



Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.



# Prevalence of serum neutralizing antibodies to adenovirus type 5 (Ad5) and 41 (Ad41) in children is associated with age and sanitary conditions



Wei-Xiong Yang<sup>a,1</sup>, Xiao-Hui Zou<sup>b,1</sup>, Shuang-Ying Jiang<sup>a</sup>, Nan-Nan Lu<sup>a</sup>, Mei Han<sup>c</sup>, Jian-Hai Zhao<sup>a</sup>, Xiao-Juan Guo<sup>b</sup>, Sheng-Cang Zhao<sup>a,\*</sup>, Zhuo-Zhuang Lu<sup>a,b,\*</sup>

<sup>a</sup> Qinghai Center for Disease Control and Prevention, Xining, Qinghai 810007, China

<sup>b</sup> State Key Laboratory of Infectious Disease Prevention and Control, National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing 100052, China

<sup>c</sup> Qinghai Provincial Kangle Hospital, Xining, Qinghai 810006, China

## ARTICLE INFO

### Article history:

Received 27 January 2016

Received in revised form 20 September 2016

Accepted 21 September 2016

### Keywords:

Adenovirus

Human adenovirus type 41

Vector

Seroprevalence

Sanitary condition

Age

China

## ABSTRACT

Neutralizing antibody (NAb) can dampen the immunogenicity of adenovirus (Ad) vector-based vaccine. Vector systems based on human adenovirus type 41 (Ad41) have been constructed and used to develop recombinant vaccines. Here, we attempted to study the seroprevalence of NAb to Ad5 and Ad41 among children and adults in Qinghai province, China. The positive rates (titer  $\geq 40$ ) of Ad5 and Ad41 NAb in adults from Xining city were 75.7% and 94.7%, respectively. The moderate/high-positive rates (titer  $\geq 160$ ) of NAb were quite close between the two viruses in adults (70.4% for Ad5 and 73.5% for Ad41). Age-dependent increase of NAb seroprevalence was observed for both viruses in children. NAb-positive rate of Ad41 reached 50% at 3.3–4.6 years of age for children from Chengxi district, Xining city, approximately 1.5 years earlier than that of Ad5 did. Interestingly, NAb level was also associated with sanitary conditions among young children. For Ad5, 8–15% children (0.2–3.0 years of age) from city or town, where the sanitations were relatively better, had moderate/high-positive NAb, while the same rate was 62% for children from villages. For Ad41, 22% children from city, 47% from town and 88% from villages possessed moderate/high-positive NAb. The possible influence of NAb titer distributions on the application of Ad41-vectored vaccines was discussed in detail. Our results suggested that children from places with poor sanitations should be included for comprehensive Ad NAb seroprevalence studies, and provided insights to the applications of Ad41 vectors.

© 2016 Elsevier Ltd. All rights reserved.

## 1. Introduction

Human adenoviruses (Ads) are classified into 7 species (A–G), including more than 50 types. Human adenovirus type 2 (Ad2) and Ad5, both of which belong to adenovirus species C (Ad-C), are the first types to be reconstructed into gene transfer vectors and are widely applied in gene therapy or recombinant vaccine studies [1,2]. After that, Ad vectors based on other human or mam-

malian Ad types were invented in succession to extend the cellular tropism since different Ads could recognize and infect different types of cell [3]. Preclinical studies and Clinical trials showed that serum neutralizing antibodies (NAbs) severely impaired the effect of Ad vector-mediated gene transfer [4–8]. The use of various Ad vectors has additional benefits besides expanded cellular tropism: it can help evade the pre-existing NAb because NAbs against different Ad species have little cross-reactivity [9,10].

Ad-F differs from other human Ad species in several aspects [11]. It is the only human Ad pathogen that can cause infant gastroenteritis (this is why Ad-F is called enteric Ad). It expresses two types of fiber protein and lacks RGD motif on the virion, which implies that Ad-F may have profound changes in cellular tropism considering fiber and RGD motif are the most important ligands Ads use to attach target cells. Ad-F is very difficult to cultivate in laboratory conditions and is called fastidious Ad. The mechanism for the fastidiousness of Ad-F remains elusive to date. It was

\* Corresponding authors at: Qinghai Center for Disease Control and Prevention, 55 Ba Yi Zhong Lu, Cheng Dong Qu, Xining, Qinghai 810007, China (S.-C. Zhao). State Key Laboratory of Infectious Disease Prevention and Control, National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, 100 Ying Xin Jie, Xi Cheng Qu, Beijing 100052, China (Z.-Z. Lu).

E-mail addresses: [qhzhaozhengcang@163.com](mailto:qhzhaozhengcang@163.com) (S.-C. Zhao), [luzz@ivdc.chinacdc.cn](mailto:luzz@ivdc.chinacdc.cn) (Z.-Z. Lu).

<sup>1</sup> Wei-Xiong Yang and Xiao-Hui Zou contributed equally to this project and should be considered co-first authors.

proposed that Ad-F had the potential to be developed as a gene transfer vector targeting gastroenteric tract [12], which was delayed by its non-cultivable feature. Ad-F contains two members: Ad40 and Ad41. Lemiale and the colleagues developed an Ad41-based gene delivery vehicle for the first time [13]. We established Ad41 packaging cell lines, constructed E1-deleted replication-defective Ad41 vector system [14,15], and improved the vector system by increasing the packaging efficiency of recombinant Ad41 [16,17]. Recently, we constructed recombinant Ad5 and Ad41 carrying Middle East respiratory syndrome coronavirus (MERS-CoV) neutralizing antigen gene, and preliminarily evaluated their function as MERS-CoV candidate vaccines in mouse model [18]. Because NAb level plays a critical role in the application of Ad as recombinant vaccine vector, here we intended to titrate serum NAbs to Ad5 and Ad41 in healthy populations of various age groups.

## 2. Materials and methods

### 2.1. Cell lines and viruses

293TE32 was a modified 293 cell line established previously to package recombinant Ad41 [16,17]. 293 cell line was purchased from Invitrogen corporation (Cat. no. R705-07; Invitrogen, Carlsbad, CA, USA); HEp-2 (ATCC No. CCL-23) cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Ad5GFP and Ad41GFP were replication-defective Ad5 and Ad41 vectors carrying GFP reporter gene. They were constructed previously in the lab [15,16].

293, 293TE32 and HEp-2 cells were cultivated with Dulbecco's modified Eagle's medium (DMEM) plus 8% fetal bovine serum (FBS; HyClone, Logan, UT, USA) and incubated at 37 °C in a humid atmosphere containing 5% CO<sub>2</sub>. For 293TE32 cells, G418 (Merck KGaA, Darmstadt, Germany) was added to a final concentration of 0.40 mg/ml during the routine passaging. The cells were 1:6 split and cultured in new flasks every 3 or 4 days when they reached 95% confluence.

Ad5GFP and Ad41GFP were amplified in 293 and 293TE32 cells, respectively. After three cycles of freeze-and-thaw, the released virus was subjected to cesium chloride ultracentrifugation, followed by three rounds of dialysis against a buffer containing 10 mM Tris-Cl (pH7.6), 1 mM MgCl<sub>2</sub>, 150 mM NaCl. Purified virus was preserved at -80 °C in dialysis buffer containing 5% glycerol. The virus particle titer was calculated by determining the concentration of virus genomic DNA, where 100 ng of genome DNA is equivalent to 2.90 × 10<sup>9</sup> vp [19]. The multiplicity of infection (MOI) was calculated from particle titer.

### 2.2. Human serum samples

Serum samples were collected from Xining city or Guinan county of Qinghai province, China. Xining is the capital city of Qinghai province, and Guinan is a county 140 km southwest of Xining. Serum samples of adults were randomly obtained from 226 healthy volunteers who participated in physical examination program in Kangle hospital, Xining city from June to October in 2015. Child serum samples were from the remnant sera of a survey program on antibody against human hepatitis B virus after vaccination. Samples of child serum were collected in May, July and August in 2014. 167 samples were collected at Chengxi district of Xining city, 74 samples were collected at Mangqu town of Guinan county, and 60 samples were collected from 8 villages in Guinan county. The demographic information of samples was summarized in Table 1. Sera were preserved at -20 °C after collection and incubated at 56 °C for 30 min to inactivate complements before NAB

assay. Informed consent was obtained from the serum donors or the legal guardians of the enrolled children. The protocols were approved by the Ethics Committee of China CDC, Beijing, China.

### 2.3. Adenovirus neutralization assay [20]

Human serum samples were 10-, 40-, 160- and 640-fold diluted in DMEM medium containing 0.2% bovine serum albumin (BSA; Sigma-Aldrich, USA) and loaded in a 96-well plate at a volume of 50 µl per well. DMEM containing 0.2% BSA and 2.5% FBS was added to 4 wells for preparation of uninfected or half-infected controls. Ad5GFP or Ad41GFP were diluted in DMEM plus 0.2% BSA to a final concentration of 4 × 10<sup>7</sup> or 1 × 10<sup>8</sup> vp/ml, respectively. The diluted virus (50 µl/well) was added to the plate and mixed thoroughly with the serially diluted human sera. DMEM plus 0.2% BSA was added to 2 wells containing no human serum, and served as uninfected controls. Equal volume of DMEM plus 0.2% BSA and virus diluent were mixed, added to the remaining 2 wells containing no human serum, and served as half-infected controls (50% amount of virus was added when compared with test wells). The mixture was incubated at room temperature for 1 h after mixing thoroughly by vibration. Exponentially growing HEp-2 cells were detached by trypsin treatment and suspended as single cell solution in DMEM plus 4% FBS at a density of 2 × 10<sup>5</sup> cell/ml. HEp-2 cells (100 µl) were added to each well of the 96-well plate. The samples of human serum were finally 40-, 160-, 640- and 2560-fold diluted because the volume of the infection system increased from 50 µl to 200 µl after addition of the virus and cells. The plate was transferred to cell culture incubator and cultivated for 40 h. Old culture medium was discarded by tapping on absorbent paper, and the cells were fixed in 1% formaldehyde in PBS (80 µl per well). GFP fluorescence intensity was determined with an EnSpire<sup>®</sup> Multimode Plate Reader (Perkin Elmer, USA). The Excitation and Emission wavelength were set to be 488 nm and 509 nm, respectively. Five horizontal points were read from each well (the distance between points was set to be 1.4 mm). The values of the two terminal points were discarded due to edge effect, and the values of the remaining 3 points were summed and recorded as the reading of the well. Uninfected wells served as blank control, reading minus the blank value was the GFP intensity of the well. The average GFP intensity of half-infected wells was used as the cut-off to determine the titer of human serum sample. The highest dilution of human serum which could inhibit more than 50% activity of the virus was defined as the NAB titer.

### 2.4. Data analysis

The NAB titers of serum samples were determined to be <40, 40, 160, 640, 2560 or >2560. The titer of <40 was defined as negative. Conversely, the titers of ≥40 were considered positive. A binary variable was established to represent Ad NAB titers ≥160 or low/negative titers ≤40. The data were firstly analyzed with multivariate binary logistic regression to see the contributions of gender, age and/or geographic locations to the percentage of NABs titer ≥160 (age was treated as a scale variable). When the effect of some factors, such as gender, could be neglected, the samples were grouped according to the age or geographic locations, and further analyzed with nonparametric method (Kruskal-Wallis test) or Chi-square test to observe the difference of NAB levels among groups. To simplify the demonstration of titer distributions with bar chart, serum samples were practically divided into 3 categories according to NAB titer: ≤40 (low), 160–640 (medium) and ≥2560 (high). SPSS 18 was used for data analysis and a P value of <0.05 was considered statistically significant. The raw data of this survey can be found in the [supplementary material](#).

**Table 1**  
Demographics of serum sample donors.

Area	Gender	Count (% of total)									Total	
		Age (year):	0.2–0.5	0.6–3.0	3.1–7.0	7.1–14	18–30	31–40	41–50	51–65		66–83
Xining	Male						54 (10.2)	18 (3.4)	13 (2.5)	15 (2.8)	1 (0.2)	101 (19.2)
	Female						52 (9.9)	22 (4.2)	36 (6.8)	11 (2.1)	4 (0.8)	125 (23.7)
Chengxi	Male	4 (0.8)	32 (6.1)	47 (8.9)	18 (3.4)							101 (19.2)
	Female	0 (0.0)	28 (5.3)	26 (4.9)	12 (2.3)							66 (12.5)
Guinan (town)	Male	0 (0.0)	20 (3.8)	19 (3.6)								39 (7.4)
	Female	0 (0.0)	14 (2.7)	21 (4.0)								35 (6.6)
Guinan (village)	Male	1 (0.2)	19 (3.6)	14 (2.7)								34 (6.5)
	Female	1 (0.2)	13 (2.5)	12 (2.3)								26 (4.9)
Total		6 (1.1)	126 (23.9)	139 (26.4)	30 (5.7)	106 (20.1)	40 (7.6)	49 (9.3)	26 (4.9)	5 (0.9)		527 (100.0)

### 3. Results

#### 3.1. Seroprevalence of Ad5 and Ad41 NABs in healthy adults

Seroprevalence of Ad5 and Ad41 NABs was first investigated in healthy adult population. As shown in Fig. 1A, the frequency distribution of samples was nearly universal among different NAB titers of Ad5 except a very small number of participants had Ad5 NAB titer of 40. In contrast, the Ad41 NAB-negative population was very small (5.3%), as well as the strongly positive ones (titer > 2560), while most of the people had a moderate Ad41 NAB titer. Actually, the log-transformed data of Ad41 NAB titer were close to a normal distribution. The independence between Ad5 and Ad41 NABs could be seen from the scatter diagram (Fig. 1B). The correlation analysis showed that the NAB titer values of Ad5 was independent with that of Ad41 (Kendall's tau-b = 0.014, P = 0.800). This phenomenon implied that there was little cross-reactivity between NAB of Ad5 and NAB of Ad41, which made it reasonable to analyze the NAB data of Ad5 and Ad41 separately.

Logistic regression analysis of Ad5 NAB data showed that gender factor had no impact on the NAB level while age did. Shown are the distributions of Ad5 and Ad41 NAB levels among age groups (Fig. 1C). The percentage of low Ad5 NAB level gradually decreased as the age grew. However, rank-sum test result showed that there was no statistically significant difference among these age groups (P = 0.531). If the NAB levels were categorized into negative/low ( $\leq 40$ ) and moderate/high positive ( $\geq 160$ ) subgroups and the data were re-analyzed with Pearson Chi-square test, statistically significant difference could only be found between age groups of 18–30 and 51–83 (P = 0.046). Logistic regression analysis of Ad41 NAB data showed that both gender and age had no impact on NAB level. The analysis of the child data also showed that gender was not a contributory factor to NAB titer. Therefore, gender factor was negligible and will not be further mentioned in following parts of the results section. Above analysis illustrated that NAB levels were relatively stable among healthy adults in Xining. The positive percentages (titer  $\geq 40$ ) of Ad5 and Ad41 NAB in adults were calculated to be 75.7% (95% confidence interval, CI: 69.9–81.4%) and 94.7% (CI: 91.6–97.3%), respectively. The moderate- and high-positive percentages (titer  $\geq 160$ ) of Ad5 and Ad41 NAB in adults were calculated to be 70.4% (95% confidence interval, CI: 64.2–76.5%) and 73.5% (CI: 67.7–79.2%), respectively (Table 2). Similar prevalence rates derived from child specimens in different age or location groups are summarized in Table 2 without further mention.

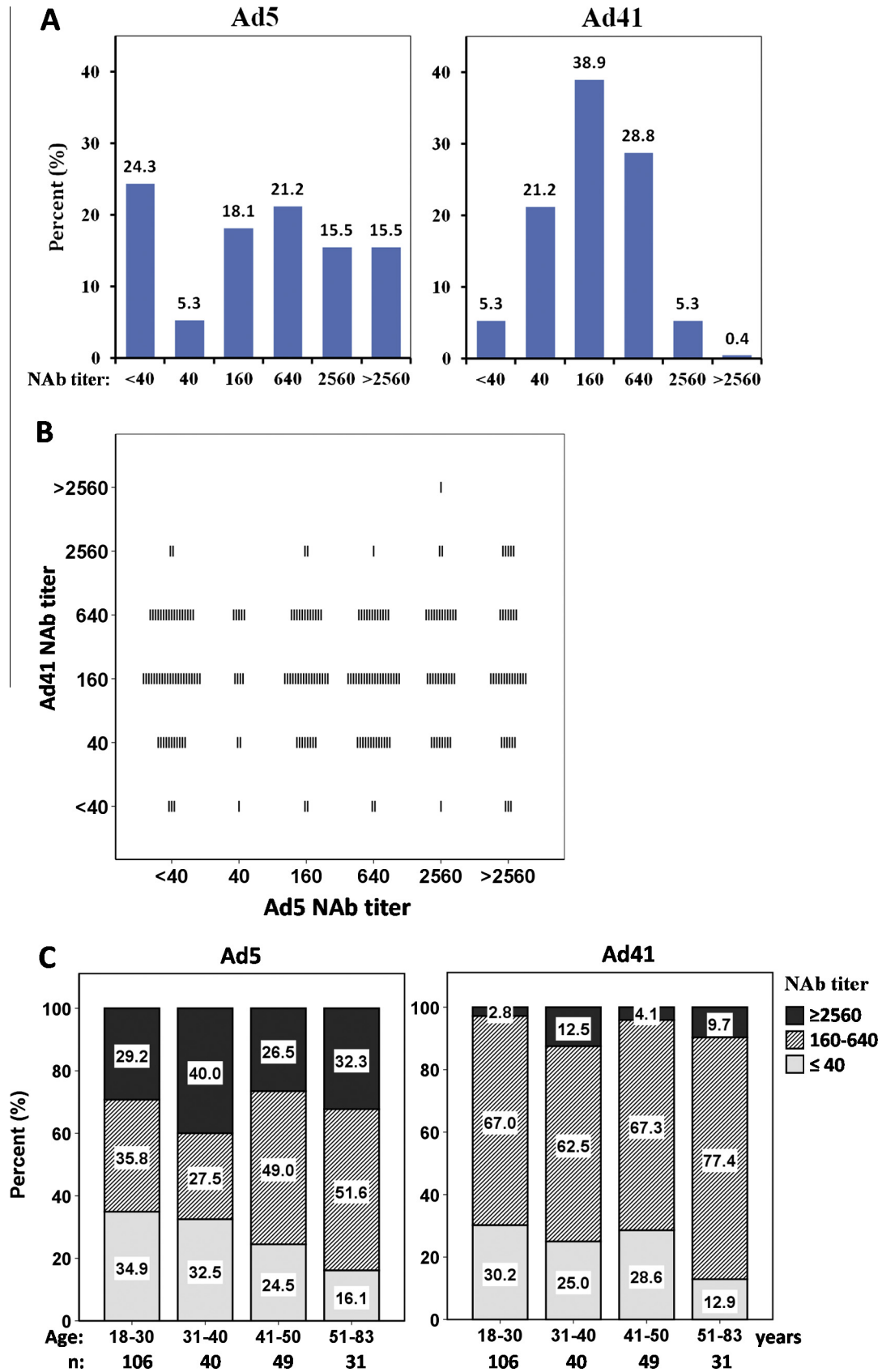
#### 3.2. Seroprevalence of Ad5 and Ad41 NABs was associated with age in children

The data of adults suggest that the infection rate of the population might be saturated in adults above 18 years of age. The trend

of Ad infection was further investigated in children from 0.2 to 14 years of age. Enrolled were serum samples collected at Chengxi district of Xining city in 2014. The NAB titer distribution histograms were very different between Ad5 and Ad41 (Fig. 2A). If the columns of <40 were removed from the histograms, we could see that more children had low or high Ad5 NAB titer while very small number of children had the middle level of Ad5 NAB. The reverse was the case for Ad41. Age was the major factor influencing NAB level for both Ad5 and Ad41 when analyzed with logistic regression. Samples were grouped to 3 categories according to the age, and the percentages of NAB levels in different age groups are shown in Fig. 2B. Data of adults were also combined to build the plots although statistically significant differences between adult and child groups were not labeled. For Ad5, the prevalence rate increased as the age grew. The pairwise comparison results showed that NAB levels were significantly different between all child age groups. The percentage of high-positive Ad5 NAB kept going up among the child groups sorted with a growing age, and decreased in the adult group. This is because saturated infection had been reached in adult group and the NAB level would wane gradually in years post infection. For Ad41, the moderate/high rate was low (22%) in age group of 0.2–3.0 years, and it grew very rapidly and reached 90% in age group of 3.1–7.0 years. The pairwise comparison results showed that Ad41 NAB titers were significantly different between group 0.2–3.0 and other groups. The difference between groups 3.1–7.0 and 7.1–14 was not significant (P = 0.193). Actually, the infection had been saturated before age 7 as the high-positive percentage started to decrease after that. To find the age when 50% NAB-positive rate could be reached, the data of children were re-categorized into 5 age groups (each group consisted of 33 or 34 cases). As shown in Fig. 3, NAB-positive rate of Ad5 reached 50% at the age of 4.7–6.7 years, approximately 1.5 years later than that of Ad41 did.

#### 3.3. Seroprevalence of Ad5 and Ad41 NABs was associated with sanitary conditions in young children

Child serum samples collected in the capital town (Mangqu) and 8 villages of Guinan county were used to study the effect of sanitary conditions on Ad NABs. The town had better sanitations than the villages although they had similar geographic environment and climate. Comparatively, Xining city had the best sanitations. Therefore, the data collected from children between 0.2 and 7.0 years of age in Chengxi district of Xining were combined. Logistic regression analysis showed that both geographic location and age had impact on NAB level of Ad5 and Ad41. The data from the 0.2- to 3.0-year age and the 3.1–7.0-year age groups were analyzed separately. As shown in Fig. 4A, children under 3 years of age in villages had an obviously higher Ad5 or Ad41 NAB titer than those living in town or city. For Ad41, children in town also had a higher



**Fig. 1.** Titer distributions of serum Ad5 NAb or Ad41 NAb among healthy adults in Xining city. (n = 226). (A) Overall frequency distributions of serum samples according to NAb titers. (B) Scatter plot to show the independence of NAb titers of Ad5 with that of Ad41. The correlation analysis results showed that the values of Ad5 NAb titer was independent with that of Ad41's (Kendall's tau-b = 0.014, P = 0.800). (C) Titer distributions of Ad5 NAb or Ad41 NAb in different age groups.

**Table 2**  
Positive or moderate/high-positive rate of Ad NAb for people in different age or location groups.

Age group (years)	Percentage (95% confidence interval)%											
	Ad5 NAb titer $\geq 40$			Ad5 NAb titer $\geq 160$			Ad41 NAb titer $\geq 40$			Ad41 NAb titer $\geq 160$		
	City	Town	Village	City	Town	Village	City	Town	Village	City	Town	Village
0.2–3.0	31 (21–44)	18 (6–32)	74 (57–88)	8 (2–15)	15 (3–29)	62 (45–77)	23 (14–34)	47 (30–65)	91 (80–100)	22 (12–32)	47 (30–65)	88 (76–97)
3.1–7.0	56 (45–68)	43 (27–58)	73 (54–91)	30 (20–41)	35 (21–50)	69 (48–88)	92 (84–97)	100 (100–100)	100 (100–100)	90 (82–97)	100 (100–100)	100 (100–100)
7.1–14	70 (53–85)			50 (32–68)			97 (90–100)			87 (72–97)		
18–83	75.7 (69.9–81.4)			70.4 (64.2–76.5)			94.7 (91.6–97.3)			73.5 (67.7–79.2)		

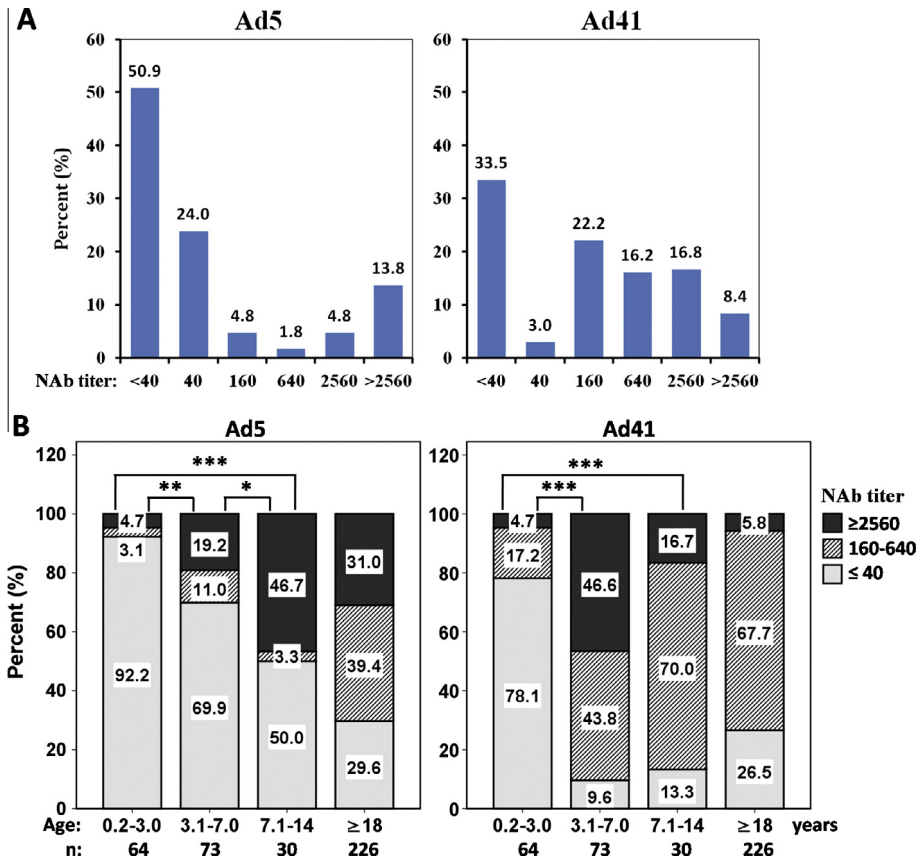
NAb seroprevalence than those in city. More than 50% children in villages had moderate/high NAb level of Ad5 or Ad41 before 3 years of age. For children between 3 and 7 years old, the difference of NAb level between city, town and village gradually diminished as shown in Fig. 4B. Statistically significant difference was only found in Ad5 NAb between city and village ( $P = 0.006$ ). The children in town even had a higher percentage of strongly positive cases than those in villages although the difference was not statistically significant. These results demonstrated that better sanitary conditions delayed the infection of Ad5 and Ad41 in children.

#### 4. Discussion

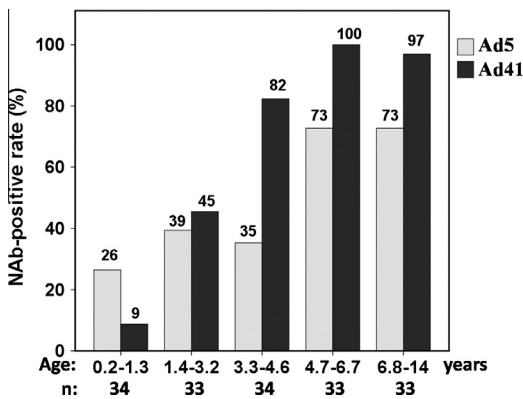
Ad5-based vector has proved to be one of the most immunogenic antigen delivery tools [6,21]. However, Ad5 NAb can dampen the immunogenicity of Ad5 vectored recombinant vaccine [4–8]. International serosurveys demonstrate that the seroprevalence of Ad5 NAb varies in different geographic regions although the reason for that is still unknown [22,23]. Prevalence rate of Ad5 NAb in China was reported to be 60–82% in healthy adults, which slightly fluctuated according to different geographic locations and age [24–26]. Our results showed that the seroprevalence of Ad5 NAb in adults in Xining was 75.7%, which was consistent with the published investigations [24–26]. Yu and the colleagues determined the Ad2 and Ad5 NAb titers of 274 children below 12 years of age in Changchun (the capital city of Jilin province, China), and found that Ad5 NAb prevalence rate was age dependent and the overall prevalence of Ad5 NAb was 43%. Our results of the Ad5 NAb prevalence in children in Xining were similar with that in Yu's report. In addition, we found that sanitary conditions might play an important role in Ad5 infection among young children less than 3 years of age. Children living in villages had higher titers of Ad5 NAb than children in town or city. This phenomenon is worth noting for Ad5-based vaccine development because children are supposed to be the major vaccination population and most of them are from rural or poor sanitation areas in China and other developing countries. Our results suggested that children from poor sanitation area should be included for comprehensive seroepidemiological surveys of Ad.

We developed a sensitive and high-throughput method to determine Ad41 NAb titer in this survey. Studies in Ad41 NAb seroprevalence are rare, and most of them were done with qualitative methods decades ago [27–30]. In previous Ad41 NAb assay, wild-type Ad41 were commonly used and the titer of NAb was determined by observing the inhibition of diluted serum specimens on the occurrence of cytopathic effect (CPE) of indicator cells. CPE method was insensitive, subjective and time-consuming [20]. The inaccuracy might be further magnified by the fastidiousness feature of Ad41. We constructed recombinant Ad41 carrying GFP reporter gene, and directly determined the expression of reporter gene with a multimode plate reader. The sensitivity was substantially improved, as evidenced by increased titer value and prevalence of Ad41 NAb. We found a prevalence rate of Ad41 as high as 94.7% among healthy adults in Xining. The growth of prevalence was age-dependent in children, and positive rate reached 50% between 3.3 and 4.6 years of age.

Considering that Ad41 is transmitted through the fecal-oral route, we intended to investigate the effect of sanitary conditions on the prevalence of Ad41 NAb. Epidemiologic studies have shown that enteric Ads have a worldwide distribution and are of comparable prevalence in developed and developing countries and in urban and rural areas [31,32]. However, recent studies suggested that geographical difference and climate might play an important role in the prevalence of Ad NAb [22,25]. To control the geographic and climate factors, serum specimens collected from town and



**Fig. 2.** Titer distributions of serum Ad5 NAb or Ad41 NAb among children at Chengxi district, Xining city. (n = 167). (A) Overall frequency distributions of serum samples according to NAb titers. (B) Titer distributions of Ad5 NAb or Ad41 NAb in different age groups. The data of adults served as controls and were not included for statistical analysis. The difference between each age groups was analyzed with nonparametric method (Kruskal-Wallis test), and a P value of <0.05 considered statistically significant. \* P < 0.05; \*\* P < 0.01; and \*\*\* P < 0.001.



**Fig. 3.** NAb-positive rate of Ad5 and Ad41 in serial age groups for children at Chengxi district, Xining city (n = 167).

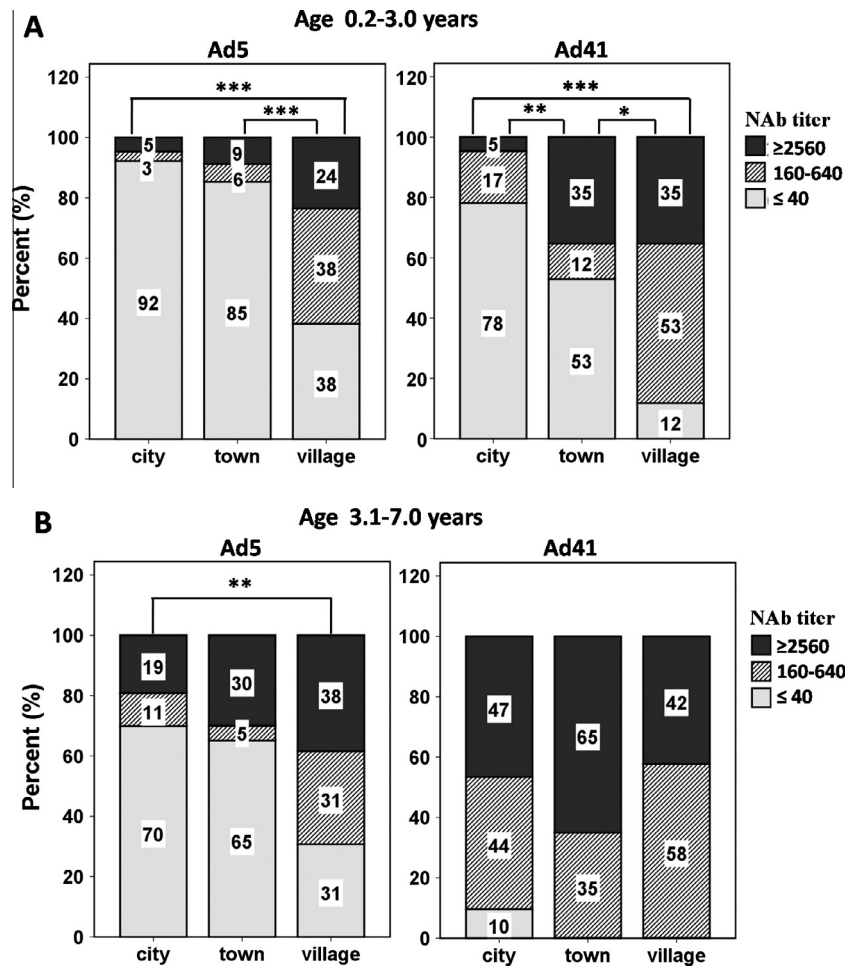
villages in Guinan county, 150 km southwest of Xining city, were used in this study. Comparatively, people living in villages tend to use more unclean water or eat more unclean food than people in towns or cities due to different life styles. We found that children living in villages did have higher NAb level than those in town below 3 years of age (Fig. 4A). Intriguingly, the difference in Ad41 NAb titer diminished in children more than 3 years of age. These results demonstrated that the sanitary condition was an important factor affecting the Ad41 NAb level at early time in life and Ad41-related pathogens existed extensively in the environment.

While our data implied that NAb of Ad5 seldom cross-reacted with that of Ad41, the NAb cross-reactivity between Ad40 and

Ad41 was not investigated in this study. Ad-F comprises two members of Ad40 and Ad41. In Kidd's report, the cross-reaction between Ad40 and Ad41 NABs was so strong that Kidd chose to use one Ad41 variant to determine the NAB of both Ad40 and Ad41 [27]. However, the strong cross-reaction was not found in Shinozaki's report [28].

Moderate prevalence of low level Ad NAb in infants less than 6 months of age was commonly detected in prior reports [22,28,33,34], which was attributed to maternal antibodies. Because of the very limited sample size for the group of <6 months of age, it was combined to neighboring groups and was not separately analyzed in this study. Human maternal antibodies can persist for 6–12 months after birth [35]. If we looked into the NAb titer data of children in Chengxi district under 1 year of age, considerable number of infants (30%, 8/27) had an Ad5 NAb titer of 40, which might partly correspond to the maternal antibody. Interestingly, none of the 27 infants had an Ad41 NAb titer of 40. It could be reasonable if infants less than 1 year of age possessed negligible maternal NAb to Ad41: few adults, including mothers, of course, had high titers of Ad41 serum NAb (Fig. 1).

The negative impact of NAb on the immunogenicity of Ad5 vectored vaccine was definite [4]. When looking into this issue from the angle of quantitative analysis, it was suggested that low levels of Ad5 NABs had no effect on the protective efficacy of Ad5 vector-based vaccine [6,36]. Ad40 or Ad41-based gene transfer vectors have been used in gene therapy or recombinant vaccine studies [18,37,38]. However, the impact of NAb on the infection of Ad40 or Ad41 is little known. In mouse model, passively transferred serum NAb to Ad conferred little resistance to intestinal challenge



**Fig. 4.** Titer distributions of serum Ad5 NAb or Ad41 NAb among children from places with different sanitations. The data were firstly stratified by age (A: 0.2–3.0 years of age; B: 3.1–7.0 years of age), and then analyzed with nonparametric method (Kruskal-Wallis test) to find the difference between each location groups. A value of  $P < 0.05$  was considered statistically significant. \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; and \*\*\*  $P < 0.001$ .

by homologous virus although IgA in intestine could protect mice from re-infection [39,40]. These findings led Kidd to comment that maternal serum NAb could not protect infants from the infection of Ad40 or Ad41 [27]. That serum NAb could not block re-infection of pathogen from mucosal route was also seen on Ad5 vector in mouse model [41]. Actually, it was Unicomb and the colleagues who dug deepest into this subject although their work is not frequently cited [42]. They found that even the NAb in intestine failed to protect infants from re-infection of enteric adenovirus. Of course, re-infection did not always mean a concomitant symptom of diarrhea. They attributed the weak neutralizing activity of NAb to the low titer. We found an obvious rise of Ad41 serum NAb prevalence in the people's early years of life. The proportion of strong positive Ad41 NAb was highest in children at 3–7 years of age and went down gradually after that age. For the healthy adults, the distribution of log-transformed Ad41 NAb titer was very close to a normal distribution, which was very different from that of Ad5 NAb (Fig. 1A). Here, we tried to give a possible explanation to the reasons behind these phenomena. For Ad41, the pathogen is widely spread. When infants without Ad41 NAb are infected, strong immune response is induced to produce high level of NAb to prevent a secondary infection. The high level of Ad41 NAb wanes as children grow to adults. When adults contact the pathogen, only those who have high titer of NAb in the intestine are protected from re-infection. However, most of adults have low level of NAb and will be re-infected (this explains why the pathogen is widely

spread). For the infected adults with a low level of NAb, the preserved immunity against Ad41 can restrict the infection to be latent without occurrence of diarrhea symptom, and immune system is re-stimulated to produce moderate level of NAb in serum. For Ad5, pathogen in the environment is relatively rare. There exists a population who haven't contacted the pathogen during their entire life time. When people are infected, strong immune response can be stimulated to produce high level of NAb, and low level of Ad5 NAb can prevent the host from re-infection (this explains why the pathogen is rare in environment). The high level of Ad5 NAb in serum wanes naturally as people get older.

If the above explanation is true, we should be optimistic about the future of Ad41 vectored vaccine. Since re-infection of Ad41 occurs naturally and frequently, such vaccines can be applied to populations of all ages and don't need to be restricted to the NAb-negative children. It also means Ad41 as an antigen carrier can be reused several times during one's life span. Furthermore, restricted infection will make the vaccines safer. On the other side, mild infection will simultaneously lead to reduced immune response to the target antigen carried by Ad41 vector. More studies need to be performed to address these assumptions and questions.

In summary, we investigated seroprevalence of Ad5 and Ad41 NAb among adult and child populations. The difference in NAb titer distribution of these two types of Ad was analyzed and discussed in detail. Our results provided insights into the possibility of using Ad41 as a vaccine vector.



## Conflicts of interest

The authors declare no conflicts of interest.

## Acknowledgements

This study was supported by Chinese National Natural Science Foundation (No. 31400156) and by National Science and Technology Major Project (No. 2012ZX10004-208 and No. 2014ZX10004002-004).

## Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.vaccine.2016.09.043>.

## References

- Mizuguchi H, Kay MA, Hayakawa T. Approaches for generating recombinant adenovirus vectors. *Adv Drug Deliv Rev* 2001;52:165–76.
- Khare R, Chen CY, Weaver EA, Barry MA. Advances and future challenges in adenoviral vector pharmacology and targeting. *Curr Gene Ther* 2011;11:241–58.
- Arnberg N. Adenovirus receptors: implications for targeting of viral vectors. *Trends Pharmacol Sci* 2012;33:442–8.
- Fausther-Bovendo H, Kobinger GP. Pre-existing immunity against Ad vectors: humoral, cellular, and innate response, what's important? *Hum Vaccines Immun* 2014;10:2875–84.
- Ledgerwood JE, Costner P, Desai N, Holman L, Enama ME, Yamshchikov G, et al. A replication defective recombinant Ad5 vaccine expressing Ebola virus GP is safe and immunogenic in healthy adults. *Vaccine* 2010;29:304–13.
- Buchbinder SP, Mehrotra DV, Duerr A, Fitzgerald DW, Mogg R, Li D, et al. Efficacy assessment of a cell-mediated immunity HIV-1 vaccine (the step study): a double-blind, randomised, placebo-controlled, test-of-concept trial. *Lancet* 2008;372:1881–93.
- Sumida SM, Truitt DM, Kishko MG, Arthur JC, Jackson SS, Gorgone DA, et al. Neutralizing antibodies and CD8+ T lymphocytes both contribute to immunity to adenovirus serotype 5 vaccine vectors. *J Virol* 2004;78:2666–73.
- Rahman A, Tsai V, Goudreau A, Shinoda JY, Wen SF, Ramachandra M, et al. Specific depletion of human anti-adenovirus antibodies facilitates transduction in an in vivo model for systemic gene therapy. *Mol Ther* 2001;3:768–78.
- Roy S, Clawson DS, Calcedo R, Lebherz C, Sanmiguel J, Wu D, et al. Use of chimeric adenoviral vectors to assess capsid neutralization determinants. *Virology* 2005;333:207–14.
- Hierholzer JC, Stone YO, Broderson JR. Antigenic relationships among the 47 human adenoviruses determined in reference horse antisera. *Arch Virol* 1991;121:179–97.
- Tiemessen CT, Kidd AH. The subgroup F adenoviruses. *J Gen Virol* 1995;76(Pt 3):481–97.
- Croyle MA, Stone M, Amidon GL, Roessler BJ. In vitro and in vivo assessment of adenovirus 41 as a vector for gene delivery to the intestine. *Gene Ther* 1998;5:645–54.
- Lemiale F, Haddada H, Nabel GJ, Brough DE, King CR, Gall JG. Novel adenovirus vaccine vectors based on the enteric-tropic serotype 41. *Vaccine* 2007;25:2074–84.
- Han BJ, Guo L, Qu JG, Wang M, Wang JW, Lu ZZ, et al. Improved replication of enteric adenovirus type 41 in Hep2 cell line expressing E1B55K. *Bing Du Xue Bao* 2007;23:258–64.
- Lu ZZ, Zou XH, Dong LX, Qu JG, Song JD, Wang M, et al. Novel recombinant adenovirus type 41 vector and its biological properties. *J Gene Med* 2009;11:128–38.
- Lu ZZ, Zou XH, Lastinger K, Williams A, Qu JG, Estes DM. Enhanced growth of recombinant human adenovirus type 41 (HAdV-41) carrying ADP gene. *Virus Res* 2013;176:61–8.
- Zou XH, Xiao X, Chen DL, Li ZL, Song JD, Wang M, et al. An improved HAdV-41 E1B55K-expressing 293 cell line for packaging fastidious adenovirus. *J Virol Methods* 2011;175:188–96.
- Guo X, Deng Y, Chen H, Lan J, Wang W, Zou X, et al. Systemic and mucosal immunity in mice elicited by a single immunization with human adenovirus type 5 or 41 vector-based vaccines carrying the spike protein of Middle East respiratory syndrome coronavirus. *Immunology* 2015;145:476–84.
- Chen DL, Dong LX, Li M, Guo XJ, Wang M, Liu XF, et al. Construction of an infectious clone of human adenovirus type 41. *Arch Virol* 2012;157:1313–21.
- Sprangers MC, Lakhai W, Koudstaal W, Verhoeven M, Koel BF, Vogels R, et al. Quantifying adenovirus-neutralizing antibodies by luciferase transgene detection: addressing preexisting immunity to vaccine and gene therapy vectors. *J Clin Microbiol* 2003;41:5046–52.
- Abbinck P, Lemckert AA, Ewald BA, Lynch DM, Denholtz M, Smits S, et al. Comparative seroprevalence and immunogenicity of six rare serotype recombinant adenovirus vaccine vectors from subgroups B and D. *J Virol* 2007;81:4654–63.
- Mast TC, Kierstead L, Gupta SB, Nikas AA, Kallas EG, Novitsky V, et al. International epidemiology of human pre-existing adenovirus (Ad) type-5, type-6, type-26 and type-36 neutralizing antibodies: correlates of high Ad5 titers and implications for potential HIV vaccine trials. *Vaccine* 2010;28:950–7.
- Barouch DH, Kik SV, Weverling GJ, Dilan R, King SL, Maxfield LF, et al. International seroprevalence of adenovirus serotypes 5, 26, 35, and 48 in pediatric and adult populations. *Vaccine* 2011;29:5203–9.
- Zhang S, Huang W, Zhou X, Zhao Q, Wang Q, Jia B. Seroprevalence of neutralizing antibodies to human adenoviruses type-5 and type-26 and chimpanzee adenovirus type-68 in healthy Chinese adults. *J Med Virol* 2013;85:1077–84.
- Yu B, Zhou Y, Wu H, Wang Z, Zhan Y, Feng X, et al. Seroprevalence of neutralizing antibodies to human adenovirus type 5 in healthy adults in China. *J Med Virol* 2012;84:1408–14.
- Sun C, Zhang Y, Feng L, Pan W, Zhang M, Hong Z, et al. Epidemiology of adenovirus type 5 neutralizing antibodies in healthy people and AIDS patients in Guangzhou, southern China. *Vaccine* 2011;29:3837–41.
- Kidd AH, Banatvala JE, de Jong JC. Antibodies to fastidious faecal adenoviruses (species 40 and 41) in sera from children. *J Med Virol* 1983;11:333–41.
- Shinozaki T, Araki K, Ushijima H, Fujii R. Antibody response to enteric adenovirus types 40 and 41 in sera from people in various age groups. *J Clin Microbiol* 1987;25:1679–82.
- Jarecki-Khan K, Unicomb LE. Seroprevalence of enteric and nonenteric adenoviruses in Bangladesh. *J Clin Microbiol* 1992;30:2733–4.
- Saderi H, Roustai MH, Sabahi F. Antibodies to enteric adenoviruses (Ad40 and Ad41) in sera from Iranian children. *J Clin Virol* 2000;16:145–7.
- Grimwood K, Carzino R, Barnes GL, Bishop RF. Patients with enteric adenovirus gastroenteritis admitted to an Australian pediatric teaching hospital from 1981 to 1992. *J Clin Microbiol* 1995;33:131–6.
- Glass RI, Bresee J, Jiang B, Gentsch J, Ando T, Fankhauser R, et al. Gastroenteritis viruses: an overview. *Novartis Found Symp* 2001;238:5–19. discussion –25.
- Appiahgari MB, Pandey RM, Vrti S. Seroprevalence of neutralizing antibodies to adenovirus type 5 among children in India: implications for recombinant adenovirus-based vaccines. *Clin Vaccine Immunol* 2007;14:1053–5.
- Yu B, Wang Z, Dong J, Wang C, Gu L, Sun C, et al. A serological survey of human adenovirus serotype 2 and 5 circulating pediatric populations in Changchun, China, 2011. *Virol J* 2012;9:287.
- Niewiesk S. Maternal antibodies: clinical significance, mechanism of interference with immune responses, and possible vaccination strategies. *Front Immunol* 2014;5:446.
- Appiahgari MB, Saini M, Rauthan M, Jyoti, Vrti S. Immunization with recombinant adenovirus synthesizing the secretory form of Japanese encephalitis virus envelope protein protects adenovirus-exposed mice against lethal encephalitis. *Microbes Infect* 2006;8:92–104.
- Yamasaki S, Miura Y, Davydova J, Vickers SM, Yamamoto M. A single intraduodenal administration of human adenovirus 40 vaccine effectively prevents anaphylactic shock. *Clin Vaccine Immunol* 2013;20:1508–16.
- Ko SY, Cheng C, Kong WP, Wang L, Kanekiyo M, Einfeld D, et al. Enhanced induction of intestinal cellular immunity by oral priming with enteric adenovirus 41 vectors. *J Virol* 2009;83:748–56.
- Hashimoto K, Yoshikawa M, Sugihara Y, Sasaki S. Intestinal resistance in the experimental enteric infection of mice with a mouse adenovirus. II. Determination of the neutralizing substance in the intestinal tract as an IgA antibody. *Jpn J Microbiol* 1971;15:499–508.
- Hashimoto K, Sugiyama T, Yoshikawa M, Sasaki S. Intestinal resistance in the experimental enteric infection of mice with a mouse adenovirus. I. Growth of the virus and appearance of a neutralizing substance in the intestinal tract. *Jpn J Microbiol* 1970;14:381–95.
- Xiang ZQ, Gao GP, Reyes-Sandoval A, Li Y, Wilson JM, Ertl HC. Oral vaccination of mice with adenoviral vectors is not impaired by preexisting immunity to the vaccine carrier. *J Virol* 2003;77:10780–9.
- Unicomb LE, Jarecki-Khan K, Hall A, Podder G. Previous enteric adenovirus infection does not protect against subsequent symptomatic infection: longitudinal follow-up of eight infants. *Microbiol Immunol* 1996;40:161–8.