Recombinant Adenovirus Expressing Vesicular Stomatitis Virus G Proteins induce both humoral and cell-medicated immune responses in Mice and Goats

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Abstract

Background: With a human type 5 replication-defective adenovirus expression vector, we constructed the three recombinant adenoviruses (rAd) and expressed the Vesicular Stomatitis Virus (VSV) Indiana serotype glycoprotein (VSV-IN-G), VSV New Jersey serotype glycoprotein (VSV-NJ-G), and the G fusion protein [two serotypes G (VSV-IN-G-NJ-G)]. Three rAds were named rAd-IN, rAd-NJ, and rAd-IN-NJ. The three rAds were inoculated into AAV-293 cells, and the AAV-293 cells were serially propagated to 20 generations until the virus titers were stable, then TCID50 was determined. In direct immunofluorescence and western blot were used for detecting the expression of the target proteins and lymphocyte proliferation test was used for immune cell numbers. Results: The results showed that G proteins we expressed with good reactogenicity. The rAds were used to subcutaneously inoculate mice three times with 2-week intervals, and goats two times with 3-week intervals, respectively. On 0, 2, 4, and 6 weeks of post-inoculation for the mice and 0, 3, 6, 9, and 12 weeks for goats, their sera were collected and NT antibodies were determined. The results showed that the rAds could induce the production of VSV antibodies in the mice, and VSV NT antibodies in the goats. The antibody levels were 1:16 to 1: 32 in mice, and 1:32 to 1: 64 in the goats. The rAds induced strong immune lymphocyte proliferations in mice and goats, which was significantly higher than those of the negative control groups.

Conclusion: The three rAds expressed VSV-G proteins at high levels, and induced humoral and cellular immune responses in both mice and goats, which laid a foundation for further studies of the recombinant adenovirus vaccines expressing VSV glycoprotein.

Background

Vesicular stomatitis (VS) is an acute and highly contagious zoonotic infectious disease in cattle, horses, pigs, and other mammals, which is caused by the vesicular stomatitis virus [1–3]. This disease is characterized by vesicular lesions on the mouth (lips, gums, tongue), nostrils, coronary band, and teats [4]. VS is in the list of the animal diseases of obligatory report, notifiable to OIE (Office International des Epizooties, World Organization for Animal Health) [5, 6]. Two main serotypes of VSV, New Jersey (NJ) and Indiana (IN), have been described by neutralization test and complement fixation test [7, 8]. These two viruses are morphologically similar and also similar in their pathology but they generate distinct neutralizing antibodies in infected animals [9]. The cross-protection between the two serotypes is poor, and so different serotypes of vaccines have to be used against New Jersey virus (VSV-NJ) and Indiana virus (VSV-IN) [10, 11]. From 2004 through 2006, 751 vesicular stomatitis outbreaks, which were caused by vesicular stomatitis virus serotype New Jersey (VSV-NJ), were reported in nine states of the northwestern United States [12]. The VS outbreaks occurred in Mexico from the 2005 to 2011 was also induced by VSV-NJ, which spread from the US to Mexico and did not produce a new variant strain [13]. The disease can be prevented and controlled by vaccinations and there have been some achievements in searching new vaccines against VSV, while a great effort is still needed in new vaccine development [10, 11, 14].
The recombinant adenovirus (rAd) vector has been used to obtain the transient inducible expression [15]. Adenovirus vector is divided into replication-defective form and replication form [16]. The replication-defective adenovirus vector with all the structure genes being removed, keeps only the vital fragments for cis-acting elements and the packaging signal sequence, so its cytotoxicity is significantly reduced. Foreign sequences of up to 37 Kb can be inserted into the vector and the vector is capable of replication in any cell and were normally permissive for human rAd. In addition, the CMV promoter is also inserted into vectors to make efficiently express foreign genes. The expression system is a main vector for the research of rAd vectored vaccine at present time [17]. rAd vector vaccines have shown some promise as a single dose vaccine in mice against respiratory syncytial virus, Mycobacterium tuberculosis, and measles virus [18–20]. Rojas J M constructed the rAd expressing the peste des petits ruminants virus (PPRV) F or H proteins, and used to vaccinate sheep by intramuscular inoculation. The results indicated that these adenovirus constructs could be a promising alternative to current vaccine strategies for the development of PPRV DIVA vaccines [21]. A replication-defective adenovirus 5 with foot-and-mouth disease virus (FMDV) capsid and 3C proteinase coding regions (Ad5-FMDV3CWT) was used to vaccinate swine, and their results showed that the construct was unable to induce protection against FMDV challenge [22–24].

VSV-G gene for vesicular stomatitis virus glycoprotein (G) which contains the glycosylation sites and the antigenic determinant [25]. VSV-G positions on the protrusions of the envelope and stimulates the production of neutralizing antibodies, as well as presenting the specific type strains. VSV-G is the preferred antigen for VSV engineered vaccine research [26]. Our study used VSV-G gene as the candidate gene, examined the rAd vaccine to express two serotypes G protein and the fusion G proteins of VSV by a replication-defective human adenovirus type 5 expression vector, and evaluated the analysis of immunogenicity in mice and goats.

**Results**

**Generation and identification of recombinant adenovirus**

The VSV-IN-G gene (1536 bp) and VSV-NJ-G gene (1554 bp) were amplified by PCR and the VSV-IN-NJ-G gene (3100 bp) was amplified by overlapping PCR with the polypeptides GlyGlyGlyGlySer successfully. The three genes were cloned into the adenovirus shuttle vector pacAd-CMV K-NpA, and we obtained three recombinant shuttle plasmids which were named with pAd-VSV-IN-G, pAd-VSV-NJ-G and pAd-VSV-IN-G-NJ-G. The recombinant shuttle plasmids were identified by PCR, restriction and DNA sequencing. The recombinant shuttle plasmids and adenovirus backbone plasmid linearized with PacI restriction enzyme were co-transfected to the AAV-293 cells. After ten-day incubation period, the CPE appeared on the cells (Fig. 1). The three kinds of recombinant adenovirus were collected and named with rAd-IN, rAd-NJ, rAd-IN-NJ, respectively. They were serially propagated to 4 generations, then the viruses were inoculated into AAV-293 cells. And the cells infected with virus had CPE after 48 h ´ incubation. The target genes were amplified rightly by PCR with the specific primers (Fig. 2).
Production And Characterization Of Recombinant Adenovirus

The recombinant adenovirus rAd-IN, rAd-NJ and rAd-IN-NJ were inoculated into the AAV-293 cells were serially propagated to 20 generations and the virus titers were stable by determining the TCID\textsubscript{50}. The rAds of 5th, 10th, 15th and 20th generation were collected to extract viral DNA using AxyPrep\textsuperscript{TM} Body Fluid Viral DNA / RNA Miniprep Kit, and then the target genes could be identified by PCR and DNA sequencing correctly, this matched the expected results((Fig. 3), while the control group without any stripe. The TCID\textsubscript{50} of the rAd-IN of 5th, 10th, 15th and 20th generation were $10^{-8.56}$/mL, $10^{-8.16}$/mL, $10^{-8.96}$/mL and $10^{-8.34}$/mL. The TCID\textsubscript{50} of the rAd-NJ of 5th, 10th, 15th and 20th generation were $10^{-7.76}$/mL, $10^{-8.16}$/mL, $10^{-7.67}$/mL and $10^{-8.2}$/mL. The TCID\textsubscript{50} of the rAd-IN-NJ of 5th, 10th, 15th and 20th generation were $10^{-6.96}$/mL, $10^{-7.16}$/mL, $10^{-7.67}$/mL and $10^{-7.2}$/mL. These results suggested that the recombinant adenovirus have a certain genetic stability.

Detection of protein expression by western blot analyses and IFA

The recombinant adenovirus infected AAV-293 cell to detect the expression of G proteins of VSV. The expression of VSV-IN-G or VSV-NJ-G proteins, as well as the fusion protein of two serotypes into recombinant adenovirus was confirmed by western blotting using anti-VSV-IN-G protein mouse McAb and Anti-VSV-NJ goat PcAb. On the blots, the presence of 57 kDa, 57 kDa, and 114 kDa bands corresponds to the VSV-IN-G, VSV-NJ-G, and the fusion proteins, respectively (Fig. 4 and Fig. 5). In the detection, the rAd-IN and the rAd-NJ groups were detected by the antibodies of corresponding serotype, respectively. As the rAd-IN-NJ contained VSV-IN-G gene and VSV-NJ-G gene, so anti-VSV-IN-G protein mouse McAb and Anti-VSV-NJ goat PcAb were used to detect the fusion protein of two G proteins.

The Vero cells were infected by rAd-IN, rAd-NJ or rAd-IN-NJ to detect the expression of VSV-G proteins in Vero Cells by IFA. And the cells were incubated at 37 °C, 5% CO\textsubscript{2} for 48 h, then the IFA experiment was conducted by the method described in 2.3. The result of IFA showed that the experiment groups of rAd-IN, rAd-NJ or rAd-IN-NJ showed specific green fluorescence that was not detected in the negative controls group of wtAd (Fig. 6 and Fig. 7). These results demonstrate that the VSV-G proteins and the recombinant fusion G protein could be expressed in vitro following the recombinant adenovirus infect Vero cells and retained its antigenic reactivity.

Neutralizing Antibody Responses

The neutralizing activity against VSV is an indicator of functional antibodies that provide immune protection. Therefore, we investigated the neutralizing antibodies of mice immunized with recombinant
adenovirus containing VSV-G genes, using a micro-neutralization assay. The results suggested that the mice can produce neutralizing antibodies after incubation. The titers of VNA in all experimental groups were low after first immunization of mice and goats, but these titers increased significantly after the booster immunization. As shown in Table1 and 3, VNA was detected in mice and goats after incubated with rAd-IN or rAd-IN-NJ, and the titers ranged from 1:16 to 1:32 after booster immunization, and the rate was greater than or equal to 50%. Also as shown in Tables 2 and 4, the titers of VNA exceeded 10 at 4 weeks after primary immunization in the rAd-NJ or rAd-VSV-NJ-G included mice groups, which was a number that represented a margin considered to be protective in the goats vaccinated with live attenuated VSV vaccines [19]. Moreover, with the stimulation of VSV-IN, the titer of the rAd-NJ group was lower compared to that of the rAd-IN or rAd-IN-NJ-vaccinated goat groups. At the same time, when using the VSV-NJ to stimulate, the titers of the rAd-NJ and rAd-IN-NJ groups were higher than the group of rAd-IN. However, all the mice that had been immunized with rAd showed VNA titers above 10 after 2 weeks booster immunization, except the group of rAd-IN stimulated by VSV-NJ. As expected, no neutralization activity against VSV was detected in the pre-immune sera or in the sera of experimental animals injected with wtAd.

![Table 1](image)

**Detection of VSV neutralizing antibodies in mice induced by recombinant adenovirus by VSV-IN**

<table>
<thead>
<tr>
<th>Group</th>
<th>Weeks after first immunization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>rAd-IN</td>
<td>0 (10/10)</td>
</tr>
<tr>
<td></td>
<td>0 (3/10)</td>
</tr>
<tr>
<td>rAd-IN-NJ</td>
<td>0 (10/10)</td>
</tr>
<tr>
<td></td>
<td>0 (6/10)</td>
</tr>
<tr>
<td>wAd</td>
<td>0 (10/10)</td>
</tr>
<tr>
<td>PBS</td>
<td>0 (10/10)</td>
</tr>
</tbody>
</table>
Table 2
Detection of VSV neutralizing antibodies in mice induced by recombinant adenovirus by VSV-NJ

<table>
<thead>
<tr>
<th>Group</th>
<th>Weeks after first immunization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>rAd-IN</td>
<td>0 (10/10)</td>
</tr>
<tr>
<td></td>
<td>0 (5/10)</td>
</tr>
<tr>
<td>rAd-NJ</td>
<td>0 (10/10)</td>
</tr>
<tr>
<td></td>
<td>0 (4/10)</td>
</tr>
<tr>
<td>rAd-IN-NJ</td>
<td>0 (10/10)</td>
</tr>
<tr>
<td></td>
<td>0 (4/10)</td>
</tr>
<tr>
<td>wtAd</td>
<td>0 (10/10)</td>
</tr>
<tr>
<td>PBS</td>
<td>0 (10/10)</td>
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</tbody>
</table>

Table 3
Detection of VSV neutralizing antibodies in goats induced by recombinant adenovirus by VSV-IN

<table>
<thead>
<tr>
<th>Group</th>
<th>Weeks after first immunization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>rAd-IN</td>
<td>0 (4/4)</td>
</tr>
<tr>
<td></td>
<td>1:4 (1/4)</td>
</tr>
<tr>
<td>rAd-NJ</td>
<td>0 (4/4)</td>
</tr>
<tr>
<td></td>
<td>1:4 (1/4)</td>
</tr>
<tr>
<td>rAd-IN-NJ</td>
<td>0 (4/4)</td>
</tr>
<tr>
<td></td>
<td>1:4 (1/4)</td>
</tr>
<tr>
<td>wtAd</td>
<td>0 (4/4)</td>
</tr>
<tr>
<td>PBS</td>
<td>0 (4/4)</td>
</tr>
</tbody>
</table>
Table 4
Detection of VSV neutralizing antibodies in goats induced by recombinant adenovirus by VSV-NJ

<table>
<thead>
<tr>
<th>Group</th>
<th>Weeks after first immunization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>rAd-IN</td>
<td>0 (4/4)</td>
</tr>
<tr>
<td>rAd-NJ</td>
<td>0 (4/4)</td>
</tr>
<tr>
<td>rAd-IN-NJ</td>
<td>0 (4/4)</td>
</tr>
<tr>
<td>wtAd</td>
<td>0 (4/4)</td>
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<tr>
<td>PBS</td>
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</table>

Lymphocyte Proliferation Response

To investigate the cell-mediated immune responses induced by the three kinds of recombinant adenovirus, the lymphocyte proliferative responses from mice and goats were analyzed at the time of 2 weeks after the third immunization. VSV-IN and VSV-NJ were two different irritants to stimulate the lymphocyte in different experiment groups. The results showed that the mice immunized with recombinant adenovirus exhibit significantly stronger lymphocyte proliferation responses whether with VSV-IN or VSV-NJ, compared with the PBS and wtAd control group (Fig. 8 and Fig. 9). When VSV-IN was used to stimulate the lymphocyte, the lymphocyte proliferative responses of rAd-IN group and rAd-IN-NJ group were stronger with the SI value about 2.5. In addition, the stimulation index (SI) value of the rAd-IN-NJ group was relatively higher than that of rAd-IN and rAd-NJ groups in mice. There was significant difference was between the rAds and the control groups, but no significant difference among mice immunized with different rAds (P > 0.05).

Discussion

VSV has a limited human pathogenicity. Vesicular stomatitis (VS) is an acute viral zoonotic disease, and also an economically important disease in cattle, horses and swine. Thus, effective vaccination is very important for VS prevention. Currently, controlling the spread of VS is mainly dependent on the immunization of susceptible animals that have received live attenuated vaccines or inactivated vaccines. However, virulence reversion of the vaccine is relying on the development of heat-stable, safe, and effective VS vaccines.
Cellular immune responses, particularly those associated with CD3 + CD8 + cytotoxic T lymphocytes (CTL), play a primary role in controlling viral infection. Recent studies in both humans and nonhuman primates suggest that CTL control virus replication and delay disease progression [27]. Adenovirus vector expression system can efficiently integrate and express the exogenous genes, and also effectively stimulate a high level of humoral and cellular immune response against the corresponding target antigens. Although the comparative researches are in the works between adenovirus vector vaccine and genetic engineering subunit vaccines, the existing data suggest that adenovirus vector vaccine is superior to the pox virus vector vaccine and DNA vaccine at the level of the specificity of antigens and the count of CD8 + T cell [28]. Human type 5 replication-defective adenovirus expression vector is a common vector used in the research of vaccine and genetic therapy, and it has good prospects for development as vaccine vector. The newly developed heat-stable technology can make adenovirus vector vaccines stored at room temperature up to 45 °C for six months, and its infection hold steady. rAd vector vaccine can be inoculated in a variety of ways. The permissive host cell range is very wide. The virus has been used to infect many mammalian cells types (both replicative and non-replicative) for high expression of the recombinant protein [29–32]. Considering these advantages, it is valuable to perform further research in developing candidate vaccines with adenovirus vector for VS prevention.

VSV-G gene encoding glycoprotein was the target gene as glycoprotein was a determinant of pathogenesis in swine, a natural host [33]. It has been reported that VSIV-GNJGIN expressed both glycoproteins stably through multiple rounds of replication in swine and induced neutralizing antibodies against both VSV serotypes, with a dominance of the Indiana serotype in the serological response [10, 33]. In this study, the rAds expressed single serotypes glycoprotein. Both glycoproteins of VSV were generated by a replication-defective human type 5 adenoviral expression system, which could provide a much faster and safer method to generate RCA (replication-competent virus)-free rAds at high titer. Two serotypes G genes were cloned by RT-PCR accurately, and at the same time the fusion G gene of 3100 bp was amplified by overlapping PCR with the polypeptides GlyGlyGlyGlySer successfully [24]. By using the principle of gene homologous recombination in AAV-293 cell that carrying the main structural gene sequence of adenovirus, the rAds containing the target genes were created. In order to determine the best time of gaining the cell cultures containing rAds, the rAds containing EGFP gene was constructed as the control in our study. We carried out many times rAd experiments, and found that the experiment needed 10 ~ 15 days. Do not keep plate more than 15 days. The time before the 7th day and after the 15th day is unfavorable to collect the cell culture supernatants. On day 10, the plate is ready for harvest. The three rAds inoculated into the AAV-293 cells were serially propagated to 20 generations, and the virus titers were stable by detecting the TCID50, up to 10 ~ 8.0/mL. In addition, the PCR identification was conducted to amplify the target genes from the cell culture supernatants containing rAds for confirming the hereditary stability of G genes in rAds. The results of western blotting and IFA suggested that three target proteins can be expressed in AAV-293 cells and Vero cells, but only AAV-293 cell could induce cytopathic effect (CPE) at 36 ~ 48 h of virus infection. All the proteins had good biological activities.

In evaluating the immune reaction of the rAd-IN, rAd-NJ and rAd-IN-NJ in mice and goats, and in virus neutralization test for detection of neutralizing antibody level, the antibody levels were 1:16 to 1:32 in
three groups, and antibody titers are not high. The results of lymphocyte proliferation test to detect levels of immune cells show that the vaccines can cause a strong immune mouse lymphocyte proliferation, and are significantly higher than the negative control group.

Conclusion

The three rAds can express foreign proteins very efficiently, and the proteins can cause a certain degree of humoral and cellular immune responses in immunized mice and goats, which laid a foundation for further studies of the recombinant adenovirus vaccines expressing VSV glycoprotein.

Methods

Cells and viruses

AAV-293, Vero and BHK-21 cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 IU/mL streptomycin at 37 °C in 5% CO₂. VSV-IN and VSV-NJ stocks were grown and titrated in Vero cells.

Construction Of Plasmids And Recovery Of Recombinant Viruses

The G gene of VSV-IN (VSV-IN-G) was amplified by reverse transcription (RT)-PCR from purified viral (VSV-IN) RNA using the following two primers: 5´-CGGAATTC_EcoR_I GCCACC_(kozak sequence) ATGAAGTGCTTTTGTA-3´ and 5´-ATTTGCAGCAGCCGCGCNot_I TTACTTTCAAGTCGTTTC-3´.

The G gene of VSV-NJ (VSV-NJ-G) was amplified by RT-PCR from purified viral (VSV-NJ) RNA using the following primers: 5´-CGGAATTC_EcoR_I GCCACC_(kozak sequence) ATGTTGTCTTATCTATTTGCA-3´ and 5´-ATTTGCAGCAGCCGCGCNot_I TTACGGAATGAGCATTCCACGACGCA-3´. We amplified the fusion G genes of VSV-IN and VSV-NJ (VSV-IN-G-NJ-G) by overlapping PCR for polypeptides Gly-Gly-Gly-Gly-Ser and the two primers (containing the linkers gene sequence) were 5´-GGTGGAGGTGGAAGCAAGClinker ATGTTGTCTTATCTATTTGCA-3´ and 5´-GCTTCCACCTCCACClinker CTTTCAAGTCGTTTC-3´.

The target genes (VSV-IN-G, VSV-NJ-G, VSV-IN-G-NJ-G) and the adenovirus shuttle vector (pacAd-CMV K-NpA) were digested with restriction enzymes EcoR I and Not I. Then the fragments were cloned into the shuttle vector ligated with T4 DNA ligase to construct the recombinant shuttle plasmids containing the target genes (pAd-VSV-IN-G, pAd-VSV-NJ-G, pAd-VSV-IN-G-NJ-G). The recombinant shuttle plasmids and adenovirus backbone plasmid linearized with PacI restriction enzymes were co-transferred to AAV-293 cell monolayer in 6-well tissue culture plates (Costar, Corning, NY, USA) using Lipofectamine-2000 reagent (Invitrogen, USA). Briefly, 2 µg of each recombinant shuttle plasmid were diluted in 250 µL of OPTI-MEM (Invitrogen) and mixed gently. Meanwhile, 10 µL of Lipofectamine-2000 was mixed with 250 µL OPTI-
MEM for 5 min. The two dilutions were then combined, incubated for 20 min, and then slowly added to 80%~90% confluent monolayer of AAV-293 cells that had been prewashed twice with OPTI-MEM. After 4 h of incubation at 37 °C, 5% CO₂, the transfection medium was removed, and 2 mL DMEM containing 10% FBS was added to each well, then incubated for 7 ~ 15d. When the AAV-293 cells showed cytopathic effect (CPE), the cell culture medium was collected. The three kinds of rAd were named with rAd-IN, rAd-NJ and rAd-IN-NJ.

The three rAds inoculated into the AAV-293 cells were serially propagated to 20 generations. The rAds of 5th, 10th, 15th, and 20th generations were collected to extract viral DNA using AxyPrep™ Body Fluid Viral DNA / RNA Miniprep Kit, and then identified by PCR. In addition, the rAds of 5th, 10th, 15th, and 20th generations were diluted 10-fold with DMEM containing 2% FBS, and then 100 µL was inoculated in AAV-293 cells. The cells were incubated at 37 °C, 5% CO₂ for 3d. The cytopathic effect (CPE) was observed and recorded on 3 d post-incubation, and TCID₅₀ was calculated by the method of Reed-Muenels.

**Detection of expressing G protein by western blot and IFA**

AAV-293 cells infected with rAds were washed twice with phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM K₂HPO₄, pH7.2) and then lysed in 4x SDS-PAGE sample buffer. For western blot analysis, 100 µg purified rAds were loaded on 10% SDS-PAGE. Separated proteins were electro-pheretically transferred to a nitrocellulose membrane. The nonspecific antibody binding sites were blocked for 12 h at 4°C with a blocking solution containing 5% skim-milk in PBST (PBS and 0.05% Tween-20), then membranes were washed with PBST three times. Then the one membrane was reacted for 1 h at 37°C with mouse anti-VSV-IN-G protein McAb diluted (1:5000) in PBST containing 5% skim-milk incubated for 1 h at 37°C. with PBST for 3 times, it was treated with peroxidase-conjugated goat anti-mouse IgG diluted (1:2000) in PBST containing 5% skim-milk incubated for 1 h at 37°C to detect the expression of the VSV-IN-G protein in rAd-IN or rAd-IN-NJ. Goat anti-VSV-NJ-G protein PcAb (1:1000) and peroxidase-conjugated rabbit anti-goat IgG (1:2000) were used to detect the expression of the VSV-NJ-G protein in rAd-NJ or rAd-IN-NJ. 3,3-Diaminobenzidine tetra hydrochloride was used as the substrate for membrane development.

For indirect immunofluorescence assay (IFA), Vero cells were seeded into six-well tissue culture plates (Costar Corning Inc., Corning, NY) at 2 × 10⁵ concentration of cells every well. When the cells reached approximately 80 ~ 90% confluence, the culture medium was removed and the cells were washed with PBS (pH 7.2) three times. Then 500 µL DMEM containing 2 × 10⁵ PFU of recombinant adenovirus were added into the six-well tissue culture plates incubated for 1 h at 37°C, 5% CO₂. After removing the virus, fresh medium was added and cultures were incubated at 37°C, 5% CO₂ for IFA. At 48 h incubation, the cells were washed with PBS three times and then fixed with 4% paraformaldehyde 500 µL /mL for 30 min at 4°C. Then the paraformaldehyde was removed and the cells were washed three times with PBST. The rAd-IN and rAd-VSV-IN-NJ were detected using anti-VSV-IN-G protein mouse McAb (1:2000) followed by fluorescein isocyanate conjugated goat anti-mouse IgG (1:200) for 1 h at room temperature. rAd-NJ and
rAd-IN-NJ were detected with Anti-VSV-NJ goat PcAb (1:500) and peroxidase-conjugated rabbit anti-goat IgG using the same method. Then the cells were visualized under a fluorescent microscope.

**Immunization And Sample Collection**

rAd antigen preparation: Stock antigens were prepared from AAV-293 cells infected with the rAd-IN, rAd-NJ, rAd-IN-NJ. AAV-293 cells were infected with rAds of 10 MOI for 2 d incubation at 37°C, 5% CO2. After two days, the cells were collected and repeatedly frozen and thawed at -20°C/room temperature three times. Then the rAds was obtained from the supernatant content centrifuged at 4°C, 3000 rpm for 10 min. The TCID\textsubscript{50} of the rAds were determined by the method of Read-Muenels. In order to evaluate the immune effects of the rAds, the study selected 50 6-8-week-old female BALB/c mice, which were purchased from Chinese Experimental Animal Resources Research Institute for food and drug control (Beijing, China) and divided randomly into five groups, each group of 10. The three groups of mice were respectively inoculated subcutaneously three times at 2-week intervals with 10\textsuperscript{8} TCID\textsubscript{50} of rAds. At last, two groups of mice were inoculated with wildtype adenovirus and PBS, and the PBS group was used as the negative control.

After 0, 2, 4, 6 weeks of the first inoculation, the blood was collected from the orbital cavity of the mice. The serum was collected from the blood incubated at 4°C and centrifuged at 4°C, 3000 rpm for 10 min. Then the serum was stored at -80°C for future use to detect the specific antibody levels by neutralization test. At the end of the experiments, the mice were euthanized by CO\textsubscript{2}. The procedure of CO\textsubscript{2} euthanasia was referenced by previously published article [34]. Briefly, the mice home cage was placed in a transparent polycarbonate euthanasia chamber (44 cm × 23.5 cm × 21 cm). The chamber was covered with an acrylic lid with ports for CO\textsubscript{2} gas inlet and outlet. Compressed CO\textsubscript{2} gas was provided from a cylinder (Weiler Welding, Moraine, OH) and controlled by the regulator (Western Medica, Westlake, OH). The flow rate of CO\textsubscript{2} was 1.7 L per minute. Before turning off the CO\textsubscript{2}, death was confirmed when the complete cessation of breathing of mice was observed for at least 3 minutes.

Goats: 20 outbred healthy Boer goats (6 months old) were were purchased from local self-supporting farmers in Fuyang (China) and divided randomly into 5 groups, 4 goats per group, and housed in separate rooms. All goats were negative VSV infection as detected by neutralizing antibodies (titers < 1:2). Groups 1, 2, and 3 were subcutaneously injected twice at 3-week interval with 10\textsuperscript{8} TCID\textsubscript{50}/mL rAd-IN, rAd-NJ, or rAd-IN-NJ, Group 4 and 5 were inoculated subcutaneously with 10\textsuperscript{8} TCID\textsubscript{50}/mL wtAd or 1 mL PBS as negative controls. All experiments were approved by the Ethics committee of Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, (Permit number:2015-014). All manipulations were carried out in accordance with the requirements of the Regulations of Experimental Animal Administration of China. On 0, 3, 6, 9, 12 weeks post first inoculation, blood was collected from the vein of each goat. Sera were collecting from the blood incubated at 4°C and centrifuged at 4°C, 3000 rpm for 10 min. Then, the sera were kept at -20°C for using to detect the specific antibody levels by neutralization test. At the end of the
experiments, the goats were euthanized by intravenous injection of the euthanasia solution, which was composed of 429 mg/kg of sodium pentobarbital and 55 mg/kg of phenytoin based on the weight of the goat [35].

**Determination Of Neutralizing Antibody Titers**

Sera were collected and incubated at 56°C for 30 min to inactivate complement. The sera were diluted 5-fold with DMEM, and then serially diluted 2-fold in DMEM. Serial dilutions of the serum were incubated with 200 TCID$_{50}$ doses of wild-type viruses (VSV-IN or VSV-NJ) for 1 h at 37°C. After incubation, samples were added to BHK-21 cells in quadruplicate assays in 96-well plates, incubated at 37°C for 3 days. The CPE was observed and recorded after 3d incubation. The virus-neutralizing antibody (VNA) titer was defined as the highest serum dilution that inhibited CPE by at least 50%. A titer equivalent to 10 or higher was considered positive.

**Lymphocyte Proliferation Assay**

The lymphocyte proliferation assay of the mice was conducted at 2 weeks after the 3rd immunization. Spleens were aseptically removed from three mice every group and splenocyte suspensions were prepared as previously described [20]. The splenocytes were extracted and purified using a Spleno-cyte Extraction kit (TBD Science, Tianjin, China) and seeded in 96-well flat-bottom plates at a density of 2 x 10$^6$ cells per well in RPMI 1640 medium (Invitrogen, Grand Island, NY, USA) containing 10% FBS. Then 100 µL of medium containing VSV-IN or VSV-NJ was added to each well of splenocytes. Concanavalin A (ConA) (Sigma Aldrich, St. Louis, MO, USA) was used as positive control, and medium was used as negative control. The plates were incubated at 37°C in 5% CO$_2$ for 3 days. After 3days, the proliferative response was determined using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay, and the Cell Titer 96 Aqueous One Solution Cell Proliferation kit (Promega, Madison, WI, USA), following the manufacturer's instructions. At the end of the incubation, the OD of the plate wells was read at 490 nm. The lymphocyte proliferation rate was quantified using the stimulation index (SI), which was calculated as the ratio of the OD$_{490}$ of the stimulated cells to the OD$_{490}$ of the negative controls.

The lymphocyte proliferation assay of the goats was conducted at week 9 after first immunization. The blood was collected from the jugular vein of goats and was heparinized. Then, peripheral blood mononuclear cells (PBMC) were separated by Filoll-Hypque density gradient centrifugation (TBD Sciences, Tianjin, China) as described by Wang et al. (2013), Cells were resuscitated at 2 x 10$^6$/mL with RPMI 1640 medium containing 10% FBS, 2 mM glutamine, 50 mM 2-mercaptoethanol, 100 IU/mL streptomycin, and 100 IU/mL penicillin. The cells were plated in 96-well plates with 100 µl per well. Subsequently, 100 µL per well of medium with or without inactivated VSV-NJ or VSV-IN were added and mixed. ConA (5 µg/mL, Sigma-Aldrich) was used as a positive control. Uninfected cells cultured only in
medium were used as a negative control. Each sample was tested in triplicate. After incubation of the samples at 37°C in 5% CO₂ for 72 h, 20 µL of MTS was added, samples were incubated at 37°C in 5% CO₂ for 4 h. At the end of the incubation, the OD₄₉₀ was measured. The stimulation index (SI) was calculated as the ratio of the average OD₄₉₀ value of stimulated cells to average OD₄₉₀ value of negative control.

**Statistical analysis**

The data were analyzed with one-way ANOVA in GraphPad Prism version 5.00 (GraphPad Software, San Diego, CA, USA); p values less than 0.05 were considered statistically significant.

** Declarations**

**Abbreviations**

Vesicular Stomatitis Virus (VSV); Vesicular Stomatitis Virus Indiana serotype glycoprotein (VSV-IN-G); Vesicular Stomatitis Virus New Jersey serotype glycoprotein (VSV-NJ-G); Recombinant adenoviruses (rAd); Kb: Kilobase; Vesicular stomatitis (VS); Office International des Epizooties, World Organization for Animal Health (OIE ); Foot-and-mouth disease virus (FMDV); Cytotoxic T lymphocytes (CTL); Fetal bovine serum (FBS); cytopathic effect (CPE); phosphate -buffered saline (PBS).

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**Ethical approval and consent to participate**

All of the animal protocols were performed in accordance with the ‘Guidelines for Experimental Animals’ of Ministry of Science and Technology (Beijing, China) and the study was approved by the Research Ethics Committee of the Chinese Academy of Agricultural Sciences. No specific permits were required for these locations and activities. The owners gave their written consent for sample collection, and the locations where we sampled are not privately owned or protected in any way.

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**Authors' contributions**

N/A
Availability of data and materials

All data generated or analyzed during this study are included in this published article and its additional files.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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References


Figure 1

CPE of AAV-293 cells infected with the recombinant adenoviruses. A: CPE of AAV-293 cells infected with the rAd-IN; B: CPE of AAV-293 cells infected with the rAd-NJ; C: CPE of AAV-293 cells infected with the rAd-IN-NJ; D: CPE of AAV-293 cells infected with the wtAd; E: CPE of AAV-293 cells infected with VSV; F: The normal AAV-293 cells.
Figure 2

**Figure 3**

PCR identification of different passages the recombinant adenovirus. M: 2K Plus DNA Marker; 1-4: PCR product from rAd-IN at passage 5th, 10th, 15th, 20th; 6-9: PCR product from rAd-NJ at passage 5th, 10th, 15th, 20th; 11-14: PCR product from rAd-IN-NJ at passage 5th, 10th, 15th, 20th; 5, 10, 15: Negative control.

**Figure 4**

Expression of VSV G protein in the rAd-IN and rAd-NJ infected 293 cells by western blot. M: Protein molecular weight Marker; 1: 293 cells infected with rAd-IN; 2, 3: 293 cells infected with wtAdV; 4: 293 cells infected with rAd-NJ.
Figure 5

Expression of fusion protein in the rAd-IN-NJ infected 293 cells by western blot. M: Protein molecular weight Marker; 2: 293 cells infected with rAd-IN-NJ (detected by VSV-IN monoclonal antibody); 3: 293 cells infected with rAd-IN-NJ (detected by VSV-NJ polyclonal antibody); 1, 4: Negative control.

Figure 6

Identification of the expression of VSV G protein by IFA. A: Vero cells infected with rAd-IN (detected by VSV-IN monoclonal antibody; B: Vero cells infected with rAd-NJ (detected by VSV-NJ polyclonal antibody); C: Vero cells control.
Figure 7

Identification of expression of VSV fusion G protein in Vero cells by IFA. A: Vero cells infected with rAd-IN-NJ (detected by VSV-IN monoclonal antibody); B: Vero cells infected with rAd-IN-NJ (detected by VSV-NJ polyclonal antibody); C: Vero cells control.

Figure 8

Cell Proliferation Assay

Stimulation Index

不同病毒

rAd-IN
rAd-NJ
rAd-IN-NJ
wtAd
PBS

VSV-IN
VSV-NJ
VSV-IN
VSV-NJ
VSV-IN
VSV-NJ
VSV-IN
VSV-NJ
VSV-IN
VSV-NJ
Proliferation of lymphocyte to virus stimulation in the immunized mice.

**Figure 9**

Proliferation of lymphocyte to virus stimulation in the immunized goats.

**Supplementary Files**

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