



## Research Article

## Seroprevalence of neutralizing antibodies against adenovirus type 26 and 35 in healthy populations from Guangdong and Shandong provinces, China

Haisu Yi<sup>a,1</sup>, Qian Wang<sup>a,1</sup>, Jiankai Deng<sup>b,1</sup>, Hengchun Li<sup>c,g</sup>, Yingkun Zhang<sup>d</sup>, Zhilong Chen<sup>e</sup>, Tianxin Ji<sup>f</sup>, Wenming Liu<sup>c</sup>, Xuehua Zheng<sup>c</sup>, Qinghua Ma<sup>b</sup>, Xinxin Sun<sup>c</sup>, Yudi Zhang<sup>c,g</sup>, Xuegao Yu<sup>b</sup>, Mengzhang He<sup>f</sup>, Ling Chen<sup>a,c,h,\*</sup>, Ying Feng<sup>c,\*</sup>

<sup>a</sup> State Key Laboratory of Respiratory Disease, National Clinical Research Center for Respiratory Disease, Guangzhou Institute of Respiratory Health, The First Affiliated Hospital of Guangzhou Medical University, Guangzhou, 510120, China

<sup>b</sup> Department of Laboratory Medicine, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou, 510080, China

<sup>c</sup> Center for Infection and Immunity, Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou, 510530, China

<sup>d</sup> Clinical Laboratory, Pingyi Hospital of Chinese Medicine, Linyi, 273399, China

<sup>e</sup> Xiamen Institutes of Respiratory Health, Xiamen, 361013, China

<sup>f</sup> Department of Respiratory and Critical Care Medicine, The Second Affiliated Hospital of Guangzhou Medical University, Guangzhou, 510260, China

<sup>g</sup> University of Chinese Academy of Science, Beijing, 100049, China

<sup>h</sup> Guangzhou Laboratory & Bioland Laboratory, Guangzhou, 510320, China

## ARTICLE INFO

## Keywords:

Human adenovirus type 26 (HAdV 26)  
Human adenovirus type 35 (HAdV 35)  
Neutralizing antibody (nAb)  
Seroprevalence  
Guangdong and shandong provinces

## ABSTRACT

Human adenoviruses type 26 (HAdV26) and type 35 (HAdV35) have increasingly become the choice of adenovirus vectors for vaccine application. However, the population pre-existing immunity to these two adenoviruses in China, which may reduce vaccine efficacy, remains largely unknown. Here, we established micro-neutralizing (MN) assays to investigate the seroprevalence of neutralizing antibodies (nAbs) against HAdV26 and HAdV35 in the general population of Guangdong and Shandong provinces, China. A total of 1184 serum samples were collected, 47.0% and 15.8% of which showed HAdV26 and HAdV35 nAb activity, respectively. HAdV26-seropositive individuals tended to have more moderate nAbs titers (201–1000), while HAdV35-seropositive individuals appeared to have more low nAbs titers (72–200). The seropositive rates of HAdV26 and HAdV35 in individuals younger than 20 years old were very low. The seropositive rates of HAdV26 increased with age before 70 years old and decreased thereafter, while HAdV35 seropositive rates did not show similar characteristics. Notably, the seropositive rates and nAb levels of both HAdV26 and HAdV35 were higher in Guangdong Province than in Shandong Province, but did not exert significant differences between males and females. The seroprevalence between HAdV26 and HAdV35 showed little correlation, and no significant cross-neutralizing activity was detected. These results clarified the characteristics of the herd immunity against HAdV26 and HAdV35, and provided information for the rational development and application of HAdV26 and HAdV35 as vaccine vectors in China.

## 1. Introduction

Human adenoviruses (HAdVs) are non-enveloped, double-stranded DNA viruses belonging to *Adenoviridae* and the genus *Mastadenovirus*. HAdVs are grouped into subgroups A–G (Madisch et al., 2005; Robinson et al., 2013; Greber, 2020), and more than 104 genotypes of HAdVs have been identified and classified (<http://hadvwg.gmu.edu/>) according to their chemical and biological properties. HAdVs can infect various tissues and organs (Ghebremedhin, 2014; Lynch and Kajon, 2016; Ismail et al.,

2018) and cause a variety of diseases in humans, including acute upper respiratory tract infections (URTIs), conjunctivitis, acute hemorrhagic cystitis, gastroenteritis and meningoencephalitis (Greber, 2020; Huang et al., 2021; Pscheidt et al., 2021). Although most HAdVs cause only mild or limited infection (Berciaud et al., 2012; Huang et al., 2021), a few of them such as HAdV3, HAdV7, and HAdV55 can cause severe infections, especially in particular populations, such as in children, the elderly, and people with severely compromised immune systems (Kajon et al., 2018; Westerberg et al., 2018; Otto et al., 2021).

\* Corresponding authors.

E-mail addresses: [chen\\_ling@gibh.ac.cn](mailto:chen_ling@gibh.ac.cn) (L. Chen), [feng\\_ying@gibh.ac.cn](mailto:feng_ying@gibh.ac.cn) (Y. Feng).

<sup>1</sup> Haisu Yi, Qian Wang and Jiankai Deng contributed equally to this work.

The infectivity and cell entry mechanism of HAdVs make them very suitable as vectors for vaccine and gene therapy, especially upon engineering to delete *E1* gene region to make them replication-defective to ensure safety (Crenshaw et al., 2019; Mennechet et al., 2019). HAdVs vectors have many advantages for transferring foreign genes, including easy manipulation, huge foreign genes compatibility and large-scale production (Tatsis and Ertl, 2004). Various HAdVs vectors have been used in numerous pre-clinical and clinical trials for many years (Baden et al., 2013; Zhang and Zhou, 2016; Logunov et al., 2020b). As pre-existing humoral immunity against HAdVs vectors may lead to reduced vaccine efficacy, many groups have developed different genotypes of HAdVs vectors with lower seroprevalence, such as HAdV26, HAdV35, or animal-derived, such as chimpanzee adenovirus (Keefer et al., 2012; Baden et al., 2015; Zhu et al., 2020).

Vaccine vectors based on HAdV26 (subgroup D) and HAdV35 (subgroup B) have been extensively studied and are listed for vaccine applications in more than 50 clinical trials (<https://clinicaltrials.gov>). HAdV26 is being advertised as a vaccine vector of low seroprevalence and is well characterized (Barouch et al., 2011; Mennechet et al., 2019). HAdV26 has been intensively evaluated in large-scale vaccination trials against HIV, Ebola virus and SARS-CoV-2 (Baden et al., 2013; Zhang and Zhou, 2016; Logunov et al., 2020b). HAdV35 also has the advantage of low seroprevalence (Barouch et al., 2011), and has been characterized and evaluated in small-scale vaccine clinical trials against HIV, Ebola, malaria and tuberculosis (Crech et al., 2013; Omosa-Manyonyi et al., 2015). Vaccine development has been accelerated since the COVID-19 pandemic. More HAdVs vectors will be employed to combat SARS-CoV-2 and other infectious diseases. Among over ten adenoviruses are being developed as COVID-19 vaccine vectors, two HAdV26 vectored COVID-19 vaccines have been approved for vaccination in the USA and in Russia (Alderson et al., 2021; Mendonca et al., 2021). However, the seroprevalence of HAdV26 and HAdV35 in China has not been well characterized, especially considering the country's large territory. Therefore, it is important to investigate the pre-existing immunity against HAdV26 and HAdV35 in the general population for guiding the rational application of HAdV-based vaccines.

In this study, we constructed replication-competent recombinant HAdV26 and HAdV35 reporter viruses expressing secreted-alkaline-phosphatase (SEAP), and established micro-neutralizing (MN) assays to investigate the seroprevalence of nAbs against HAdV26 and HAdV35 in general population of Guangdong and Shandong provinces, China. We analyzed the distributions of seropositive rates and nAb titers among different age, gender and regions.

## 2. Materials and methods

### 2.1. Human sample collection

Serum samples were randomly collected from 1184 healthy participants who received health examinations between 1 January to April 30, 2021. Among them, 260 were collected from Shandong Province, China, while 924 from Guangdong Province, China. The volunteers aged from 2 months to 97 years old. The percentages of males to females were 45.7% and 54.3%. They were divided into seven groups according to their age: < 20, 21–30, 31–40, 41–50, 51–60, 61–70 and > 70 years old. We summarized the demographic information of the serum samples in [Supplementary Table S1](#). A total of 4 mL of fresh blood sample was collected with non-anticoagulant tube, and centrifuged at 1000×g at room temperature for 30 min after blood coagulation. Then the serum samples were separated, inactivated at 56 °C for 90 min, and stored at –80 °C.

### 2.2. Animal immunized serum collection

Six-week-old female BALB/c mice (n = 5) were purchased from Beijing Vital River Laboratory Animal Technology Co. Ltd. Eight-week-

old New Zealand rabbits (n = 5) were purchased from Guangdong Medical Laboratory Animal Center. The mice were housed and immunized in the Animal Experimental Center of GIBH, CAS, and the rabbits were housed and immunized in the Experimental Animal Center of Guangdong Province. HAdV26 and HAdV35 were inactivated by treatment with 0.2% of β-propiolactone (Tokyo Chemical Industry, Cat: 57-57-8, Tokyo, Japan) overnight at 4 °C. The mice were immunized twice at 2 weeks apart with  $2 \times 10^9$  viral particles (vp) of inactivated HAdV35, and the mice were bled and euthanized at 2 weeks after booster immunization. The rabbits were inoculated twice at 2 weeks apart with  $2 \times 10^{11}$  vp of inactivated HAdV26 in complete Freund's adjuvant (Sigma-Aldrich, Cat: F-5506, Missouri, America), and blood samples were collected at 2 weeks after booster immunization. Serum samples were isolated, inactivated and preserved at –80 °C.

### 2.3. Generation of recombinant HAdV26 and HAdV35 reporter viruses

Wild-type HAdV26 (ATCC: VR-1104) and HAdV35 (ATCC: VR-718) strains were cultured in HEK293 cells (ATCC: CRL-1573). Recombinant HAdV26 and HAdV35 expressing SEAP were constructed according to our previously described methods (Zheng et al., 2017; Ye et al., 2018). In brief, the viral genomic DNA was extracted by sodium-dodecyl-sulfonate lysis (Sigma-Aldrich, Cat: L-4509, St Louis, MO, USA) followed by phenol-chloroform extraction. The terminal regions of the HAdV26 and HAdV35 genomes were cloned into pMD18T vectors (TaKaRa, China) by PCR to gain shuttle plasmids. Through the recombination between shuttle plasmids and viral genomes in *Escherichia coli* BJ5183 competent cells (Agilent Technologies, Santa Clara, CA, USA), the genomic plasmids pAd26 and pAd35 were obtained. Subsequently, the E3 regions were deleted by homologous recombination to obtain pAd26ΔE3 and pAd35ΔE3. The coding sequences for SEAP were amplified by PCR and inserted into p26E3LR and p35E3LR to obtain the shuttle reporter plasmids. The pAd26-SEAP and pAd35-SEAP were constructed by homologous recombination between pAd26ΔE3 or pAd35ΔE3 and shuttle reporter plasmids. The pAd26-SEAP and pAd35-SEAP were linearized and transfected into HEK293 cells to be rescued and propagated. Finally, HAdV26-SEAP and HAdV35-SEAP gained, and the infectious virus titers were determined as described (Aste-Amezaga et al., 2004).

### 2.4. HAdV26 and HAdV35 micro-neutralizing (MN) assays

The serum neutralizing antibody (nAb) titers against HAdV26 and HAdV35 were detected by MN assays as previously established in our lab. In brief, the HEK293 cells were seeded into 96-well plates at  $3 \times 10^4$  cells per well and cultured for 36 h. Human serums were serially three-fold diluted in DMEM from 1:18 to 1:18,432, and incubated with HAdV26-SEAP or HAdV35-SEAP at  $5 \times 10^6$  vp per well for 1 h at 37 °C. After that, the mixtures were added to the HEK293 cells and incubated at 37 °C for 24 h. At last, the supernatants were harvested to detect SEAP activity with the Phospha-Light System (Thermo Fisher Scientific, Cat: T1016, Massachusetts, USA) according to the manufacturer's instructions. Relative light units (RLU) were recorded by a luminometer (MLX Microtiter®, Dynex Technologies, Inc., USA). nAb titers from duplicated wells were calculated as the dilutions that inhibited 50% RLU values.

### 2.5. Statistical analysis

The statistical significance of seroprevalence in different groups was performed using the Chi-squared test, and Chi-square test for trend. The correlation of the nAb titers among groups were evaluated using the Mann-Whitney test or the Kruskal-Wallis test. All statistical analyses were computed with SPSS version 17.0 (SPSS Inc., Chicago, IL, USA) and *P* values of <0.05 were considered statistically significant.

### 3. Results

#### 3.1. Establishment and validation of MN assays based on HAdV26-SEAP and HAdV35-SEAP

To establish neutralization assay based on reporter viruses, we constructed recombinant HAdV26 and HAdV35 expressing SEAP (Fig. 1A). The SEAP reporter genes were efficiently expressed in HEK293 cells that were infected by these viruses. The SEAP activity positively correlated with the infection dosage of HAdV26-SEAP and HAdV35-SEAP (Fig. 1B).

The specificity and dose-dependency of MN assays based on HAdV26-SEAP and HAdV35-SEAP was validated by using serum samples from animals immunized with HAdV26 or HAdV35. The neutralization curves of animal anti-sera to HAdV26-SEAP and HAdV35-SEAP were shown (Fig. 1C and D). SEAP activities decreased with increasing concentration of respective serum samples, reflecting the neutralization activity of the animal anti-sera. The results showed that the anti-sera from the rabbit immunized with HAdV26 had high neutralization titers against HAdV26-SEAP but no detectable neutralizing activities against HAdV35-SEAP (Fig. 1C), while the anti-sera from mouse immunized with HAdV35 had high neutralization titers against HAdV35-SEAP but no neutralizing activity against HAdV26-SEAP (Fig. 1D). This result suggested that MN assays based on HAdV26-SEAP and HAdV35-SEAP could specifically detect the neutralizing activities of the anti-sera against HAdV26 and HAdV35.

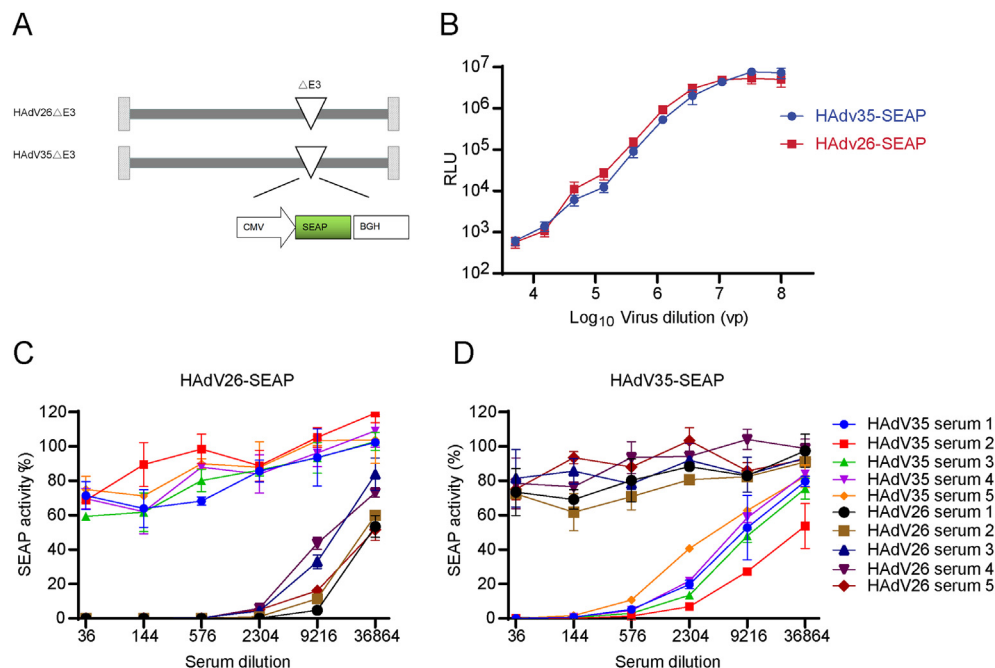
#### 3.2. The overall seroprevalence and titer of nAbs against HAdV26 and HAdV35 in the populations from Guangdong and Shandong provinces, China

Using the reporter adenoviruses HAdV26-SEAP and HAdV35-SEAP, we next investigated the nAb titers among 1184 blood donors

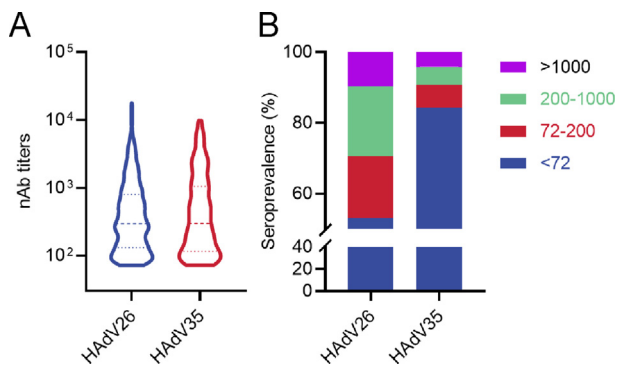
(Supplementary Table S1). The overall positive rate of HAdV26 nAb was 47.0% [95% confidence interval (CI) 44.1%–49.8%], which was significantly higher than that of HAdV35 nAb (15.8%, 95% CI 13.7%–17.9%) (Chi-square test,  $P < 0.001$ , Supplementary Tables S2 and S3). Across the cohort, a similar trend in the distribution of nAb titers against HAdV26 and HAdV35 was shown, with numbers decreasing from low to high titers (Mann-Whitney test,  $P = 0.788$ , Fig. 2A). The individuals were divided into four levels according to nAb titers (Fig. 2B): negative ( $< 72$ ), low titers (72–200), moderate titers (201–1000), and high titers ( $> 1000$ ). For HAdV26, low and moderate nAb titers were detected more frequently than high nAb titers, which were 17.5% (95% CI 15.3%–19.6%), 19.8% (95% CI 17.5%–22.0%), 9.7% (95% CI 8.0%–11.4%), respectively. For HAdV35, seropositive rates were much lower and evenly distributed among low, moderate, and high groups, which were 6.4% (95% CI 5.0%–7.8%), 5.2% (95% CI 4.0%–6.5%), and 4.1% (95% CI 3.0%–5.3%), respectively. These results revealed that HAdV26 has a higher seroprevalence level than HAdV35 in Guangdong and Shandong provinces, China. Nevertheless, HAdV26 nAb titers were mostly maintained at low to moderate levels.

#### 3.3. The seroprevalence and titer of nAbs against HAdV26 and HAdV35 in different age groups

The seropositive rates of HAdV26 and HAdV35 in different age groups were analyzed. The overall seropositive rates of HAdV26 increased with age before 70 years old and decreased thereafter (Trend Chi-square test,  $P < 0.001$ , Fig. 3A), while the overall seropositive rates of HAdV35 among different age groups did not reach statistical significance (Trend Chi-square test,  $P = 0.065$ , Fig. 3A). The number of individuals with moderate HAdV26 nAb titer was also progressively increased with age before 60 years old and decreased thereafter (Fig. 3B). Individuals with low and high HAdV26 nAb titers were dominant in age



**Fig. 1.** Establishment and validation of neutralization assays based on HAdV26 and HAdV35 reporter viruses. **A** A schematic diagram for the construction of the genomic structures for HAdV26-SEAP and HAdV35-SEAP. **B** The SEAP activity detected in cell culture media from HEK293 cells at 24 h after infection with HAdV26-SEAP and HAdV35-SEAP. The relative light units (RLU) were recorded by a luminometer. Six parallel repetitions were made for each well. **C, D** Specificity of HAdV26-SEAP and HAdV35-SEAP in detecting specific neutralization. HAdV26-immunized rabbit serum samples ( $n = 5$ ) and HAdV35-immunized mouse serum samples ( $n = 5$ ) were serially diluted and incubated with  $5 \times 10^6$  vp of HAdV26-SEAP (**C**) or HAdV35-SEAP (**D**). After incubation for 1 h, the mixtures were added to HEK293 cells in 96-well plates in duplicates. One day later, the SEAP activity in the culture supernatants were detected. Cells infected with HAdV26-SEAP and HAdV35-SEAP without anti-sera were used as 100% SEAP activities. The percentages of SEAP activity in the presence of anti-sera versus viral infection controls are shown. One representative result from two independent experiments is shown. Error bars stand for mean  $\pm$  standard deviation (SD).



**Fig. 2.** The overall seroprevalence of nAb against HAdV26 and HAdV35. **A** Distribution of nAb titers against HAdV26 and HAdV35 in seropositive individuals. The data were analyzed with the Mann-Whitney test. **B** Distribution of different levels of nAb titer against HAdV26 and HAdV35. The individuals were divided into four levels according to nAb titers: negative (< 72), low titers (72–200), moderate titers (201–1000), and high titers (> 1000).

group of 60–70 years, and nAb titers reached the lowest level in individuals younger than 20 (Fig. 3B). The frequency of serum samples with low, moderate and high HAdV35 nAb titers did not increased with age (Fig. 3C). The frequency of low, moderate, and high HAdV35 nAb titers maintained relatively low in individuals older than 20 (seropositive rates ranging from 4.4% to 6.8%, Fig. 3C). HAdV26 nAbs were the lowest in the age group younger than 20 (Kruskal-Wallis test,  $P = 0.004$ , Fig. 3D), while no significant difference was observed for the HAdV35 nAb titers in the seven different age groups (Kruskal-Wallis test,  $P = 0.322$ , Fig. 3E). These results suggested that seropositive rates and nAb titers for HAdV26 and HAdV35 had different patterns. Individuals younger than 20 years had a lower seroprevalence than other age groups, implying that these people might be more susceptible to HAdV26 and HAdV35 infection, and they might have a better response to HAdV26 and HAdV35 vector vaccines.

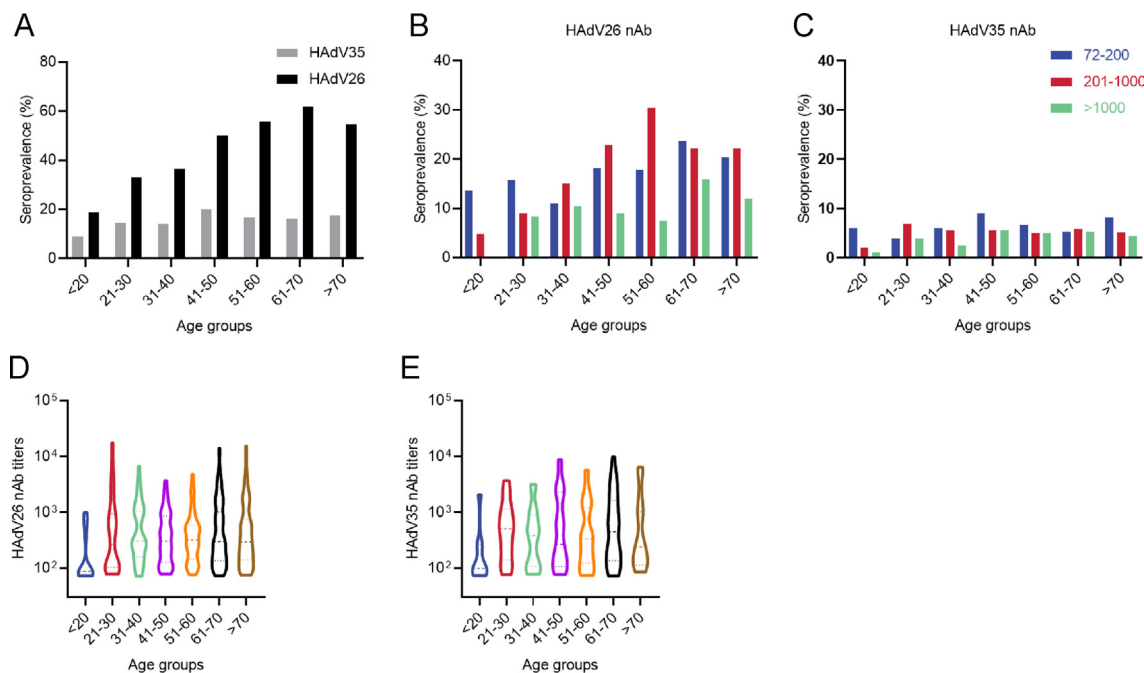
**3.4. The seroprevalence and titer of nAbs against HAdV26 and HAdV35 in different gender and region groups**

We next evaluated the seroprevalence and titer distributions of HAdV26 and HAdV35 nAbs in different gender and region groups. The seropositive rates of HAdV26 nAb in males were higher than that in females (male: 51.1%, 95% CI 46.9%–55.3%; female: 43.5%, 95% CI 39.6%–47.3%; Chi-square test,  $P = 0.009$ ; Fig. 4A). However, no statistical difference was observed for the seroprevalence of HAdV35 nAb between males and females (male: 16.1%, 95% CI 13.0%–19.2%; female: 15.6%, 95% CI 12.8%–18.4%; Chi-square test,  $P = 0.823$ ; Fig. 4B). The titers of neither HAdV26 nAb nor HAdV35 nAb had significant differences in males and females (Mann-Whitney test,  $P = 0.735$  for HAdV26 nAb and  $P = 0.408$  for HAdV35 nAb, respectively; Fig. 4C and D). Therefore, gender impacted the seropositive rates of HAdV26, but did not affect the nAb titers of HAdV26, while both the seropositive rates and nAb titers of HAdV35 were not associated with gender.

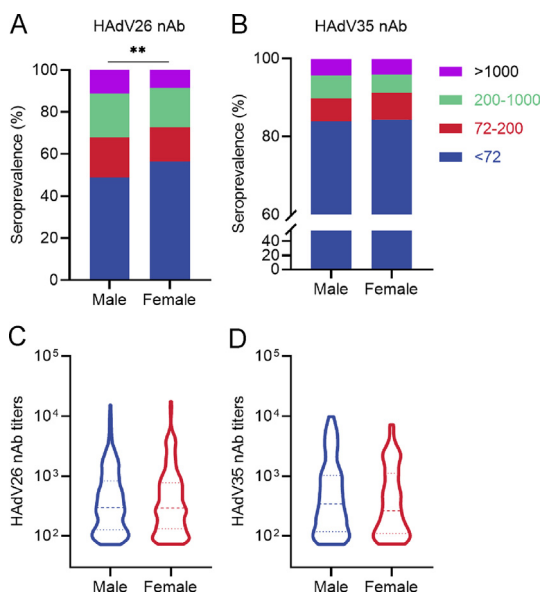
Notably, the seropositive rates of both HAdV26 nAb and HAdV35 nAb were significantly higher in Guangdong Province than that in Shandong Province. The seropositive rates of HAdV26 in Guangdong and Shandong provinces were 48.7% (95% CI 45.5%–51.9%) and 40.8% (95% CI 34.8%–46.8%), respectively (Chi-square test,  $P = 0.024$ , Fig. 5A), and the seropositive rates of HAdV35 in Guangdong and Shandong provinces were 17.8% (95% CI 15.4%–20.3%) and 8.5% (95% CI 5.1%–11.9%), respectively (Chi-square test,  $P < 0.001$ , Fig. 5B). The HAdV26 nAb titers in Guangdong Province were higher than that in Shandong Province (Mann-Whitney test,  $P = 0.012$ , Fig. 5C), while the titers of HAdV35 nAb tent to be higher in Guangdong Province but had no statistical difference as compared to Shandong Province (Mann-Whitney test,  $P = 0.367$ ; Fig. 5D).

**3.5. The seroprevalence and titer of nAbs against HAdV26 and HAdV35 in double-positive and single-positive samples**

The seropositive rates of HAdV26 nAb in HAdV35-seropositive individuals was slightly higher than that in HAdV35-seronegative



**Fig. 3.** The distribution of seroprevalence and titer of nAb against HAdV26 and HAdV35 in age groups. **A** The distribution of seropositive rates against HAdV26 and HAdV35 nAb in different age groups. The data were analyzed with Chi-square test for trend. **B, C** The distribution of different nAb levels (low, 72–200; moderate, 201–1000; high, > 1000) against HAdV26 and HAdV35 in different age groups. **D, E** The seropositive nAb titers to HAdV26 and HAdV35 in different age groups. The data was analyzed with the Kruskal-Wallis test.

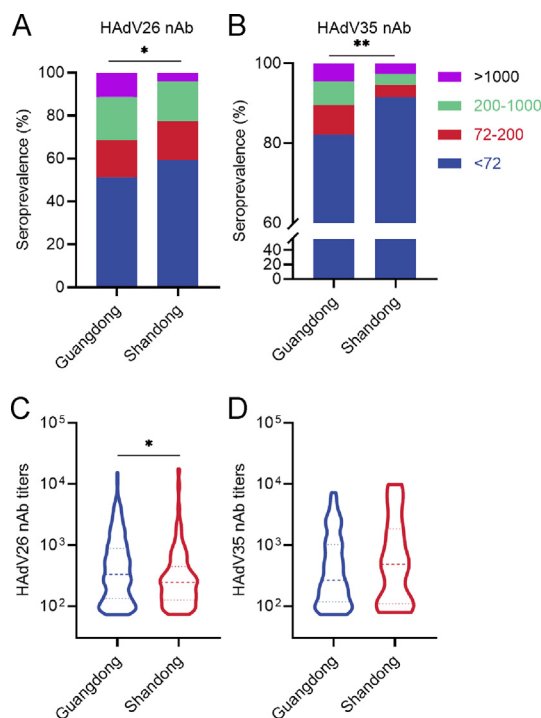


**Fig. 4.** The distribution of seroprevalence and titer of nAb against HAdV26 and HAdV35 in gender groups. **A, B** The seropositive rates of different nAb titer levels in different genders. The difference of overall seropositive rate between males and females was analyzed by Chi-square test, and a  $P$  of  $<0.05$  was considered statistically significant.  $**P < 0.01$ . **C, D** The seropositive nAb titers against HAdV26 and HAdV35 in different genders. The data were analyzed by Mann-Whitney test.

individuals, but did not reach a statistical difference (51.9%, 95% CI 44.6%–59.1% versus 46.0%, 95% CI 42.9%–49.1%; Chi-square test,  $P = 0.142$ ; Fig. 6A). Similarly, there was no statistical difference for the seropositive rates of HAdV35 nAb between HAdV26-seropositive and seronegative individuals (17.4%, 95% CI 14.3%–20.6% versus 14.3%, 95% CI 11.6%–17.1%; Chi-square test,  $P = 0.142$ ; Fig. 6A, Supplementary Table S4). The percentages of serum samples with high HAdV26 nAb titer in HAdV35-seronegative individuals were higher than that in HAdV35-seropositive individuals (Chi-square test,  $P < 0.001$ ; Fig. 6B). In contrast, the frequency of sera with low, moderate and high HAdV35 nAb titers in HAdV26-seropositive individuals were similar to those in HAdV26-seronegative ones (Chi-square test,  $P = 0.952$ ; Fig. 6C). Moreover, the titers of HAdV26 nAb in HAdV35-seropositive individuals were comparable to those in HAdV35-seronegative ones (Mann-Whitney test,  $P = 0.609$ ; Fig. 6D). Similarly, the titers of HAdV35 nAb among HAdV26-seropositive and HAdV26-seronegative individuals also did not reach a statistical difference (Mann-Whitney test,  $P = 0.616$ ; Fig. 6E). These results suggested that the HAdV26 infection in individuals had little correlation to the infection of HAdV35 and vice versa. Interestingly, HAdV35-seronegative individuals tend to have a higher HAdV26 nAb titer.

#### 4. Discussion

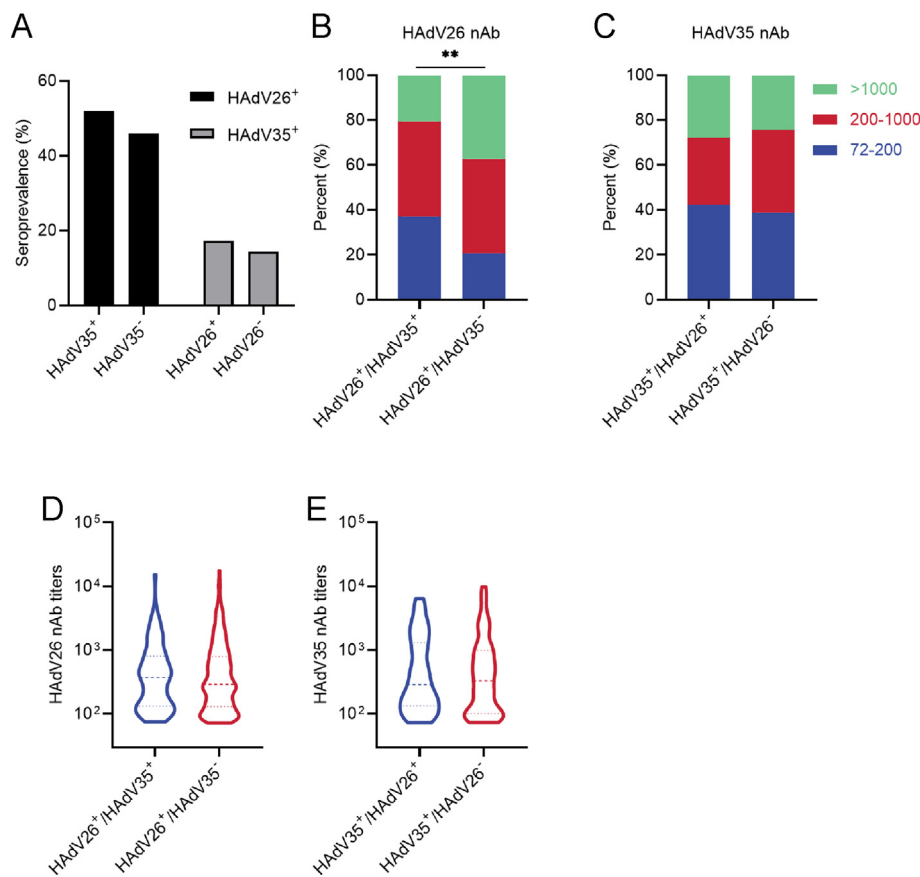
In this research, we established a neutralization assay based on the reporter virus HAdV26-SEAP and HAdV35-SEAP (Fig. 1). This method has been validated using the corresponding HAdV26- and HAdV35-immunized animal sera. For adenovirus neutralization assay, different research teams have used different methods, such as CPE-based methods (Zhang et al., 2013), green fluorescent protein (Xiang et al., 2006), luciferase (Barouch et al., 2011),  $\beta$ -galactosidase reporter virus (Kuriyama et al., 1998). These could cause variation of the values from different studies performed on similar populations (Supplementary Tables S5 and S6). The technical conditions, sample size, and cutoff values for determining positivity can also lead to the variations. These disparities make the comparison among different studies very difficult.



**Fig. 5.** The distribution of seroprevalence and titer of nAb against HAdV26 and HAdV35 in region groups. **A, B** The seropositive rates of different nAb titer levels in Guangdong and Shandong provinces, respectively. The difference of overall seropositive rate between Guangdong and Shandong provinces was analyzed by Chi-square test, and a  $P$  of  $<0.05$  was considered statistically significant. **C, D** The seropositive nAb titers against HAdV26 and HAdV35 in different region groups. The data were analyzed by Mann-Whitney test.  $*P < 0.05$ ,  $**P < 0.01$ .

Some studies used the serum titer of  $>16$  as cutoff value (Abbink et al., 2007; Omosa-Manyonyi et al., 2015), but from our results, distortions at high serum concentrations are often observed, possibly resulting from nonspecific neutralization. On the other hand, a high cutoff value may lead to the loss of serum samples with weak neutralization titers. In this study, we make the cutoff value of 72 to best reflect the real situation. In our previous work, we have also investigated nAb titers for HAdV5, HAdV4, HAdV7, HAdV14, HAdV55 using the similar method (Sun et al., 2011; Zheng et al., 2017; Ye et al., 2018) and the same cutoff value.

HAdV26 and HAdV35 have been developed as adenovirus vector for years. HAdV26-based vaccine expressing HIV-1 envelope protein (Env) can elicit broad and diverse antigen-specific humoral and cellular immune responses in humans (Baden et al., 2013; Barouch et al., 2013; Logunov et al., 2020a; Sadoff et al., 2021b; Vergnes, 2021). HAdV26-based respiratory syncytial virus (RSV) vaccine (Sadoff et al., 2021a), Ebola virus vaccine (Goldstein et al., 2020) and Zika virus vaccine (Salisch et al., 2021) exerted good immunogenicity and even protective efficacy in human clinical trials, making HAdV26 vector a promising candidate for further development. The ongoing COVID-19 pandemic calls for the development of new vaccination platforms. HAdV26 vector vaccine was a logical choice and has shown good immunogenicity and protection effect (Logunov et al., 2020a; Sadoff et al., 2021b; Skowronski and De Serres, 2021). The HAdV35 vector also has been explored for Ebola virus vaccine (Geisbert et al., 2011), RSV vaccine (Widjoatmodjo et al., 2015), HIV-1 vaccine (Xin et al., 2005), and malaria vaccine (Ouédraogo et al., 2013), either alone or combined with other adenoviruses vectors. In our study, the seropositive rate of HAdV26 nAb were 47.0% (Fig. 2A), which was much lower than that of HAdV4, HAdV5, HAdV7 nAb but higher than that of HAdV14 and HAdV55 in China. The seropositive rates of HAdV35 nAb



**Fig. 6.** The distribution of seroprevalence and titer of nAb against HAdV26 and HAdV35 in double-positive and single-positive samples. **A** The seropositive rates of HAdV26 and HAdV35 nAbs in double-positive and single-positive samples. The data were analyzed by Chi-square test. **B, C** The distribution of seropositive rates of different nAb titer levels in double-positive and single-positive samples. The data were analyzed by Chi-square test, and a  $P$  of  $<0.05$  was considered statistically significant.  $**P < 0.01$ . **D, E** The seropositive nAb titers against HAdV26 and HAdV35 in double-positive and single-positive samples. The data were analyzed by Mann-Whitney test.

were 15.8%, which was the lowest among the seven HAdVs investigated based on our previous studies (Sun et al., 2011; Zheng et al., 2017; Ye et al., 2018). Previous studies from other researchers demonstrated that, the seroprevalence of nAbs against HAdV26 and HAdV35 varied by regions, methods and cutoff value (Supplementary Tables S5 and S6). HAdV26 seroprevalence were moderate in adults in the developing world, ranging from 21.0% to 67.8% (Supplementary Table S6) (Abbink et al., 2007; Barouch et al., 2011), whereas HAdV26 seroprevalence in USA and Europe was 8.0%–25.4% (Barouch et al., 2011). In China, there was a report on HAdV26 with seroprevalence of 35.3% based on the methods of GFP reporter virus in 2013 (Zhang et al., 2013). There were no reports on the seroprevalence of HAdV35 in China. So far, HAdV5 has been the most commonly used vaccine vector, but the seroprevalence ranges from 74.2% to 94.0% in the developing world (Wang et al., 2014; Widjoatmodjo et al., 2015). Our finding demonstrated that HAdV35 had advantages over HAdV26 as vaccine vectors due to lower pre-existing immunity. These results revealed that pre-existing immunity should be considered when selecting an adenovirus as the vaccine vector in a region.

In our study, the titers of HAdV26 nAb were comparable with that of HAdV35 (Fig. 2A). The HAdV26-seropositive individuals mainly possessed low and moderate nAb titers, while HAdV35-seropositive individuals of the low, moderate and high nAb titer groups distributed similarly at a lower positive rate (Fig. 2B). The different distribution trends of nAb titers against the two viruses in healthy people suggest that the two viruses have different epidemiological history in the population.

Several studies revealed that seroprevalence of HAdV4, HAdV5, HAdV7, HAdV14 and HAdV55 were increased with age (Sun et al., 2011; Yu et al., 2012; Zheng et al., 2017; Ye et al., 2018). However, our data suggest that the seropositive rates of HAdV26 increase before age of 70 years old and decrease thereafter (Fig. 3A), and the occurrence of moderate and high nAb titers against HAdV26 tends to be high in people older than 40 years old (Fig. 3B). Neither the seropositive rates nor the nAb titer level of HAdV35 increased with age, comparing with previous results from Southeast Asia (Barouch et al., 2011; Mennechet et al., 2019). Both HAdV26 and HAdV35 had a very low seroprevalence rate in people younger than 20 years old. The irregular distribution of HAdV35 nAb may correlate with low seroprevalence rate and short circulation time in these population. These results suggested that young people were more suitable for adenovirus vector vaccine when taking pre-existing immunity into account.

From our results, the seroprevalence of HAdV26 in males was slightly higher than that in females (Fig. 4A), but the nAb titers of HAdV26 were not affected by gender (Fig. 4C). Similar to the results of other HAdVs (Barouch et al., 2011; Tian et al., 2016; Zheng et al., 2017; Ye et al., 2018), gender is not an important factor influencing either seropositive rates or nAb titers of HAdV35 (Fig. 4B and D). Intriguingly, our results showed that both HAdV26 and HAdV35 nAb seropositive rates were higher in Guangdong Province than in Shandong Province (Fig. 5A and B), and the nAb titers of HAdV26 were also higher in Guangdong Province (Fig. 5C). Together with the previous studies (Schmitz et al., 1983; Wadell et al., 1985; Barouch et al., 2011), these results demonstrated the

regional differences in the distribution of adenovirus seroprevalence. The possible reasons for this difference are that the temperate climates in Guangdong Province are more suitable for HAdV26 and HAdV35 than those in Shandong Province.

We found that the HAdV26 infection in individuals had little correlation to the infection of HAdV35 and vice versa (Fig. 6), and the anti-sera from animals immunized with HAdV26 also showed little cross-neutralizing activity to that immunized with HAdV35 (Fig. 2). These results suggested that a small portion of people might be more susceptible to adenovirus infection. They might be infected with HAdV26 and HAdV35 alone or simultaneously. The correlation of cross-reactivity may be attributed to the fiber protein, as some adenovirus originated from the recombination of other adenovirus (Roberts et al., 2006; Bradley et al., 2012).

## 5. Conclusions

In summary, we established MN assays based on reporter virus HAdV26-SEAP and HAdV35-SEAP, and investigated the seroprevalence of HAdV26 and HAdV35 in general population from Guangdong and Shandong provinces, China. HAdV vectors, as the vaccine and gene therapy platforms, are expected to become more applicable in the future. Our results provide insightful information for the rationale of HAdV26 and HAdV35 vector in different regions and populations.

## Data availability

All data relevant to the study are included in the article or uploaded as supplementary information.

## Ethics statement

Blood samples usage was approved by the Ethics Committee of the Second Affiliated Hospital of Guangzhou Medical University (No. 2020-hs-35). The mice immunization experiments were approved by the Institutional Animal Care and Use Committee (No. 2018027) of GIBH, CAS. The rabbit immunization experiments were approved by the Experimental Animal Center of Guangdong Province (No. B202102-2).

## Author contributions

Haisu Yi: designed the experiments, data curation, writing - original draft. Qian Wang: conceptualization and methodology. Jiankai Deng: blood samples preparation and characterization. Hengchun Li: methodology. Yingkun Zhang: blood samples preparation and characterization. Zhilong Chen: methodology. Tianxin Ji: data curation. Wenming Liu: formal analysis. Xuehua Zheng: supervision and validation. Qinghua Ma: blood samples preparation and characterization. Xinxin Sun: methodology. Yudi Zhang: software. Xuegao Yu: blood samples preparation and characterization. Mengzhang He: Project administration. Ling Chen: designed the experiments, funding acquisition. Ying Feng: visualization, editing, funding acquisition.

## Conflict of interest

The authors declare no conflict of interest.

## Acknowledgements

This work was supported by the Strategic Priority Research Program of the Chinese Academy of Sciences (XDB29050701), the Emergency Key Program of Guangzhou (EKPG21-20), the China Evergrande Group funding for SARS-CoV-2 (2020GIRHHMS22), the Zhongnanshan Medical Foundation of Guangdong Province (ZNSA-2022009), and the China Postdoctoral Science Foundation (2020M682942).

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.virs.2022.06.006>.

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