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Immunogenicity and antigenicity of a recombinant chimeric protein containing epitopes of poliovirus type 1

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Summary. – To design a vaccine that simultaneously prevents both rotavirus (RV) and poliovirus (PV), a PV type 1 (PV1) chimeric protein using RV VP6 as a vector (VP6F) was constructed, expressed in *Escherichia coli* expression system and characterized by SDS-PAGE, Western blot, immunofluorescence assay and neutralization test. The results showed that the chimeric protein reacted with anti-VP6F and anti-PV1 antibodies and elicited production of serum antibodies against the chimeric protein in guinea pigs. Antibodies against the chimeric protein neutralized RV Wa and PV1 infection *in vitro*. The results provided a relevant possibility of developing novel approaches in the rational design of vaccines effective against both RV and PV.

Keywords: chimeric protein; neutralizing activity; rotavirus; poliovirus epitope

Introduction

Group A rotavirus (RVA), belonging to the *Reoviridae* family, is the most important etiologic agent of acute gastroenteritis in infants and young children worldwide, and responsible for 453, 000 deaths annually, primarily in developing countries (Parashar *et al*., 2006; Tate *et al*., 2012). Significant reduction of RV hospitalizations has been observed in industrialized and developing countries after implementation of oral live RV vaccines, Rotarix and Rotateq. However, lower immunogenicity, efficacy and potential adverse of these vaccines should not be ignored (Cunliffe *et al*., 2012; Eng *et al*., 2012; Kollaritsch *et al*., 2015; Sow *et al*., 2012). Moreover, the RV vaccines at present are mainly based on the serotype-specific neutralizing antigens VP4 and VP7, which are not sufficient enough to protect against infections from a variety of RV genotypes. VP6, as the group antigen of RV, is the major structural protein forming the middle layer in the triple-layered viral capsid. For the last few years, studies have shown that VP6 could stimulate a protective immune response (Bugli *et al*., 2014; Li *et al*., 2014; Marashi *et al*., 2014; Pastor *et al*., 2014; Shoja *et al*., 2015) and a short fragment of VP6 could provide significant reduction in virus infectivity *in vitro* (El-Senousy *et al*., 2013). Previous experiments demonstrated that anti-VP6 llama-derived single-chain antibody fragments (VHH) had neutralizing activity against VP6 *in vitro* (Garaicoechea *et al*., 2008). Using RV VP6 as a vector (VP6F), the chimeric proteins carrying epitopes derived from the VP4 of RV were constructed and demonstrated that these chimeric proteins had good antigenic reactivity and immunogenicity (Teng *et al*., 2014).

Poliovirus (PV) (the genus *Enterovirus*, the *Picornaviridae* family) is the causative agent of acute paralytic poliomyelitis; and is classified into three serotypes (type 1, type 2, and type 3) (Bannwarth *et al*., 2015; Hogle and Filman, 1989). WHO and its partners set out in 1988 to eradicate PV by the year 2000 through the effectiveness of vaccination strategies, but in recent years, the virus had staged a comeback and leaped across the border into yet another countries that had been polio-free for years (Chumakov and Ehrenfeld, 2008; Roberts, 2014). Wild PV still remains endemic in Pakistan,

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Abbreviations: CPE = cytopathic effect; PV = poliovirus; RV = rotavirus; $TCID_{50}$ = 50% tissue culture infectivity doses

Afghanistan and Nigeria and during 2014 a total of 359 wild PV cases were detected in nine countries worldwide (Hagan *et al*., 2015). Together with the emergence of vaccine-derived PV (VDPV) strains and recombinant circulating vaccine-derived PV (cVDPVs), the campaign of eradicating PV will have a long way to go.

The neutralizing antigenic structure of PV has been characterized by monoclonal antibodies (Blondel *et al*., 1986; Diamond *et al*., 1985; Minor *et al*., 1983; Page *et al*., 1988). Three important neutralizing antigenic sites involved in virus neutralization of PV were distributed on surface-exposed loops of structural proteins VP1, VP2, and VP3 (Van der Marel *et al*., 1983). Neutralizing antigenic site 1, is composed of amino acids 91–102, 254, 168 of VP1; neutralizing antigenic site 2, is a complex site which includes residues 166–170 and 270 of VP2, and residues 221–226 of VP1; and neutralizing antigenic site 3 is also a complex site which includes residues 285–289 of the VP1, 58–60, 71, 73 of the VP3, and residue 72 of the VP2. The amino acid residues 89 to 100, 220 to 222, 286 to 290 of the VP1 were important cross neutralizing epitopes (Minor *et al.,* 1986) and could induce cross neutralizing antibody, protecting adults from infections by different PV serotypes (Herremans *et al.,* 2000).

RV and PV both replicate in intestinal duct and elicit both humoral mucosal responses to viruses with the production of secretory immunoglobulin A (IgA) antibody after natural infection and vaccine immunization (Fiore *et al*., 1997; Giammarioli *et al*., 1996; Ogra *et al*., 1971; Savilahti *et al*., 1988). Since RV and PV share similar transmission routes, simultaneous prevention of RV and PV has great importance for optimal utilization of the limited resources in developing countries. Some studies showed that PV vaccines were evaluated in co-administration with oral RV vaccines according to the Expanded Program on Immunization (EPI) schedule were proved effective and safe (Steele *et al*., 2010; Zaman *et al*., 2009).

In this study, using VP6F, three foreign epitopes derived from PV1 were inserted into the surface loops of VP6F to construct a chimeric protein, and then the chimeric protein was expressed in *E. coli* BL21 (DE3) cells. The expressed chimeric protein was confirmed by immunoblot and immunofluorescence assay; and was used to immunize guinea pigs to analyze the epitope-specific humoral response. The results obtained in the present study provide a new direction of exploration for the development of vaccines against both RV and PV.

Materials and Methods

Cells and viruses. MA104 cells (fetal rhesus monkey kidney cells) were grown in Eagle's Minimum Essential medium (MEM; Sigma-Aldrich Inc., USA) supplemented with 10% fetal bovine serum. Vero cells (African green monkey kidney cells) were grown in Dulbecco's Modified Eagle's medium (DMEM; Sigma-Aldrich Inc., USA) supplemented with 5% fetal bovine serum. MEM medium (without calf serum) was used when cells were inoculated and cultured with RV or PV. All cells were grown at 37°C in a 5% CO_2 atmosphere. The human RV strain Wa (G1P[8]) was maintained at the Institute of Medical Biology, Peking Union Medical College, and adapted to grow in cell culture by serial passages in MA104 cells. Three types of attenuated PV, type 1 Sabin SO+1 (PV1), type 2 Sabin SO+1 (PV2), type 3 457 Pfizer RSO1 (PV3) were provided from WHO and adapted in Vero cells.

Preparation of viruses and purification. Confluent cell monolayers were cultivated in Roche bottle (210 cm^2) and maintained in MEM medium followed by the infection with the viruses at 37°C in an atmosphere of 5% CO_2. When cytopathic effect (CPE) appeared among ≥75% of cells, cells and supernatant were harvested and then frozen, and thawed three times. After centrifugation at 3,000 × g at 4°C for 30 min, the viral supernatant was collected. For purification of the virus, polyethylene glycol 20000 (PEG 20000; Amresco, USA) to a final concentration of 5% (w/v) was added to the viral supernatant and stirred at low speed overnight at 4°C. The precipitate was collected after centrifugation at 10,000 × g at 4°C for 1 hr, and dissolved in 20 volumes of TNMC buffer (10 mmol/l Tris-HCl [pH 7.0], 150 mmol/l NaCl, 1 mmol/l $MgCl_2$, 10 mmol/l $CaCl_2$) for 30 min at room temperature. Then the precipitate was collected after centrifugation at 50,000 × g at 4°C for 1 hr, resuspended in TNMC buffer and followed by extraction with equal volume of trifluorotrichloroethane. After centrifugation at 12,000 × g at 4°C for 10 min, the aqueous phase was collected and the organic phase was extracted twice with the 1/2 volume of TNMC buffer. Samples of the aqueous phase were pooled. The virus solution containing 10% sucrose (4 ml) was placed onto the bottom of the centrifuge tube containing 15% sucrose cushion (1 ml) and centrifuged at 45,000 × g at 4°C for 18 hr. Fractions of the target virus were collected and dissolved in 20 times the volume of TNMC buffer; then centrifuged at 50,000 × g at 4°C for 1 hr. The virus pellet was collected, dissolved in TNMC buffer and stored at -20°C.

Virus titration. Determination of infectious titers of strain RV Wa and PV were carried out in MA104 and Vero cells, respectively. Briefly, RV strain Wa was pre-treated with acetylated trypsin (10 μg/ml; Sigma-Aldrich Inc., USA) for 1 hr at 37°C, viral serial ten-fold dilutions in MEM medium were titrated on confluent cells grown in 96-well microtiter plates using four replicates per dilution (100 μl/well). The plates were incubated in the presence of 5% CO_2, and cells were observed for CPE regularly under microscope. Virus infectivity titer was quantified by estimating the 50% tissue culture infectivity doses ($TCID_{50}$) and end points were calculated as previously described (Reed and Muench, 1938).

Construction of recombinant plasmid. To construct expression plasmid for chimeric protein, three neutralizing antigenic epitopes derived from the neutralizing antigenic site 1, 2 and 3 of the PV were selected and inserted into a foreign epitope presenting system based on RV vector protein VP6F (Fig. 1). Three pairs of

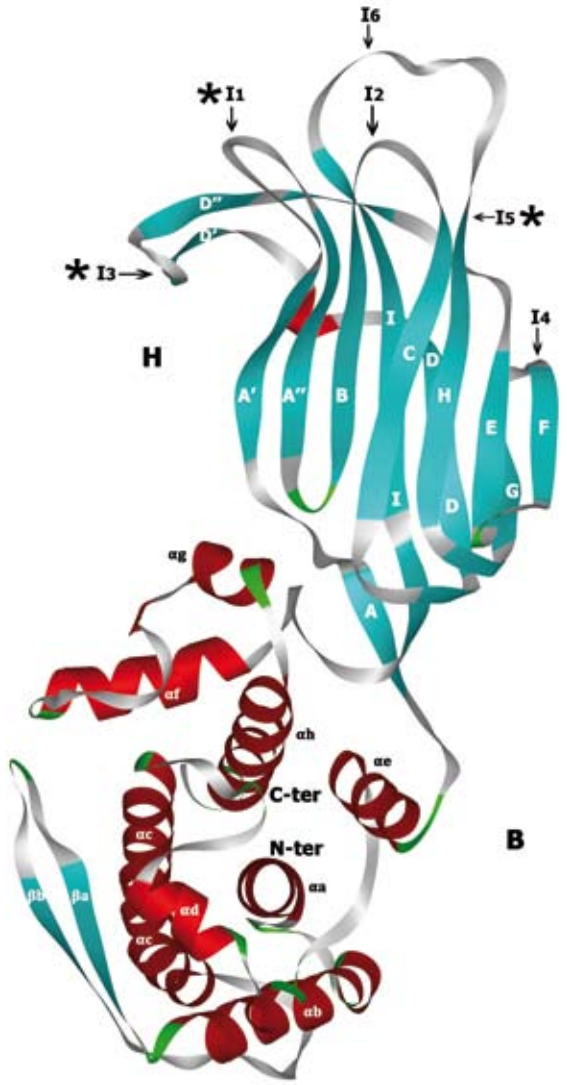

Fig. 1

3D visualization of the VP6F protein as the vector
Six foreign epitope insertion sites (I1-I6) on the outer surface that can be chosen for construction of chimeric protein. Sites I1*, I3*, and I5* sites were used for PV1 epitope insertion in this study.

specific oligonucleotide primers for three epitopes of PV1 were designed (Table 1). PV1 strain Mahoney was used as a template for the design of these primers representing the epitopes (Kitamura and Wimmer, 1980). Epitope PV1N1, TVDNPASTTNKDET, corresponded to amino acid residues 91–102, 254, 168 of VP1; epitope PV1N2, QTSPALSAALGD corresponded to amino acid residues 166–170 and 270 of VP2, and residue 221–226 of VP1; and epitope PV1N3, DYKDGSATRSPHTDT corresponded to amino acid residues 285–289 of the VP1, 58–60, 71, 73 of the VP3, and residue 72 of the VP2. The complementary oligonucleotides of each primer pair were annealed and inserted into the corresponding cloning site on the VP6F protein vector, followed

Table 1. Primers used in this study

Epitope		Primer pair (5'-3')
PV1N1	Fw1	GGACCGTGGATAACCCAGCTTCCA CCACGAATAAGGATGAGCCGC
	Rev1	GGCTCATCCTTATTCGTGGTGGAAGC TGGGTTATCCACGGTCCGC
PV1N2	Fw2	CCAGACATCACCTGCCTTATCGGCA GCACTAGGTGACGGTAC
	Rev2	CGTCACCTAGTGCTGCCGATAAGGCA GGTGATGTCTGGGTAC
PV1N3	Fw3	CGATTACAAGGATGGTAGTGCCACAC GGAGTCCACATACAGACACGGAGCT
	Rev3	CCGTGTCTGTATGTGGACTCCGTG TGGCACTACCATCCTTGTAATCGAGCT

by cloning into *Nde*I/*BamH*I site of the expression plasmid pETL that was derived from pET-3a (Novagen, USA). The resultant expression plasmids were designated pETP6F and pETP6F/PV1N123. The pETP6F contained the vector protein VP6F gene; pETP6F/PV1N123 contained the VP6F gene with epitope PV1N1 inserted in *Sac*II site (I5), epitope PV1N2 inserted in *Kpn*I site (I3), and epitope PV1N3 inserted in *Sac* I site (I1). The recombinant plasmids were verified by restriction endonuclease digestion and DNA sequencing.

Protein expression and purification. The plasmids of pETP6F and pETP6F/PV1N123 were transformed separately into *E. coli* BL21 (DE3) competent cells (Biovector Co., LTD, China) for expression. The transformed *E. coli* cells were cultured in Luria–Bertani (LB) medium supplemented with 200 μg/ml of ampicillin, incubated at 37°C until reaching absorbance A_{600} of 0.6–0.8. Cells were collected by centrifugation and resuspended in lysis buffer (150 mmol/l NaCl, 50 mmol/l Tris [pH 7.5], 5% glycerol, 1% Triton X-100, 2 mmol/l EDTA, 0.2% β-mercaptoethanol). After sonication and centrifugation, proteins were dissolved in 8 mmol/l urea and analyzed by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Purification of the target proteins VP6F and 6F/PV1N123 were carried out as previously described (Chen *et al.*, 2006), and the protein concentrations were determined using the Lowry's method (Lowry *et al.*, 1951).

Animal immunization. For production of antibodies against chimeric protein, guinea pigs (5–7 weeks of age, about 200 g) were purchased from China Medical Primates Center, Kunming, China. None of the animals had evidence of antibodies against RVA and PV by neutralization test. Guinea pigs were housed in microisolation cages and all procedures were conducted in accordance with protocols approved by the Institute of Medical Biology Animal Care and Use Ethic Committee (approval No.: YISHENGLUNZI [2011] 15). Recombinant vector protein VP6F, chimeric protein 6F/PV1N123, and reference strains RV Wa, PV1, PV2, PV3 were used as immunogens. Four guinea pigs were used for each immunogen inoculation. Guinea pigs in each group were immunized in the hind leg with 120 μg of recombinant proteins in 100 μl of dilution

solution (15 mmol/l Tris; 150 mmol/l NaCl [pH 7.0]). For RV Wa and PV1, PV2, PV3, 1×10^7 $TCID_{50}$ of the virus was administrated to each animal at each injection. At the same time, four guinea pigs were used as negative control and inoculated with 100 µl of dilution solution. Each animal was inoculated three times at 0, 14 and 28 days. Guinea pigs were bled by heart puncture at the fifth day after the last immunization. The blood samples were incubated at 37°C for 30 min, 4°C for 3 hr, and centrifuged at 8,000 × g at 4°C for 20 min. Antibody levels were detected by Western blot and neutralization test.

Indirect immunofluorescence assay (IFA). To detect PV1 antigen, Vero cells were grown on glass coverslips and when confluent monolayers were attained, the cells were washed three times with phosphate buffered saline (PBS) and infected with PV1. The coverslips were taken out 12 hr after infection, washed twice with PBS, fixed with pre-chilled methanol, and rehydrated at 4°C for 10 min with 70%, 30%, and 10% of pre-chilled ethanol. After washing with PBS, coverslips were incubated for 1 hr at 37°C with antisera against chimeric protein (1:400 dilution in 0.1% of bovine serum albumin). The unbound antibodies were removed by washing with PBST (PBS containing 0.2% Tween 20). The cells were then incubated at 37°C for 1 hr with FITC-labeled goat anti-guinea pig IgG (1:100 dilution; Sigma, USA), followed by washing with PBST. Fluorescence was detected under microscope (Nikon Eclipse E600, Japan). Meanwhile, as controls, fluorescence analysis of noninfected cells with pre-inoculated sera, virus inoculated or negative (inoculated with PBS) guinea pig sera were carried out. To detect RV antigen, MA104 cells were infected with RV strain Wa, and immunofluorescence assay was performed as described above.

Western blot of chimeric protein. Expressed samples diluted in gel loading buffer (2 mmol/l EDTA, 50 mmol/l Tris [pH 6.8], 10% glycerol, 1% SDS, 1% β-mercaptoethanol, 0.05% bromophenol blue) were heated at 95°C for 5 min, cooled at room temperature, and then subjected to SDS-PAGE. The separated proteins in the SDS-PAGE gel were blotted onto PVDF membranes for 20 min at 50 V using Trans-Blot® SD semi-dry electrophoretic transfer cell (Bio-Rad Laboratories, USA). After blocking with 5% skimmed milk in TBS (25 mmol/l Tris-HCl [pH 7.5], 0.9% NaCl), the membranes were incubated with antiserum (1:400 dilution) from immunized guinea pigs. After washing with TBST (0.1% Tween 20 in TBS), the membranes were incubated with goat anti-guinea pig IgG conjugated with horseradish peroxidase (1:2000 dilution; Sigma, USA). The membranes were washed and then incubated with 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma, USA) to visualize bound antibodies.

Neutralization test. To determine the sera neutralization titer, a micro-neutralization assay was carried out. Briefly, 50 µl of virus solution containing 100 $TCID_{50}$ was mixed with an equal volume of the guinea pig antisera at 2-fold serial dilutions and incubated in a 5% CO_2 humidified incubator for 1 hr at 37°C. The mixture was then added onto cell monolayers in a 96-well tissue culture plate with four replicates per dilution. The plates were further incubated at 37°C in a humidified CO_2 incubator for 48 hr and regularly observed for CPE presence. The appropriate cell and serum controls were included for each plate. Neutralizing titers were defined as the reciprocal of the highest dilution of antiserum that protected 50% of cells from virus-associated CPE.

Results

Expression of chimeric protein in E. coli

Three epitopes PV1N1, PV1N2, and PV1N3 of PV1 were introduced together into VP6F. The recombinant plasmid, designated as pETP6F/PV1N123, was confirmed by sequencing and then transformed into competent BL21 (DE3) cells for expression. The plasmid pETP6F was used as a control. The expressed chimeric protein 6F/PV1N123 carrying epitopes PV1N1, PV1N2, and PV1N3 of PV1 was approximately 48 kDa (Fig. 2a,b, lane 2), and the vector protein VP6F was about 43.2 kDa (Fig. 2a,b, lane 1) as expected. The expressed proteins were retained in precipitate after sonication, indicating 6F/PV1N123 and VP6F were mainly in the form of inclusion bodies.

Immunoreactivity of chimeric protein

The immunological reactivity of the chimeric protein was detected by Western blot. Western blot results showed that the chimeric protein could be specifically recognized

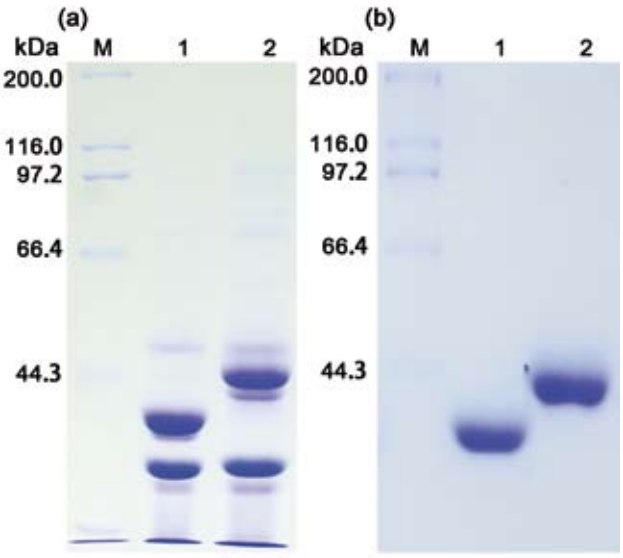

Fig. 2

10% SDS-PAGE of recombinant chimeric protein before (a) and after purification (b)

Lane M: Protein molecular weight standard (kDa); lane 1: vector protein VP6F; lane 2: chimeric protein 6F/PV1N123.

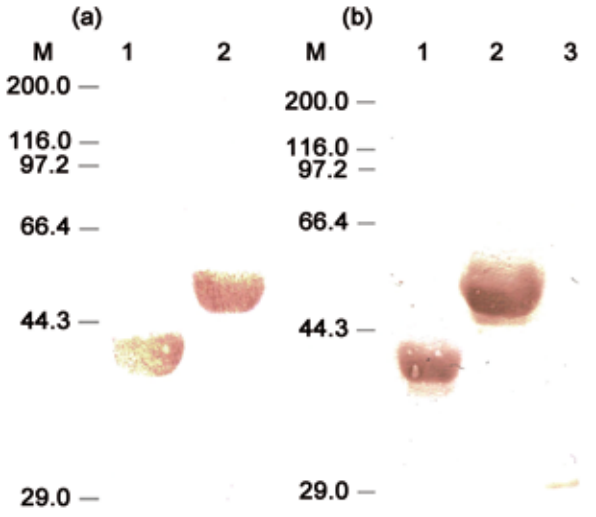

Fig. 3

Western blot of recombinant proteins with antibodies from guinea pigs immunized with the vector proteins (a) and chimeric protein (b) Lane M: Protein molecular weight standard (kDa); lane 1: vector protein VP6F; lane 2: chimeric protein 6F/PV1N123; lane 3: virus PV1. Four micrograms of recombinant protein (lanes 1–2) was loaded; and 3×10^6 $TCID_{50}$ of virus PV1 (lane 3) was loaded.

by antibodies derived from guinea pigs inoculated with vector protein VP6F (Fig. 3a). There was no immunoreactivity of sera derived from pre-immune or negative control animals observed with VP6F or the chimeric protein (data not shown).

The antibodies derived from guinea pigs immunized with chimeric protein both reacted with the vector protein VP6F and chimeric protein 6F/PV1N123 (Fig. 3b). The VP1 but not VP2 or VP3 protein of PV1 could be recognized by the antibodies from guinea pigs immunized with the chimeric protein (Fig. 3b, lane 3).

IFA of PV1 antigen in PV1 infected Vero cells

PV antigen synthesized in PV1 infected Vero cells and RV antigen synthesized in RV Wa infected MA104 cells were detected by IFA (Fig. 4). The results showed that PV1 antigen in PV1 infected Vero cells could be detected by antibodies from guinea pigs inoculated with PV1 (Fig. 4a), and chimeric protein 6F/PV1N123 (Fig. 4c). No fluorescence was detected in PV1 infected cells when detected with antibodies against the vector VP6F (Fig. 4b), and pre-inoculation sera (Fig. 4d). RV antigen in RV Wa infected MA104 cells could be detected by antibodies against VP6F (Fig. 4f), and chimeric protein 6F/PV1N123 (Fig. 4g). No fluorescence was detected in RV Wa infected cells with antibodies against PV1 (Fig. 4e), PV2 or PV3 (data not shown), mock inoculated or pre-inoculation sera (Fig. 4h).

Neutralizing antibody activity

Neutralization test has been applied to examine the sensitivity of virus-specific antibodies produced in tested animals, or the reduction of infectious units of the RV and PV. Results showed that antibodies against the chimeric protein neutralized infection of RV and PV1, in MA104 and Vero cells, respectively (Table 2).

Using PV and RV Wa as infection viruses, the neutralizing titers in antisera against the chimeric protein 6F/PV1N123 were 1:320 against Wa infection, and 1:400 against PV1 infection (Table 2). No neutralizing activity against PV1 infection was detected in antisera from guinea pigs inoculated with vector protein VP6F (lower than 1:4), and no neutralizing activity against PV1 or RV infections was detected in antisera from guinea pigs mock-inoculated with PBS as negative control (lower than 1:4). In addition, no neutralizing activities were detected in all sera from all the guinea pigs used in this study when PV2 or PV3 were used as infection viruses (lower than 1:4).

Discussion

Despite the decrease of RV and PV infections due to efficient use of inactivated and live attenuated vaccines, both diseases still remain important public health problem in some areas. In recent years, research and development of chimeric protein vaccines which were used to overcome some concerns that exist in live attenuated virus vaccines has made a great progress. It has been demonstrated that if a foreign epitope was inserted in an appropriate position on the vector protein, the chimeric protein could elicit antibodies that recognized the vector protein and neutralize the infection by the epitope-derived virus (Teng *et al.*, 2014; Tisminetzky *et al.*, 1994). In this study, a chimeric protein-based vaccine that could simultaneously prevent both RV and PV infection was designed and produced. Three neutralizing epitopes derived from the VP1 of PV1 were inserted into the surface of VP6F. The insertion sites were chosen in three different outer loops on the surface of VP6F. Results showed that the chimeric protein had specific immunogenicity and could elicit high antibody titers against both RV and PV1 in guinea pigs, suggesting that the chimeric protein based on the VP6F vector system may be a useful vaccine approach for the multimeric presentation of immunogenic epitopes.

RV VP6, as a highly immunogenic and the most significant immunodiagnostic protein for RVA detection (Svensson *et al.*, 1987), shares a high degree of antigenic cross-reactivity and could potentially provide heterotypic protection from RV infection. In recent years, the VP6 became a hot topic as it was studied in attempts to develop VP6-based vaccines, and some studies demonstrated that antibodies directed to

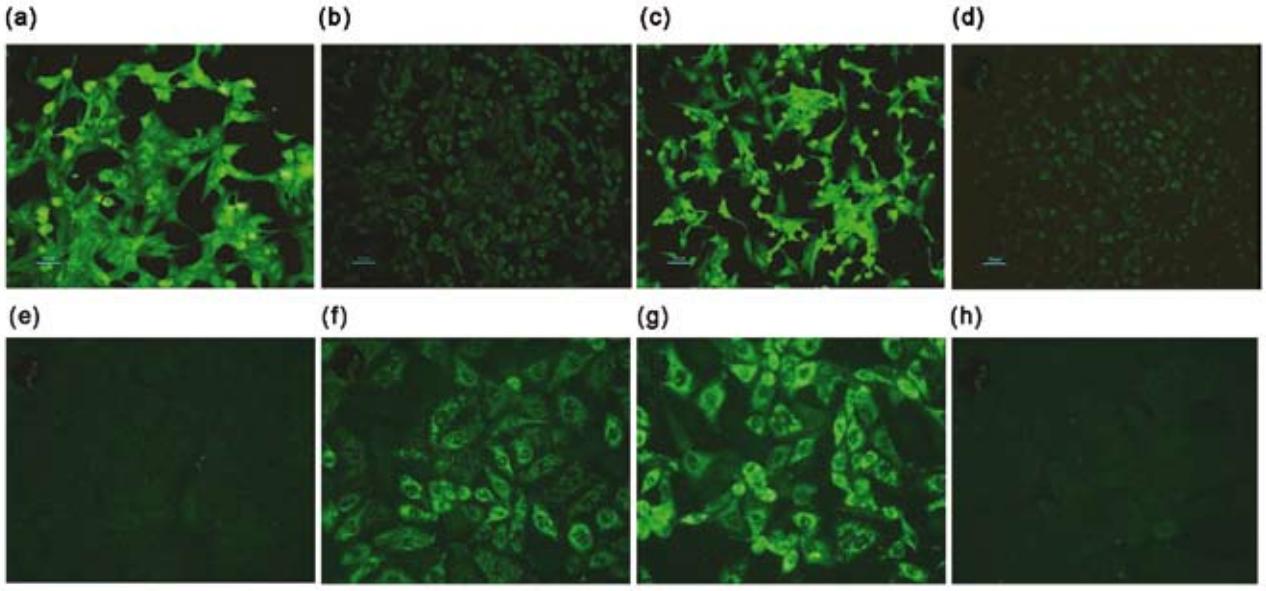

Fig. 4

Immunofluorescence analysis

VP1 antigen in PV1 infected Vero cells detected with antibodies from guinea pigs inoculated with PV1 (a), vector protein VP6F (b), chimeric protein 6F/PV1N123 (c), mock inoculated with PBS (d); and RV antigen in RV Wa infected MA104 cells detected with antibodies from guinea pigs inoculated with PV1 (e), vector protein VP6F (f), chimeric protein 6F/PV1N123 (g) and mock inoculated with PBS (h). Bar: 50 μm (a–d), 20 μm (e–h).

VP6 possess broad neutralizing activity *in vitro* and confer protection against diarrhea in mice (Garaicoechea *et al.*, 2008) and neonatal gnotobiotic piglets (Vega *et al.*, 2013).

In previous study a foreign epitope-presenting system using the RV VP6 as a vector was constructed (Teng *et al.*, 2014). The molecular structure of VP6F showed that the bone structure of VP6 was fully maintained, and the six foreign epitope insertion sites, designated I1, I2, I3, I4, I5 and I6, were as expected on exposed surface. In this study, using this vector protein, three highly conserved epitopes derived from the PV1 were selected, and inserted into epitope insertion sites I1, I3 and I5. The results demonstrated that these epitopes presented in this form could cross-react with anti-VP6F and anti-PV1 antibodies. Antibodies against the chimeric protein could react with VP6F and VP1 of PV1 and neutralize both RV strain Wa and PV1 infection *in vitro.*

The epitopes of PV and the VP6 of RV are both highly conserved in different genotypes of the PV and RV respectively (Kitamura and Wimmer, 1980; Matthijnssens *et al.*, 2012). Results showed that the recombinant chimeric protein 6F/PV1N123 could react with antibodies against VP6F and PV1, and induce production of neutralizing antibodies in animals. The antibody against chimeric protein could recognize VP1 antigen in PV1 infected cells, implying that the conformational structure of the epitopes remains in the infected cells. Cross neutralizing antibodies against the chimeric protein could neutralize infections by RV and PV1 *in vitro*, implying that the chimeric protein may be used as a candidate epitope-based vaccine. However, only epitopes derived from PV1 were included in the chimeric protein, the chimeric proteins carrying epitopes from other serotypes of the PV should be further addressed. In addition, virus-

Table 2. Neutralization titers of antibodies from chimeric protein carrying PV1 epitopes inoculated guinea pigs

Virus	Neutralization titer of antibody*						
	NC**	VP6F	6F/PV1N123	PV1	PV2	PV3	Wa
PV1	<4	<4	400	10240	<4	<4	<4
PV2	<4	<4	<4	<4	10240	<4	<4
PV3	<4	<4	<4	<4	<4	2560	<4
Wa	<4	320	320	<4	<4	<4	10240

*Arithmetic mean NT titers; **NC, negative control. In this group, sera were derived from animals that were pre-immune or mock immunized with PBS. Neutralizing titers are defined as the reciprocal of the highest dilution of antiserum that protected 50% of cells from virus-associated CPE.

like particles should be produced in order to further study the evaluation of the effectiveness of the combined vaccine candidates. Anyway, this VP6-based epitope presenting system and the recombinant VP6-based PV epitope chimeric protein will be valuable for the development of a novel RV/PV chimeric vaccine and vaccine vector.

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