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Characterization of a replication-competent vector encoding DsRed based on a human adenovirus type 4 a-like strain

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ABSTRACT

Human adenovirus type 4 (HAdV4) is an etiological agent of acute respiratory disease (ARD) in pediatric and adult patients. HAdV4 strains can be divided into two major genomic clusters, namely prototype (p)-like viruses and a-like viruses. Here, the complete genome sequence of HAdV4 strain GZ01, isolated from a child with ARD in southern China, is first reported and analyzed. This strain was determined to be of the 4a1 genome-type based on *in silico* restriction profiles. Then, a replication-competent rAd4DsRed virus, containing the HAdV4 GZ01 infectious genome and expressing the reporter molecule DsRed, was generated and characterized. Recombinant rAd4DsRed can infect AD293, hamster, and mouse cells in which DsRed protein was expressed. No changes in antigenicity and genome replication were detected for rAd4DsRed and wild-type HAdV4. Mice immunized with rAd4DsRed was elicited a marked antibody response to DsRed. A rapid method of testing neutralizing antibodies against HAdV3 and HAdV4 was also established using a mixture of rAd4DsRed and rAd3EGFP. Our results provide the foundation to develop HAdV4 vaccines, potential vector platforms for vaccine and gene therapy, and rapid methods for serological and antiviral screening.

1. Introduction

Human adenovirus (HAdV), first identified in 1953 (Rowe et al., 1953), is an important pathogen associated with acute respiratory disease (ARD), acute gastroenteritis, epidemic keratoconjunctivitis, and genitourinary illnesses in both children and adults (Bhumbra and Wroblewski, 2010; Lenaerts et al., 2008; Sandkovsky et al., 2014). To date, at least 90 HAdV genotypes have been reported and defined using a new paradigm based on genomics, and these have been divided into seven species (A–G) (Ismail et al., 2018; Pan et al., 2018; Seto et al., 2011). Specific genotypes are often associated with particular clinical manifestations. HAdV4 is the only member of species E and is associated with severe ARDs in both pediatric and adult patients (Barrero et al., 2012; Chen et al., 2015; Guo et al., 2012; Houng et al., 2006; Kajon et al., 2018; Narra et al., 2016). HAdV4 (Ad4) is the principal etiological agent of febrile ARDs in US military recruits in the post-vaccination era and a vaccine was provided from 1971 to 1994 and resumed in 2011 (Gray et al., 2000; Hoke and Snyder, 2013; Kajon et al., 2007). HAdV4 also causes outbreaks of conjunctivitis and swimming pool-acquired pharyngoconjunctival fever (Artieda et al., 2009; D'Angelo et al., 1979; Kandel et al., 2010; Levandowski and Rubenis, 1981; Li et al., 2018; Muzzi et al., 1975; Schepetiuk et al., 1993; Van Der Veen and Van Der Ploeg, 1958). However, there is currently no vaccine approved for general use in children and civilian adults, and no efficient antiviral therapy is available for adenoviruses despite some candidates that are under development (Tian et al., 2018; Toth et al., 2018).

Conventional cell-based antiviral assays include plaque assays, cytopathic effect measurements, and quantitative PCR (or RT-PCR), which are time-consuming and labor-intensive. Accordingly, various reporter viruses have been generated to develop alternative rapid and reliable cell-based methods for high throughput antiviral assays (Zhang et al., 2017; Zheng et al., 2017). Reporter viruses can also be used to study the molecular mechanism of viral replication, pathogenicity, and fitness. Many recombinant HAdVs expressing enhanced green fluorescence (EGFP) or luciferase as a reporter have been developed (Pan et al., 2018; Zheng et al., 2017). Adenovirus vectors are promising candidates for vaccination and gene therapy against cancers and infectious

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diseases and some of them have entered clinical testing (Grobusch and Goorhuis, 2017; Gurwith et al., 2013). However, the clinical applications of current HAdV5-based vectors are limited by the high prevalence of pre-existing anti-vector immunity. Thus, developing new adenoviral vectors is necessary (Alonso-Padilla et al., 2016; Guo et al., 2018). HAdV4 could be developed as a safe option for gene therapy and as vaccine vectors for long-term use in military recruits in the USA (Alonso-Padilla et al., 2016; Bullard et al., 2018; Guo et al., 2018; Gurwith et al., 2013; Hsu et al., 1992; Schuldt et al., 2012; Weaver, 2014). Further, HAdV4 seroprevalence is 58.4% in healthy populations from southern China (Ye et al., 2018).

HAdV4 strains can be divided into two major genomic clusters, namely p-like and a-like viruses (Houng et al., 2006; Kajon et al., 2007; Li and Wadell, 1988). The genetic differences between the two branches were also previously found to be reflected by their neutralization titers (Crawford-Miksza et al., 1999). Over the past five decades, a-like variants have become markedly predominant in HAdV4-positive respiratory specimens from individuals with ARD in civilian and military populations in the United States (Kajon et al., 2018, 2007). Moreover, some studies have indicated that HAdV4 is an important type, causing ARD and pharyngoconjunctival fever in China (Chen et al., 2015; Guo et al., 2012; Li et al., 2018). Unfortunately, in recent years no studies have characterized HAdV4 isolates and associated genome data in China. Further, it is necessary to develop an HAdV4 vector based on an a-like strain. In this study, we report the first whole-genome sequence of HAdV4, an a-like strain, in China. We also developed and characterized a stable HAdV4 vector expressing the red fluorescent protein DsRed2 (DsRed), based on this GZ01 strain, which could be used for antiviral screening, the quantification of neutralizing antibodies, or as a vaccine vector.

2. Materials and methods

2.1. Viruses and cells

HAdV4 strain GZ01 (GenBank accession no. KF006344.1), a clinical isolate from a child with ARD in Guangzhou, China in 2008, was maintained in the State Key Lab of Respiratory Disease (Tian et al., 2018). The genome of HAdV4 GZ01 (35,960 bases) was extracted and amplified by PCR using the genomes of HAdV4 strains in GenBank as references, and then sequenced by the Sanger method. The HAdV4 GZ01 strain was cultured in AD293 cells from the ATCC, which were maintained in our lab (Tian et al., 2018). Recombinant rAd3EGFP (rAd3) expressing enhanced green fluorescent protein (EGFP) was previously generated based on the HAdV-3 GZ-01 strain (GenBank accession no. DQ099432) (Zhang et al., 2009). Sublines of AD293 cells, maintained in our laboratory, were cultured in Dulbecco's modified Eagle's medium (; Gibco, USA) supplemented with penicillin (100 IU/ml), streptomycin (100 μg/ml), and 10% foetal bovine serum (Gibco, USA). Adenoviral particles were purified by standard CsCl gradient centrifugation, as previously described (Tian et al., 2011). The virus particle (VP) titers were determined spectrophotometrically, using a conversion factor of 1.1×10^{12} VPs per absorbance unit at 260 nm (Tian et al., 2011). Viral genomic DNA was extracted with a TaKaRa MiniBEST Viral RNA/DNA Extraction Kit Ver.5.0 (Takara, Dalian, China) according to the manufacturer's instructions.

2.2. Bioinformatics

The complete genome sequences of HAdV4 strains from GenBank (GenBank Accession Numbers) were used for comparative analyses as follows: NHRC90339 (EF371058.2), V2029E/Georgia USA/1986 (KX384946.1), RI-67 (AY594253.1), vaccine (AY487947.1), HAdVE/USA_New Jersey/RU2533/1966/P4H4F4 (MF002043), RDU2954/New Jersey USA/1966 (KX384948.1), V0014/France/1978 (KX384956.1), V1933/New Mexico USA/1985 (KX384955.1), 186/Ft Jackson South Carolina USA/1998 (KX384952), NHRC 42,606 (AY599835.1), HAdVE/USA_New York/1418/2015/P4H4F4 (MF002042), ZG/Indio California USA/1995 (KX384951.1), NHRC 3 (AY599837.1), GZ01 (KF006344.1). Simian adenovirus 26 (FJ025923.1) and HAdV-3 GZ01 (DQ099432.4) were used as control sequences.

CLUSTAL_W was used for multiple sequence alignments of adenovirus genome sequences using default parameters. Phylogenetic trees were constructed by the neighbor-joining (NJ) method. Bootstrap analysis with 100 replicates was performed to estimate the robustness of specific tree topologies. The genome type of the HAdV4 GZ01 strain was determined by comparing the *in silico* restriction profiles with those of prototype and other genome types described in the literature, and according to the genome-type denomination system (Kajon et al., 2018, 2007; Li and Wadell, 1988).

2.3. Construction of the plasmid pBRAd4 harboring the full-length genome of HAdV4 GZ01

The plasmid pBRAd4 harboring the HAdV4 GZ01 genome was constructed with a previously described strategy using the highly efficient homologous recombination system in *E. coli* BJ5183 (Zhang et al., 2009) (Fig. 1). Briefly, the rescue plasmid pBRA4LR was constructed by cloning the PCR-generated left and right end fragments of the HAdV4 genome, namely A4L and A4R, respectively, into pBR322 (Takara, Dalian, China). The resultant pBRA4LR plasmid was linearized using the restriction enzyme *Hind*III and then dephosphorylated with calf alkaline phosphatase (NEB, MA, USA). The linearized rescued plasmid and the purified HAdV4 genomic DNA were mixed and transformed into *E. coli* BJ5183 (Stratagene, CA, USA) competent cells by electroporation. Plasmids from PCR-positive clones were then transformed into Top10 competent cells. The recombinant plasmids were prepared and identified using suitable restriction enzymes and DNA sequencing analyses. The positive plasmid pBRAd4 was digested with *Asi*SI to release HAdV4 genomic DNA, which was transfected into AD293 cells using LTX with Plus reagent (Invitrogen, CA, USA), according to the manufacturer's instructions. The transfected cells were cultured for 6 to 10 days and examined daily for evidence of cytopathic effects (CPEs). The cells were harvested, and then frozen and thawed for three cycles. The viral suspensions were collected and then used to infect newly-cultured AD293 cells. At 96 h post-infection, the virus was harvested and designated rAd4.

2.4. Generation of the E3-defective replication-competent DsRed expression vector rAd4DsRed

The plasmid pBR-Ad4-dE3-DsRed was constructed as shown in Fig. 1. First, pBRAd4 was digested with *Pac*I and *Sfi*I to extract the ~15-kb fragment, which was then blunted with the Quick Blunting Kit (NEB, MA, USA) and self-ligated with T4 DNA ligase to obtain the plasmid pBRAd4-PS. The plasmid pDsRed2-N1 (also named pDsRed-N1; Takara, Dalian, China) was digested by restriction enzymes *Xho*I and *Sal*I to construct pDsRed-N1XS and delete the *Eco*RI restriction sites. Then, pBRAd4-PS was digested with *Hpa*I and then dephosphorylated, whereas pDsRed-N1XS was cut with *Ase*I and *Afi*II and then blunted. The shuttle vector ps-Ad4-DsRedXS was obtained by ligating the two fragments with T4 DNA ligase. Finally, pBRAd4 and ps-Ad4-DsRedXS were digested with *Eco*RI. Both large fragments (approximately 25 and 12 kb, respectively) were extracted and then ligated to generate the plasmid pBR-Ad4-dE3-DsRed, which was screened by PCR using three pairs of primers (Pad4-25268u: 5′-gcccaattgcaagccatccaa-3′, Cmvr: 5′-taccgtaaatactccacccattg-3′; Pad4-26401u: 5′-cctaaaacagtcagcgctcacc-3′, Pad4-32194r: 5′-ttgccagccattatagctcctgt-3′; Ad4-17833u: 5′-actgggcctgcccaccacgcgtccca-3′, Ad4-20204r: 5′-ctctttggtcttgagacgcgtgaag-3′). The successful creation of pBR-Ad4-dE3-DsRed was confirmed by restriction digestion and DNA sequencing analyses. The recombinant rAd4DsRed virus was then rescued and purified using

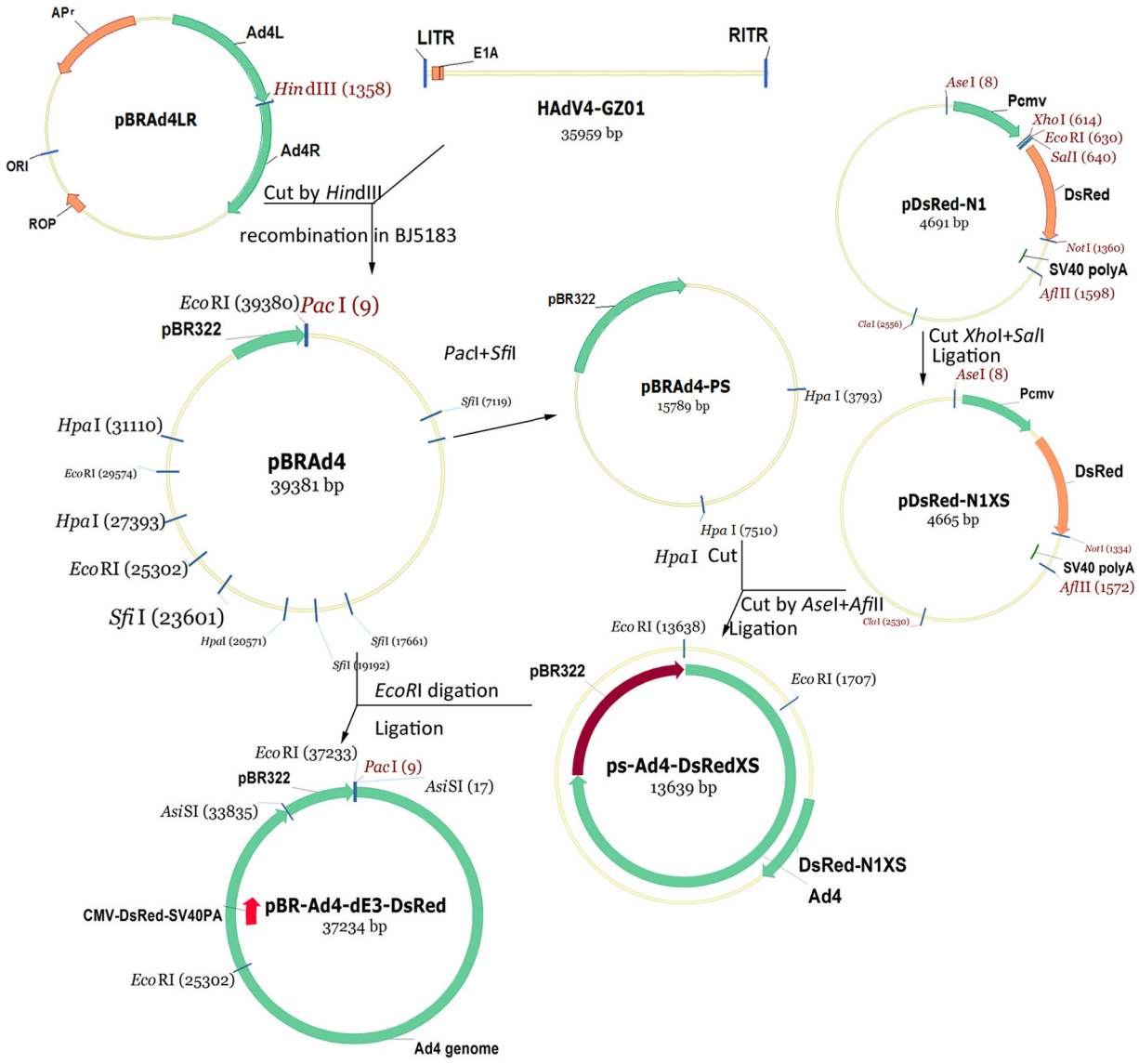

Fig. 1. Schematic depiction of construction of the infectious clone pBRAd4 and HAdV4 reporter vector pBR-Ad4-dE3-DsRed encoding the DsRed reporter gene.

AD293 cells, as described previously herein.

2.5. Growth characteristics of rAd4 and rAd4DsRed

To evaluate the genome DNA replication efficiency of rAd4 and rAd4DsRed, quantitative PCR (Q-PCR) was performed using a commercially available adenovirus Q-PCR kit (HYSMed, Guangzhou, China). The viral genomic DNA was extracted with a TaKaRa MiniBEST Viral RNA/DNA Extraction Kit Ver.5.0 (Takara, Dalian, China) according to the manufacturer's instructions. AD293 cells were cultured in 24-well plates and infected with 1×10^7 DNA copies of wild-type Ad4, rAd4, and rAd4DsRed. The infected cells were harvested with medium at various time points post-infection over 4 days and viral genomic DNA copy numbers were determined by Q-PCR.

AD293 cells were infected with serially-diluted rAd4DsRed to determine infectious virus titers by detecting fluorescence cell numbers (IFU). Mouse or hamster primary kidney cells were also infected with rAd4DsRed at 5 IFU/cell and observed with a fluorescence microscope for 5 days post-infection.

2.6. Recombinant peptides and polyclonal antisera

Recombinant DsRed protein was expressed in *E. coli* using the pET28a vector and purified with affinity chromatography using Ni-NTA His-Bind Resin (Novagen, EMD Millipore Corp., Billerica, MA, USA) under native conditions. Mouse sera against wild-type HAdV4, against rAd3EGFP, and the monoclonal antibody (MAb) MN4b, with neutralizing activity against HAdV4, were prepared as described previously (Tian et al., 2018).

Female Balb/c mice aged 4–6 weeks were immunized intramuscularly (i.m.) with 1×10^{10} VPs per mouse (approximately 10 μg of total protein) of rAd4DsRed or rAd4 without inactivation, followed by two additional booster injections at 2-week intervals with the same dose of antigen. Phosphate buffered saline (PBS) was injected into mice in the control group. Blood was collected from anesthetized mice via the retro-orbital lobe 10 days after the final immunization, and the sera were isolated, heat inactivated, and stored frozen for serology tests. The animal procedures used in this work were evaluated and approved by the Ethics Committee of the First Affiliated Hospital of Guangzhou Medical University (Guangzhou, China). They complied with all relevant guidelines and the National Law for Laboratory Animal Experimentation of China. The animal experiments were conducted in

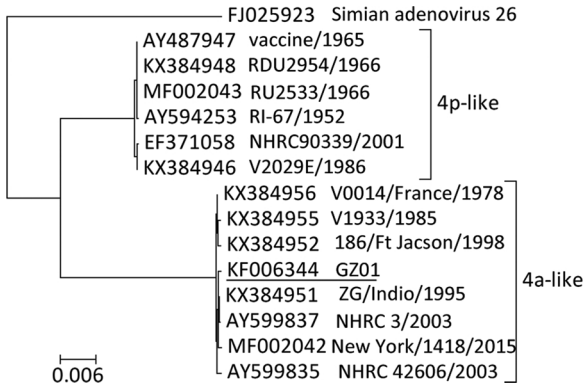

Fig. 2. Phylogenetic analysis of complete genomic sequences of human adenovirus type 4 reference strains and GZ01 strain. The phylogenetic tree was generated using the neighbor-joining algorithm. The sequence of Simian adenovirus 26 was included as the out-group control. GenBank accession numbers are in parentheses. Scale bar indicates substitutions per site.

strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health of United States (Council, 2011). All animals were housed individually and received humane care. During injection and sample collection, the mice were anesthetized with 1.5% isoflurane or 1 ml/kg body weight of 3% pentobarbital sodium to minimize suffering.

2.7. Immunoblot analysis

Purified rAd4DsRed, rAd4, Ad4, and rAd3EGFP were mixed with 5× loading buffer (10% sodium dodecyl sulfate [SDS], 5% 2-mercaptoethanol, 0.5% bromophenol blue, and 50% glycerol in 250 mM tris-HCl [pH 6.8]), heated for 5 min at 98 °C, and then separated by 12% SDS-polyacrylamide gel electrophoresis (PAGE) for Coomassie brilliant blue staining.

AD293 cells were infected with wild-type adenovirus or recombinant adenoviruses. At 72 or 96 h post-infection, cells were harvested and freeze-thawed three times. The suspensions were mixed with loading buffer and heated for 5 min at 98 °C (denatured). The samples were then fractionated on an SDS-polyacrylamide gel and transferred electrophoretically onto a polyvinylidene difluoride membrane. The membrane was blocked with 5% skim milk in PBS and then incubated with an anti-DsRed monoclonal antibody (GNI, Tokyo, Japan) at a dilution of 1:1000. The membrane was washed four times with 0.05% tween 20 in PBS (PBST) and exposed to a 1:5000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG secondary antibody (CWBio Inc., Beijing, China). After washing, the blots were developed with the 1-Step Ultra TMB Blotting Solution substrate (Thermo Scientific, Rockford, IL, USA) for 5–10 min at room temperature.

2.8. Indirect enzyme-linked immunosorbent assay (ELISA)

For ELISAs, 96-well Nunc MaxiSorp™ flat-bottom plates (Nunc, Roskilde, Denmark) were coated overnight with purified recombinant virus rAd4 or rAd4DsRed, rAd3EGFP, or wild-type Ad4 (approximately 10^{10} VPs/ml) in carbonate–bicarbonate buffer (pH 9.6) at 4 °C. They were then washed once with PBST and blocked for 2 h with 2% bovine serum albumin in PBST. The HAdV4-specific MAb MN4b and antiserum at a dilution of 1:10,000 were then added to each well and incubated for 1 h at 37 °C. The plates were washed four times with PBST and incubated for 1 h with a 1:8000 dilution of HRP-conjugated goat anti-mouse IgG (H + L) affinity-purified secondary antibody (CWBio Inc., Beijing, China). After the plates were washed four times with PBST, the products were visualized with TMB substrate (Thermo Scientific, Rockford, IL, USA). The reaction was stopped with 2 M H_2SO4, and the results were analyzed with an ELISA plate reader (Multiskan MK3; Thermo Scientific, USA) at 450 nm.

2.9. Virus neutralization tests

For in vitro adenovirus neutralization experiments, antiserum pretreated at 56 °C for 30 min and MAb MN4b were serially diluted 2-fold with DMEM, and 50-μl aliquots of each dilution were mixed with an equal volume (50% tissue culture infective doses [$TCID_{50}$] = 100) of wild-type or recombinant adenovirus. The antibody-virus mixtures were incubated for 1 h at 37 °C and transferred to 96-well plates containing 85–95% confluent monolayers of AD293 cells. After culture for 96 h, the monolayers were observed microscopically, and the neutralization titers were determined as the reciprocal of the highest dilution of mouse ascites or antiserum that protected the monolayer from visually observable CPEs.

2.10. Assay for the simultaneous detection of antibodies against Ad3 and Ad4

Designed to establish a rapid method to test antibodies against HAdV3 (Ad3) and Ad4 at the same time, equivalent amounts of rAd3-EGFP and rAd4-DsRed were mixed as a reporter virus mixture. Mouse serum anti-rAd3EGFP, anti-rAd4, or a mixture of both was serially diluted 10-fold with DMEM, and mixed with an equal volume of the reporter virus mixture ($TCID_{50}$ = 100 for each virus). The antibody–virus mixtures were incubated for 1 h at 37 °C and transferred to 96-well plates containing 85–95% confluent monolayers of AD293 cells. After culture for 24 h, the monolayers were observed under a fluorescence microscope.

3. Results

3.1. Phylogenetic analysis reveals that the HAdV4 GZ01 strain is of the 4a1 genome-type

HAdV4 strains could be divided into two branches, specifically namely 4p-like and 4a-like by restriction enzyme analysis, consistent with the result of whole genome phylogenetic analysis (Fig. 2). The 4a-like branch was further subdivided into two lineages, 4a1 and 4a2. The field strain HAdV4 GZ01 and some strains isolated from 1978 to 2015 were clustered in the 4a-like branch, which was further subdivided into two lineages. The genome-type of the HAdV4 GZ01 strain was determined to be 4a1 by comparing its restriction profiles with those of prototype and other genome types described in the literature and according to the genome-type denomination system (data not shown). The HAdV4 NHRC3 strain was used as the reference strain, and has been described as a 4a1 genome-type (Kajon et al., 2018). Comparing HAdV4 GZ01 and NHRC3 genomes, identities were 35928/35966 with nine gaps. Further, a 5-bp deletion was found in the noncoding region of the HAdV4 GZ01 L1 gene.

3.2. Generation of recombinant HAdV4 vectors

The plasmid pBRAdV4 was obtained by homologous recombination in *E. coli* BJ5183 using purified, linear HAdV4 genomic DNA and the shuttle vector pBRAd4LR (Fig. 1). pBRAdV4 and the shuttle plasmid ps-Ad4-DsRedXS (Fig. 3A) were then digested with the restriction enzyme *Eco*RI and then ligated using T4 DNA ligase. Finally, two positive pBRAd4-dE3-DsRed recombinants were obtained from 20 clones using this screening method. The plasmids pBRAdV4 and pBRAd4-dE3-DsRed were confirmed by restriction enzyme digestions (Fig. 3B and 3C).

The recombinant viruses rAd4 and rAd4DsRed were successfully rescued by transfecting AD293 cells with enzyme-digested, linearized pBRAdV4 and pBRAd4-dE3-DsRed, respectively. After 24 h, red fluorescence was detected in cells transfected with the linearized pBRAd4-dE3-DsRed DNA fragment. rAd4DsRed was used to infect AD293 cells,

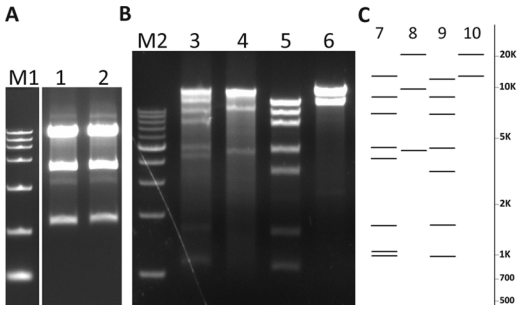

Fig. 3. Generation of recombinant HAdV4 plasmids pBRAd4 and pBRAd4-dE3-DsRed. (A) The shuttle plasmid ps-Ad4-DsRedXS was identified by restriction digestion using BamHI. M1: DNA marker DL15000. Lane 1, 1# clone, lane 2, 2# clone; (B) Restriction enzyme analysis of plasmid pBRAd4 and pBRAd4-dE3-DsRed. M2: 1-kb DNA ladder marker. Lane 3, pBRAd4/*Bam*HI; lane 4, pBRAd4/*Eco*RI; lane 5, pBRAd4-dE3-DsRed/*Bam*HI; lane 6, pBRAd4-dE3-DsRed/*Eco*RI; (C) The *in silico* restriction map of pBRAd4 and pBRAd4-dE3-DsRed genomes produced by Vector NTI 10.3.0 software. Lane 7, pBRAd4/*Bam*HI; lane 8, pBRAd4/*Eco*RI; lane 9, pBRAd4-dE3-DsRed/*Bam*HI; lane 10, pBRAd4-dE3-DsRed/*Eco*RI.

hamster kidney primary cells, and Balb/c mouse kidney primary cells. Intense red fluorescence was observed not only in AD293 cells, but also in hamster and Balb/c cells, at 72 h post-infection with rAd4DsRed (Fig. 4).

SDS-PAGE analysis demonstrated that the protein compositions of the purified rAd4 and rAd4DsRed virions were similar to those of wild-type Ad4 (Fig. 5A). Indirect ELISAs (Fig. 5B) indicated that purified rAd4, rAd4DsRed, and Ad4 virions could be detected by an anti-Ad4 polyclonal antibody. Further, whole rAd3EGFP particles were also detected by an anti-Ad4 polyclonal antibody, but the reaction was much weaker. MN4b, a neutralizing MAb that specially detects the Ad4 hexon, recognized rAd4, rAd4DsRed, and Ad4, but not rAd3EGFP; anti-PBS did not react with these virions. Further neutralization tests (Fig. 5C) demonstrated that rAd4, rAd4DsRed, and Ad4 could be neutralized by MN4b and antisera against Ad4. However, the control rAd3EGFP was not neutralized by MN4b and anti-rAd4 serum. Moreover, anti-PBS did not neutralize these viruses. To confirm the expression of DsRed2 by rAd4DsRed, AD293 cells were infected with rAd4DsRed and analyzed by western blotting with a DsRed2-specific MAb (Fig. 5D). A band was visible in rAd4DsRed-infected cell extracts (lane 2), but not in rAd4-infected or Ad4-infected cell extracts. There was also a visible band with the purified recombinant rDsRed protein, as the control.

The replication of Ad4, rAd4, and rAd4DsRed was compared through the quantification of genomic DNA using Q-PCR (Fig. 6). The replication kinetics of rAd4DsRed was almost identical to that of rAd4 and wild-type Ad4. Further, the E3 deletion had no apparent effect on the replication efficiency of rAd4DsRed.

3.3. Antibody responses in mice immunized with rAd4DsRed

Wild-type Ad4 and rAd4DsRed induced comparable antibody responses against wild-type Ad4, rAd4, and rAd4DsRed in mice after a

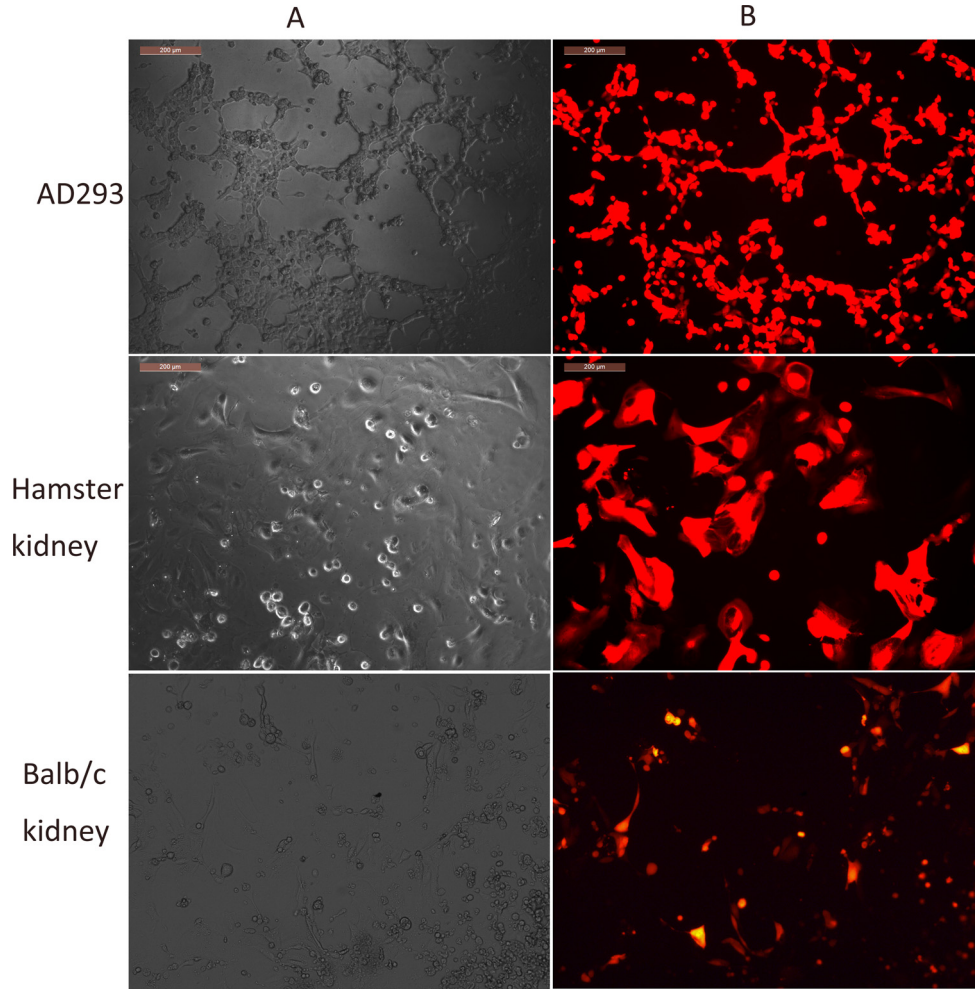

Fig. 4. HAdV4-mediated-DsRed expression in AD293 cells, hamster primary kidney cells, and Balb/c mouse primary kidney cells. Cells were observed under a fluorescence microscope 72 h post-infection with rAd4DsRed. (A) Visible light (200×). (B) Fluorescent light (200×).

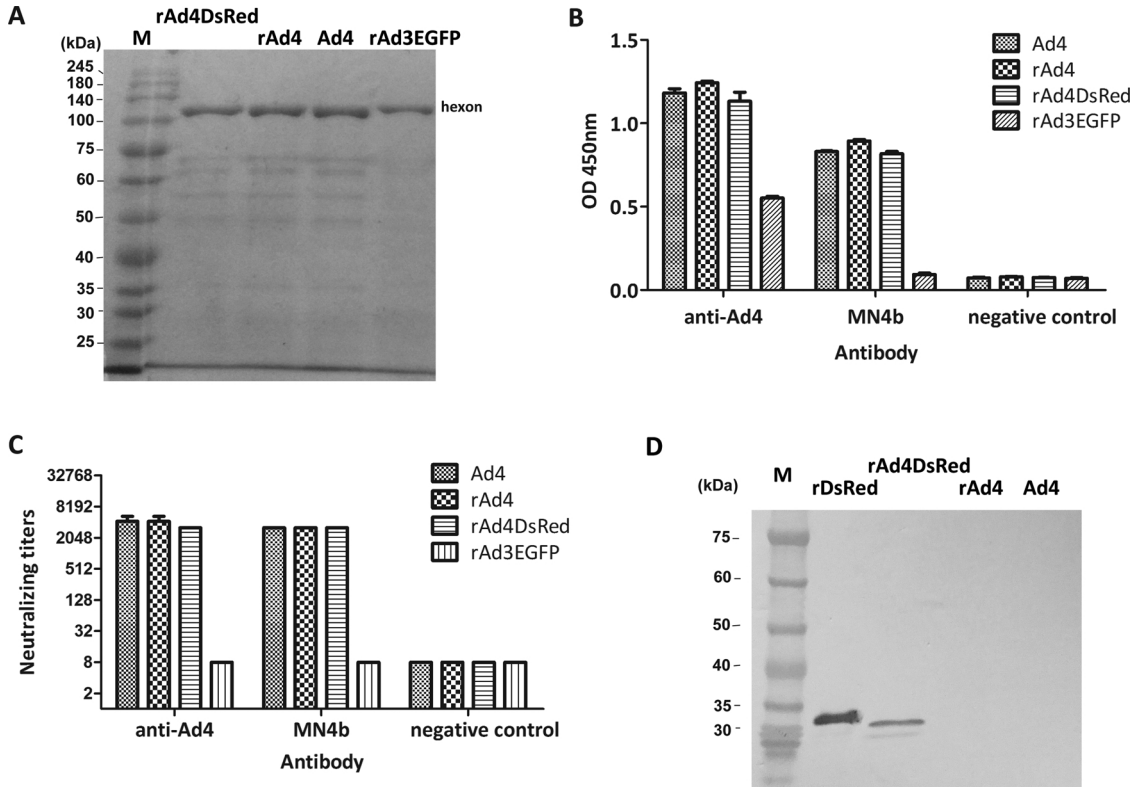

Fig. 5. Confirmation of recombinant rAd4 and rAd4DsRed viruses. (A) Confirmation of purified rAd4 and rAd4DsRed virions by SDS-PAGE. M: protein marker (kDa). (B) Indirect ELISAs. (C) Neutralization tests were performed to assess the reactions between MN4b or anti-Ad4 and purified rAd4 or rAd4DsRed virions. Whole viral particles of wild-type HAdV4 (Ad4) and rAd3EGFP were used as controls. Anti-serum from mice immunized with PBS was used as the negative control. Each experiment was repeated independently at least three times, and the means and standard deviations are shown. (D) Immunoblot analyses of AD293 cells infected with rAd4DsRed (lane 2) or rAd4 (lane 3), as well as recombinant DsRed protein (lane 1), as performed using a monoclonal antibody to detect DsRed (GNI; Tokyo, Japan). M: pre-stained protein marker.

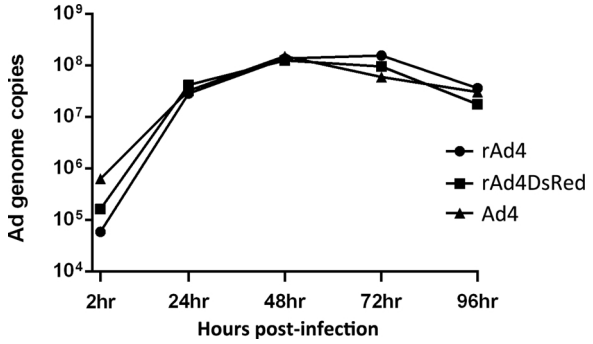

Fig. 6. Genome replication curves of Ad4, rAd4, and rAd4DsRed. Cells were harvested at 2, 24, 48, 72, and 96 h post-infection. The numbers of viral genomic DNA copies were determined by quantitative PCR.

third immunization (Fig. 7A and 7B). The antibodies in mice immunized with Ad4 or rAd4DsRed were also found to partially cross-react with rAd3EGFP, as evaluated by ELISA (Fig. 7A). However, these antibodies did not neutralize rAd3EGFP (Fig. 7B). Immunization with rAd4DsRed induced marked antibody responses against the transgene product DsRed in mice (Fig. 7C). However, no antibody against DsRed was detected in mice inoculated with wild-type Ad4 (Fig. 7C).

3.4. Assay for the simultaneous detection of neutralizing antibodies against Ad3 and Ad4 based on green and red fluorescence

As shown in Fig. 8, at 24 h post-infection, control cells infected with 100 $TCID_{50}$ of rAd4-DsRed or rAd3-EGFP did not show obvious CPEs, but fluorescence could be observed and measured in these cells. The excitation and emission wavelengths of DsRed are 554 and 586 nm, whereas those of EGFP are 488 and 507 nm. There was no cross-detection between DsRed and EGFP. As expected, anti-rAd3 was found to neutralize rAd3EGFP, but not rAd4DsRed, whereas anti-rAd4DsRed neutralized rAd4DsRed, but not rAd3EGFP; moreover, the mixture of anti-rAd3 and anti-rAd4DsRed could neutralize both rAd3EGFP and rAd4DsRed. To detect neutralizing antibodies against Ad3 and Ad4 in the same well, the equivalent amount rAd3-EGFP and rAd4-DsRed were mixed as a reporter virus mixture. Based on green fluorescence, the neutralizing antibody against Ad3 could be detected with anti-rAd3 or anti-rAd3 + anti-rAd4, whereas no neutralization was detected with anti-rAd4 as a control. Based on red fluorescence, the neutralizing antibody against Ad4 could be detected with anti-rAd4 or anti-rAd3 + anti-rAd4, but neutralization was not detected with anti-rAd3 as a control. Therefore, this assay provides a quick method to simultaneously assess neutralizing antibodies against Ad3 and Ad4 by measuring DsRed and EGFP fluorescence 24 h after infection with a rAd4-DsRed and rAd3-EGFP mixture.

4. Discussion

In this study, we constructed the adenovirus vector rAd4DsRed, based on the genome of strain HAdV4 GZ01, which encodes the reporter gene DsRed in the E3 region. This represents the first whole-genome sequence of HAdV4 in China. This field HAdV4 GZ01 strain was determined to be of the 4a1 genome-type based on *in silico* restriction profiles. The HAdV4 GZ01 strain was isolated from a child with ARD in Guangzhou, China in 2008. Some reports have indicated that a-like variants have become markedly predominant among ARD-

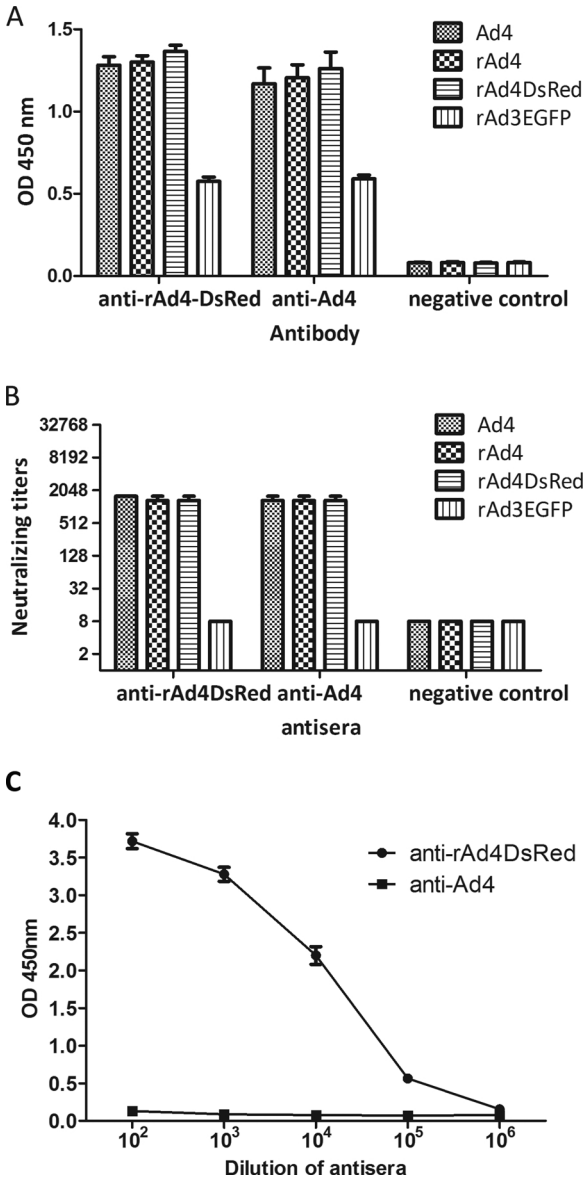

Fig. 7. Antibody responses in mice immunized with rAd4-DsRed. (A) Anti-rAd4-DsRed and anti-Ad4 antibodies in mouse sera were detected by ELISAs with purified adenoviruses as the coating antigen. Wild-type HAdV4 (Ad4) and rAd3EGFP were used as controls. Anti-sera from mice immunized with PBS were used as the negative control. (B) In vitro neutralization tests of anti-sera from mice immunized with rAd4-DsRed. Anti-sera from mice immunized with wild-type Ad4 were used as the positive control; anti-sera from mice immunized with PBS were used as the negative control. (C) Antibody responses to DsRed in mice immunized with rAd4DsRed were detected by ELISA. Anti-sera of wild-type Ad4 were used as the control.

causing HAdV4 strains in civilian and military populations in the United States over the past five decades (Hang et al., 2017; Houng et al., 2006; Kajon et al., 2018, 2007). Unfortunately, in recent years no studies have characterized HAdV4 isolates and associated genome data in China. Therefore, it is necessary to maintain continuous surveillance of field strains. It might also be important to develop HAdV4 vectors based on a-like strains for antiviral screening and the quantification of neutralizing antibodies.

Wild-type HAdV4 GZ01, recombinant rAd4, or rAd4DsRed induced similar high-titer neutralizing antibody responses in mice. These results indicate that these viruses could be developed as HAdV4 vaccine candidates. Such candidates might include live, oral, or attenuated recombinant vaccines. The rAd4DsRed vector was also found to express the reporter DsRed in cells and induced a high-level antibody response against DsRed in mice. This vector could be further developed as a gene therapy and vaccine vector. In recent years, some novel adenoviral vectors have been developed to circumvent pre-existing anti-vector immunity (Alonso-Padilla et al., 2016; Guo et al., 2018). HAdV4 uses the coxsackie and adenovirus receptor (CAR) as a cell receptor that is expressed in diverse cells and tissues. The HAdV4 seroprevalence is 58.4% in healthy adults of southern China (Ye et al., 2018). Live, oral adenovirus vaccines based on HAdV4 have been used in the US military for many years, and no severe side effects have been reported. Based on a test of live, oral vaccines based on HAdV4 and HAdV7 in volunteers, the infection was limited to the intestinal tract and did not cause illness, except for in some volunteers who apparently developed conjunctivitis caused by the self-transmission of fecal material (Couch et al., 1963; Zhu et al., 2008). An oral, replication-competent recombinant HAdV4 vector vaccine expressing influenza H5 hemagglutinin was also proven to be safe and effective using an animal model, and a further phase 1 clinical trial demonstrated its safety and immunogenicity (Alexander et al., 2012; Gurwith et al., 2013). In this study we also found that rAd4-DsRed could infect hamster and mouse primary kidney cells, express DsRed in these cells, and induce high-level antibody responses in mice, which could indicate that mice or hamsters might be a suitable animal model for HAdV4 vector research.

Many fluorescent proteins, luciferases, or alkaline phosphatases have been developed as viral reporter genes. Reporter adenoviruses can be used to establish rapid and reliable cell-based methods for high-throughput antiviral assays (Aste-Amezaga et al., 2004; Sprangers et al., 2003; Zheng et al., 2017). Here, rAd4DsRed was generated using DsRed, a red fluorescent protein, as the reporter. Fluorescence could be observed after only 24 h, and was used to assess neutralization tests based on rAd4DsRed; alternatively, the CPE method requires 2–7 days. Thus, our assay provides a rapid method and an objective readout to determine HAdV4-neutralizing antibody status. We also established a rapid method to test neutralizing antibodies against Ad3 and Ad4 by using a mixture of rAd4DsRed and rAd3EGFP. These two viruses did not affect each other during concurrent infections. This method might also be useful for assays on antiviral drugs. Furthermore, it will also be possible to develop antiviral assays by mixing more recombinant adenovirus types based on different reporter genes. In conclusion, this study provides the basis for the development of recombinant live HAdV4 vaccines, vector platforms for vaccine and gene therapy, and rapid anti-viral screening methods.

Author contributions

TXG designed and drafted the paper and performed most experiments; CY performed some experiments and edited the paper; LHT performed genome sequencing; FY and CSY prepared some viruses; ZZC and LX performed some animal experiments; ZR supervised the work and edited the final version of this manuscript. All authors read and approved the final version of the manuscript.

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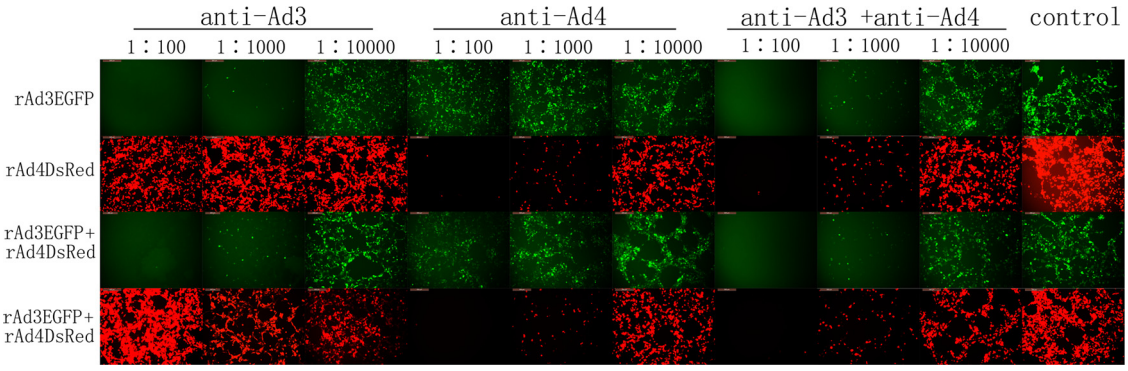

Fig. 8. Neutralization tests with rAd4DsRed and rAd3EGFP. Fluorescence of AD293 cells was observed by fluorescence microscopy 24 h post-infection with rAd4DsRed, rAd3EGFP, or a mixture of rAd4DsRed and rAd3EGFP after incubation with serially-diluted corresponding anti-sera. Shown is fluorescent light (200×).

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