this is the first report describing the serotypes of HAdVs in Mongolia.

MATERIALS AND METHODS

From January 2010 to May 2011, a total of 6,774 nasopharyngeal or eye swabs were collected from patients who visited outpatient clinics and hospitals in Mongolia and were clinically diagnosed with ILI within 3 days of disease onset. ILI was defined as sudden onset of fever (>38.0°C) with cough or sore throat in the absence of other diagnoses. Nasopharyngeal swabs were collected from patients with ILI whereas eye swabs were collected from patients with ILI and eye symptoms, such as tear shedding, eyelid swelling, and festering. The swabs were placed into 2 ml of Hanks (–) balanced salt solution (pH 7.2) with bovine serum albumin (0.5%), penicillin (500 U/ml), streptomycin (500 µg/ml), and amphotericin B (Fungizone) (2.5 µg/ml). The specimens were transported under refrigeration with ice packs to the National Influenza Center, National Center of Communicable Diseases in Ulaanbaatar within 48 h of collection.

At least 22 samples in each month were randomly selected from among those samples that were collected from patients <4 years or >60 years of age and tested negative for influenza virus using real-time reverse transcription polymerase chain reaction (rt-RT PCR) (14). Selected samples were screened for HAdVs either using
the D<sup>1</sup> Ultra<sup>™</sup> DFA Respiratory Virus Screening & ID Kit (Diagnostic Hybrids, Athens, Ohio, USA) immunofluorescence assay together with influenza virus A, and B, or using multiplex rt-PCR with the FTD Respiratory 21 test (Fast-track Diagnostics, Luxembourg, Germany), which also detects influenza virus A, B, coronavirus NL63, coronavirus 229E, coronavirus OC43, coronavirus HKU1, para-influenza virus 1, 2, 3, and 4, human metapneumovirus A and B, rhinovirus, respiratory syncytial virus A and B, enterovirus, parechovirus, bocavirus, and Mycoplasma pneumoniae. The immunofluorescence assay was performed between January and October 2010, and the multiplex rt-PCR assay was performed between October 2010 and May 2011.

Samples identified as positive using the HAdV screening tests were serotyped by amplifying and sequencing loops 1 and 2 of the hexon gene. Briefly, the viral DNA of the HAdV-positive samples was extracted from the clinical samples using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), following the manufacturer’s protocol. Using Ex Taq (TaKaRa Bio Inc., Shiga, Japan), the first PCR was performed with hexon gene-specific primers HX5-3 and HX3-4 (15) and nested PCR was performed with Adhex-GT2F and ADHEX2R (16,17). Electrophoresis was performed in 1.0% agarose gel containing ethidium bromide, and bands were visualized under ultraviolet light. Following PCR, the cycle sequence reaction was performed using the BigDye Terminator Cycle Sequencing Kit (Applied BioSystems, Foster City, Calif., USA), and the sequences of loops 1 and 2 were analyzed by an ABI PRISM 3700 DNA Analyzer (Applied BioSystems) using loop 1-specific primers (S29 and S52) (15) and loop 2-specific primers (S51 and S53) (15). Loops 1 and 2 of the hexon gene contain hypervariable regions (HVRs) 1–6 and 7, respectively, which determine the antigenicity for the neutralizing antibody against each serotype (18). Therefore, the loop 1 and loop 2 sequences were analyzed to determine serotypes according to the phylogenetic relationships of these sequences with the reference strains in GenBank. To compare the fiber gene of HAdV-D8, fiber gene-specific PCR was also performed, and sequences were determined as described previously (19). The sequences were aligned, and phylogenetic trees were constructed by the maximum likelihood method using MEGA 5 software (20).

The sequence data obtained in this study is available in the GenBank database under accession numbers AB685342-AB685377.

Statistical analysis was performed by chi-square (χ<sup>2</sup>) analysis using PASW Statistics 17.0 (International Business Machines Corp., N.Y., USA), and P-values less than 0.05 were considered to be statistically significant.

## RESULTS

A total of 6,774 samples were tested using rt-RT PCR for influenza viruses, and 6,193 samples were negative. Among the 1,950 samples randomly selected and screened for HAdVs using either multiplex rt-PCR or immunofluorescence assay, 40 (2.1%; 95% confidence interval, 1.5–2.7) were HAdV-positive. There were 31 HAdV-positive samples with hexon genes that were amplified by the first or nested PCR.

As a result of phylogenetic analysis of the 31 HAdVs, 7 different serotypes belonging to 3 species of HAdVs were detected. Patient information and the serotyping results of the 31 samples used for serotyping are summarized in Table 1. Among them, 4 samples were eye swab samples from patients with ILI and eye symptoms (1 HAdV-C6, 1 HAdV-B7, and 2 HAdV-D8). The most common serotypes among patients with ILI were HAdV-B7, followed by HAdV-B3 and -D8, although there was no statistically significant difference in the frequency of these serotypes (P = 0.175). Among these 31 samples, there were 2 cases of co-infection with other respiratory viruses (1 with HAdV-B7 and influenza A virus, and 1 with HAdV-D8 and human metapneumovirus). The frequency distribution of the 31 HAdV-positive cases by month is shown in Fig. 1. Each serotype was detected sporadically, and no apparent seasonality was observed for any serotype.

HAdV-B7 and -B3 were the common serotypes in Mongolia and cause severe acute respiratory infection (21–25). Therefore, phylogenetic trees of the hexon gene for HAdV-B7 and -B3 were constructed in order to compare the phylogenetic relationship between Mongolian strains and those in other countries, which were available in GenBank (Figs. 2A and 2B). The Mongolian strains were located in the same cluster, and all Mongolian strains of both HAdV-B7 and -B3 shared identical sequences within each serotype. When Mongolian HAdV-B7 and -B3 strains were compared with those detected in other countries, Mongolian HAdV-B7 strains were clustered with other HAdV-B7 strains that
were detected in other parts of the world, even though Mongolian strains formed a single cluster in the phylogenetic tree. HAdV-B7 strains, the most common serotype in Mongolia, were more closely related to strains detected before 2000 in different parts of the world. These strains were distinct from recent Asian strains detected in Korea, China, and Japan in the 2000s. In addition, HAdV-B3 strains in Mongolia were more closely associated with the strains detected in Japan or Korea in the 1990s than the strains detected in Japan, Korea, Taiwan, and Germany in the 2000s. Among the 5 samples positive for HAdV-D8, 3 were nasopharyngeal swabs collected from patients with ILI. Generally, HAdV-D8 causes EKC, and there are a few reports of acute respiratory infection caused by HAdV-D8 (24). To determine whether Mongolian HAdV-D8 strains from patients with ILI exhibited any mutations that may alter tissue tropism, the amino acid sequences of loops 1 and 2 of the hexon gene, and those of the fiber knob and shaft domains were compared between
Mongolian strains and other EKC-causing strains in GenBank (HAdV-D8p, 8b, and 8e [26]) (Figs. 3A and 3B). Two strains (10_03377 and 10_04126) were detected in nasopharyngeal swabs and 2 strains (10_03019 and 10_03192) were detected in eye swabs in Mongolia. In loops 1 and 2 of the hexon protein, all HAdV-D8 strains in Mongolia, including those collected from nasopharyngeal and eye swabs, were almost identical. Only 1 strain from a nasopharyngeal swab sample (11_00695) exhibited a single amino acid substitution, as compared to other Mongolian strains. This substitution occurred between HVRs 3 and 4, where the amino acid was sub-
that 5.4% of ILI infections were caused by HAdVs. However, previous reports from Mongolia indicated 1.5–2.7% of ILI infections were caused by HAdVs, as determined by multiplex rt-PCR and direct immunofluorescent test (27–29). The rate of HAdVs in our study was also lower than those of studies conducted in different parts of the world (5–10%). However, this data should be cautiously interpreted because each study used different enrollment criteria and detection methods.

A total of 7 different serotypes (HAdV-C1, -C2, -B3, -C5, -C6, -B7, and -D8) were detected from patients with ILI in Mongolia. HAdV-B7, followed by HAdV-B3 and -D8, were the most commonly detected serotypes. A similar distribution of serotypes was observed in Korea (10). HAdV-B3 and -B7 are likely to be associated with serious acute respiratory infection. Moreover, HAdV-D8 might be associated with serious respiratory infection, even though it is rarely detected in patients with respiratory infection (24). Our data emphasize the importance of monitoring these serotypes in Mongolia.

In general, there is no clear-cut seasonality for HAdV infection, and HAdVs are usually detected in all seasons (30–33). Our study in Mongolia also did not reveal any seasonal pattern to HAdV infection. Mongolia has an extreme continental climate with wide daily temperature fluctuations and long, cold winters and short summers. This may imply that HAdV infection is not affected by climatic factors. However, more HAdV-positive cases were detected between September and December 2010. Various serotypes were detected during this period, and this increase was not caused by an outbreak of any one serotype. In this study, an immunofluorescent test was performed for screening between January and October 2010. Multiplex rt-PCR was performed after October 2010, which coincided with the increased incidence of positive cases. Therefore, this change in screening method may have affected the detection rate and resulted in the observed increase in the number of positive cases after October 2010.

HAdV-B3 and -B7 is known to cause severe respiratory infection (21–25). In our phylogenetic analysis, Mongolian HAdV-B7 strains formed a single cluster and were similar to strains detected before 2000. On the other hand, Mongolian HAdV-B3 strains were also clustered into a single cluster in the phylogenetic tree and were closely related to strains detected in other Asian countries in the 1990s. This may suggest that these Mongolian strains were imported from other countries more than 10 years ago and have been circulating in Mongolia since that time. However, more sequence data is needed to support this hypothesis.

HAdV-D8 was detected in patients with ILI in this study. There was only one case of co-infection with another respiratory virus, and there were 4 ILI cases that might have been caused by HAdV-D8. HAdV-D8 is one of the most important causes of EKC, and there are few reports of acute respiratory infection that was caused by HAdV-D8 (24). The sequences of loops 1 and 2 of the hexon gene and those of the fiber knob and shaft domains were compared to detect mutations that may be associated with tissue tropism and acute respiratory infection. However, Mongolian HAdV-D8 strains were similar to other EKC-causing HAdV-D8 strains, and no specific mutations were found in loops 1 and 2, which are responsible for the antigenicity to the neutralizing antibody. Similarly, no specific mutations were found in the fiber knob and shaft domains, which are responsible for receptor binding. Moreover, HAdV-D8 is reported to cause serious acute respiratory infection (24). However, in this study, there was no clinical information available regarding the severity of the acute respiratory infection caused by HAdV-D8. Further studies are needed to determine the phenotypic or genetic changes in these HAdV-D8 strains.

Restriction fragment length polymorphism (RFLP) analysis has been commonly used to identify genotypes in the epidemiological study of HAdVs (34–42). This method can divide genotypes that cannot be differentiated by sequence analysis of the hexon gene (35). Although RFLP analysis is a useful method, we were unable to analyze the samples we collected using RFLP because the concentration of DNA was too low. All of the samples used in this study were clinical samples and RFLP requires the isolation of HAdVs. Therefore, it is necessary to isolate HAdVs in order to conduct more in-depth analyses of circulating HAdVs in Mongolia.

Only 31 samples were analyzed in this study, and the study period was only 1 year. To clarify the more detailed epidemiology of HAdVs in Mongolia, a longer study period with more samples is needed.

In conclusion, a diverse number of HAdVs serotypes were circulating throughout the year in Mongolia. Therefore, serotyping of HAdV is an important component of ILI surveillance and should be performed regularly.

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Conflict of interest None to declare.

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