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Study of a chimeric Foot-and-mouth disease virus DNA vaccine containing structural genes of serotype O in a genome backbone of serotype Asia 1 in guinea pigs

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Summary. – Since Foot-and-mouth disease virus (FMDV) serotypes display a great genetic and antigenic diversity, there is a constant requirement to monitor the performance of FMDV vaccines in the field with respect to their antigenic coverage. To avoid possible antigenic changes in field FMDV isolates during their adaptation to BHK-21 cells, a standard step used in production of conventional FMDV vaccines, the custom-made chimeric conventional or DNA vaccines, in which antigenic determinants are replaced with those of appropriate field strains, should be constructed. Using this approach, we made a plasmid-based chimeric FMDV DNA vaccine containing structural genes of serotype O in the genome backbone of serotype Asia 1, all under the control of Human cytomegalovirus (HCMV) immediate early gene promoter. BHK-21 cells transfected with the chimeric DNA vaccine did not show cytopathic effect (CPE), but expressed virus-specific proteins as demonstrated by ^{35}S-methionine labeling and immunoprecipitation. Guinea pigs immunized with the chimeric DNA vaccine produced virus-specific antibodies assayed by ELISA and virus neutralization test (VNT), respectively. The chimeric DNA vaccine showed a partial protection of guinea pigs challenged with the virulent FMDV. Although the chimeric DNA vaccine, in general, was not as effective as a conventional one, this study encourages further work towards the development of genetically engineered custom-made chimeric vaccines against FMDV.

Keywords: Foot-and-mouth disease virus; DNA vaccine; chimeric vaccine; immune response

Introduction

Foot-and-mouth disease (FMD) affects domestic cloven-hoofed animals including cattle, swine, sheep, and goats as well as more than 70 species of wild animals including deer. The disease is characterized by fever, lameness, and vesicular lesions on the tongue, feet, snout, and teats. In the sheep and goats the disease is generally mild. FMDV (the genus *Aphthovirus,* the family *Picornaviridae*) includes seven serotypes. The virus contains a single-stranded positive sense RNA genome of approximately 8,300 bp enclosed by an icosahedral capsid composed of 60 copies each of the 4 structural proteins signed 1a, 1b, 1c, and 1d (Rueckert and Wimmer, 1984; Rueckert, 1996). In FMDV-infected cells, the genome is translated as a single long ORF and the synthesized polyprotein is co-translationally processed by viral-encoded proteinases (L^{pro} and $3C^{pro}$) into 4 structural proteins and a number of non-structural proteins (Vakharia *et al.,* 1987).

The inactivated conventional FMDV vaccines have been used successfully as a part of eradication program in disease-free countries and furthermore, they are the only option available in the endemic areas. All currently avail-

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Abbreviations: FMD = foot-and-mouth disease; FMDV = FMD virus; HCMV = Human cytomegalovirus; VNT = virus neutralization test; CPE = cytopathic effect; ID_{50} = infective dose 50

able conventional FMDV vaccines are based on the cell culture-derived, chemically-inactivated whole virus mixed with a suitable adjuvant (Doel, 2003). These vaccines elicit a strong humoral response with high titers of antiviral neutralizing antibodies against antigenic sites identified on the viral capsid (Brown, 1995; Mateu, 1995). In spite of the fact that existing vaccines have been associated with a notable success, some recurrent problems have been still linked with its use, such as shortcomings in the vaccine manufacture, development of a carrier state in some vaccinated animals following contact with FMDV, (Bachrach, 1968; Salt, 1993) and relatively short-lived immunity (Bachrach, 1968; Woolhouse *et al.*, 1996). Because of these concerns, the alternative FMDV vaccines that do not require infectious virus have been attempted over the past 20–25 years (Ward *et al.*, 1997; Wong *et al.*, 2002).

Since the FMDV serotypes display a great genetic and antigenic diversity, there is a constant need to monitor current circulating strains of the FMDV to determine suitability of the available vaccines (Mateu *et al.*, 1994). For effective control of the disease under such conditions, the development of a new vaccine strain requires a screening of numerous field strains to identify isolates suitable for the large-scale growth in BHK-21 cells. The screening process is cumbersome, laborious, and expensive. To circumvent this process and possible antigenic variations during passage, it is promising to replace the antigenic determinants of an infectious genome-length cDNA copy of a vaccine strain with those of appropriate field strains. Resulting custom-made FMDV chimeras are suitable for the use as a vaccine (van Rensburg *et al.*, 2004).

In vitro production of the infectious virus from transfected plasmid carrying full-length cDNA was first described for poliovirus (Racaniello and Baltimore, 1981). For FMDV, genome-length cDNA was constructed for several serotypes including type O1K (Zibert *et al.*, 1990), type A_{12} (Rieder *et al.*, 1993), type SAT2 (ZIM/7/83), (van Rensburg *et al.*, 2004) and type OH99 (Liu *et al.,* 2004). An infectious cDNA for the attenuated strain of FMDV serotype Asia 1 (ZB/CHA/58) was constructed recently (Xin *et al.*, 2009). Using a genome-length cDNA copy of FMDV, a development of the recombinant virus containing manipulated and/or exchanged antigenic determinants for the novel vaccine is achievable.

In this work, we constructed a plasmid-based chimeric FMDV DNA vaccine containing structural genes of serotype O in the genome backbone of serotype Asia 1. Further, we examined its non-infectivity and potential to express the virus-specific proteins *in vitro* together with the ability to induce antibodies *in vivo* and to protect the immunized animals against virulent FMDV.

Materials and Methods

Cells and virus. BHK-21 cel1s were maintained in DMEM/F12 Ham mixture supplemented with 2% fetal bovine serum. FMDV strain O IND/R2/75 at passage level 5 was plaque purified, passaged in cattle tongue once and used as a source of the virus (Ravikumar, 2004). Clones of *Escherichia coli* containing genome-length cDNA (pFAs) of FMDV serotype Asia 1 (IND63/72) in pBSKS+ vector was used (Saravanan, 2005).

DNA vaccine constructs. All PCR amplified gene fragments were gel purified and cloned. The positive clones were characterized by restriction digestion analysis and nucleotide sequence analysis of the inserted fragments. The Fig. 1 illustrates the genome organization of the different DNA constructs. The multistep amplification strategy was followed to construct the empty DNA vaccine (pcFAsXP) clone downstream of HCMV immediate early gene promoter in pcDNA3.1+ vector. In order to introduce the structural gene of desired FMDV serotype into the full-length genome devoid of structural (P1) gene, a unique restriction site *Bam*HI was introduced at both ends of the structural protein (P1-2A) gene region. Total RNA from FMDV-infected BHK-21 cell supernatant (Asia 1 IND 63/72) or from cattle tongue material (O IND/R2/75) was used to amplify the structural gene (P1) by RT-PCR. The amplified structural gene (P1) from serotype Asia 1 and O were introduced into the empty DNA vaccine (pcFAsXP) construct to get the homologous DNA vaccine (pcFAs) and chimeric DNA vaccine (pcFAsOP) constructs, respectively (primer sequences available upon request).

Transfection of cells. Expression of the *Not*I linearized or uncut plasmid pcFAsOP/pcFAs driven by the HCMV promoter was verified by transfection of BHK-21 cells (in 10 cm^2 wells) using Lipofectamine® 2000 (Invitrogen) as described by Rieder *et*

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                    FMDV genome:  5′-UTR-P1-P2-P3-UTR-poly(A)-3′
   Empty DNA vaccine (pcFAsXP):  -plasmid-HCMV pro-FMDV/Asia 1 (P2+P3)-plasmid-
Homologous DNA vaccine (pcFAs):  -plasmid-HCMV pro-FMDV/Asia 1 (P1+P2+P3)-plasmid-
Chimeric DNA vaccine (pcFAsOP):  -plasmid-HCMV pro-FMDV (P1/O) (P2+P3/Asia 1)-plasmid-
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Fig. 1

Structure of FMDV genome and DNA vaccines

HCMV pro – HCMV promoter; UTR – untranslated region; P1, P2, P3 – genome regions.

al. (1993). The transfected cells were incubated at 37°C with 5% CO_2 for 48–72 hrs. If CPE was not observed in the transfected cells, the cells were freeze-thawed and 10% of the cell lysate was used to infect fresh BHK-21 cells and incubated for 72–96 hrs at 37°C. This process was repeated three times and aliquots of the lysate obtained from each step were frozen at -80°C and used for further analyses.

Labeling and immunoprecipitation of virus-specific proteins. 48 hrs after transfection with the plasmids, the BHK-21 cell monolayers were analyzed for expression of structural protein gene by ^{35}S-methionine labeling and immunoprecipitation as described by Suryanarayana *et al.* (1992) with modifications. Briefly, the cells were labeled with 50 μCi/well of ^{35}S-methionine in methionine-free DMEM for 36 hrs for plasmid DNA-transfected cells and for 5 hrs for serotype O virus-infected cells at 37°C. The ^{35}S-methionine labeled proteins were incubated overnight at 4°C with hyper-immune serum raised against recombinant VP1 gene of serotype O and Asia 1. The immunoprecipitated proteins were resolved by electrophoresis on 10% SDS-PAGE and autoradiographed.

Immunizations of guinea pigs. Plasmid DNAs to be used as vaccines were purified from *E. coli* DH5α cells using Machery-Nagel's endotoxin-free plasmid giga kit. Thirty guinea pigs were randomly divided into five groups: (1) empty DNA vaccine (pcFAsXP), (2) homologous DNA vaccine (pcFAs), (3) chimeric DNA vaccine (pcFAsOP), (4) conventional FMD vaccine, and (5) PBS control. The animals were injected intramuscularly with 0.2 ml of conventional FMDV vaccine or intradermally with 100 μg (100 μl) of DNA as primary immunization. Booster injections at 21st and 35th day were given only to the animals of DNA vaccine groups. The guinea pigs were bled on day 0 prior to the first inoculation and later 14 days after the 2nd booster. The sera were heat inactivated at 56°C for 30 mins and stored at -20°C until tested in ELISA and VNT.

ELISA of virus-specific antibodies. Detection of serum antibodies to FMDV was performed by sandwich ELISA using 96-well flat-bottomed plates (Nunc) using serotype specific antigen and antibodies. The plates were coated with polyclonal rabbit anti-146S antibodies (against type O and Asia 1) in 0.1 mol/l carbonate/bicarbonate buffer, pH 9.6, incubated at 37°C for 1 hr, and later transferred to 4°C overnight. The plates were incubated with 50 μl/well of blocking buffer containing FMDV antigen at a dilution of 1:5. Further, the plates were incubated with two-fold serially diluted test sera (from 1: 8 to 1:512) in duplicate wells for 1 hr at 37°C. Rabbit anti-guinea pig IgG peroxidase conjugate (Sigma) at 1:1,500 dilution was added for 1 hr at 37°C followed by the substrate, ortho-phenylenediamine (OPD) and 0.01% hydrogen peroxide in phosphate/citrate buffer. The spectrophotometer reading was done at A_{492} nm.

Virus neutralization test. All serum samples from guinea pigs were analyzed for the presence of neutralizing antibodies by micro-neutralization assay in BHK-21 cell monolayers. Serial two fold dilutions of sera were incubated with 100 $TCID_{50}$ of serotypes O (IND R2/75) or Asia 1 (IND 63/72) at 37°C for 1 hr. Cells were then added as indicators of residual infectivity and the plates were incubated at 37°C for 48–72 hrs. The endpoint titers were calculated as $\log_{10}$ of the highest serum dilution that showed CPE in 50% of the cell monolayers.

Challenge of immunized guinea pigs. The guinea pig infective dose 50 (ID_{50}) of the guinea pig-adapted O and Asia 1 serotypes were calculated in seronegative guinea pigs. All guinea pigs were intradermally challenged with 0.2 ml of 100 ID_{50} on the left hind pad 5 weeks after the final vaccination. Control animals were given 0.2 ml of DMEM only. All guinea pigs were kept in isolated cages and examined for 7 days. The appearance of vesicles on the virus-inoculated pad was considered as an indicator of primary infection and the appearance of lesions on non-inoculated pad as a secondary infection. In such case, the animal was recorded as not protected.

Results

Non-infectivity of the chimeric DNA vaccine in vitro

BHK-21 cells transfected with the genome-length cDNA constructs showed no apparent CPE after 48 to 72 hrs of incubation. Wild viral RNA-transfected cells showed 75% CPE after 16–20 hrs of transfection. The blind passage of the DNA-transfected cell supernatant or lysate obtained after three freeze-thaw cycles in fresh BHK-21 cells showed no apparent CPE observed after 72 to 96 hrs of incubation. The negative controls like vector-transfected or mock-transfected cells were intact, showing no change in morphology (results not shown).

Expression of virus-specific proteins from DNA vaccine constructs in vitro

The expression of the inserted FMDV serotype O and Asia 1 structural proteins and other non-structural proteins of FMDV Asia 1 backbone from the DNA vaccine constructs were determined by ^{35}S-methionine labeling and immunoprecipitation of the labeled proteins. The fluorography of the radiolabeled immunoprecipitated proteins prepared from O virus (wild type) infected cells (Fig. 2a), cells transfected with homologous DNA construct (pcFAs) (Fig. 2b), and chimeric DNA construct (pcFAsOP) (Fig. 2c) showed the presence of FMDV specific viral proteins.

Antibody response to DNA vaccines

Specific total antibody response was determined by ELISA in serum obtained from day 0 (prior to the vaccination) and two weeks after the last immunization (day 49). As expected, the highest antibody titers were observed for respective serotype viruses (Asia 1 and O) in the homologous DNA construct, chimeric DNA construct, and conventional vaccines (Fig. 3a, b). Sera from the chimeric DNA-vaccinated animals contained antibodies that were crossreactive with Asia 1 virus (Fig. 3a). The conventional vaccine group showed highest serum antibody titers as compared to the other DNA vaccine constructs-vaccinated animals

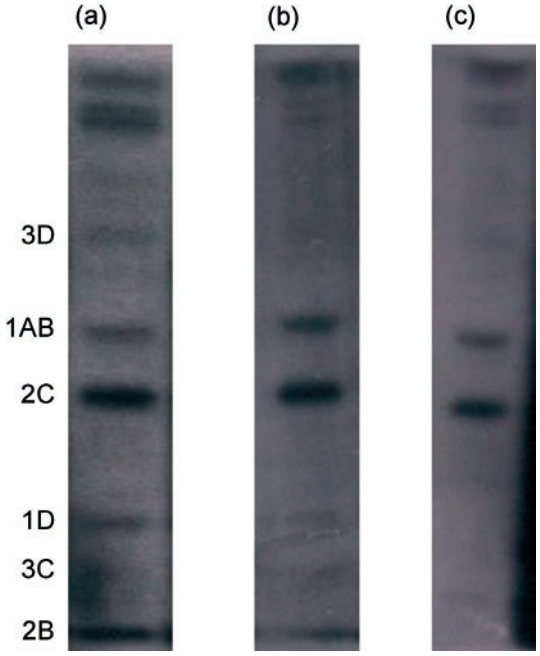

Fig. 2

Fluorography of immunoprecipitated ^{35}S-methionine labeled proteins separated by SDS-PAGE

BHK-21 cells were infected with FMDV serotype O (a), or transfected with homologous Asia 1 (b) or chimeric O/Asia 1 (c) DNA vaccine. Virus-specific proteins are presented on the left.

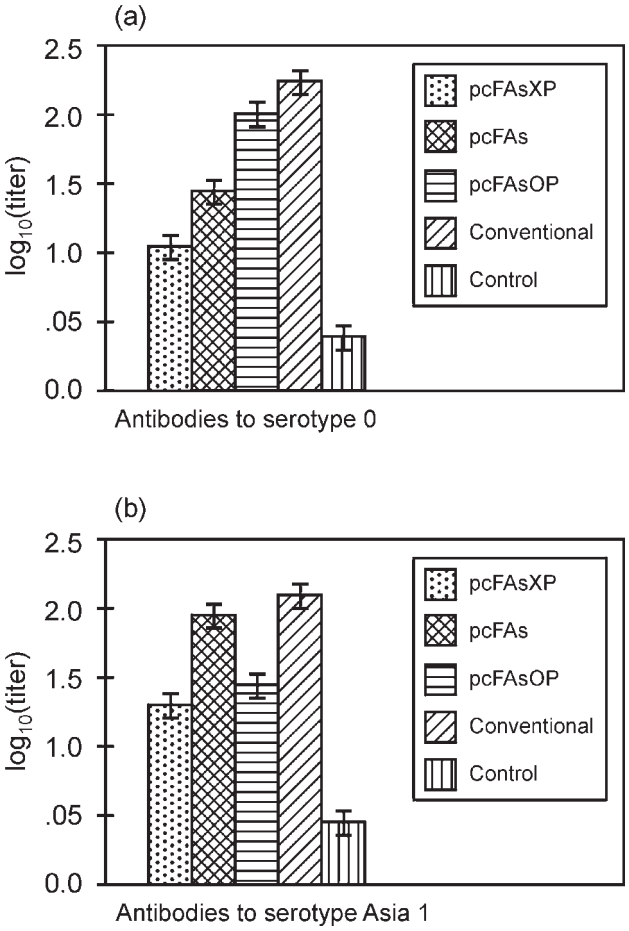

Fig. 3

ELISA antibody levels to FMDV serotypes O (a) and Asia 1 (b) elicited by DNA vaccines

Titers were expressed as $\log_{10}$ mean values ± SE.

(Fig. 3a, b). No antibody response to FMDV was detected in the negative control animals.

Table 1 summarizes the virus neutralizing antibody titers elicited against different vaccine constructs. The neutralizing antibody titers of chimeric DNA construct (pcFAsOP)-vaccinated animals titrated against the homologous virus was comparable to the homologous DNA construct (pcFAs)-vaccinated animals. From the empty DNA construct (pcFAsXP)-vaccinated animals, two of them showed some neutralizing antibody response. The highest neutralizing antibody titers against O and Asia 1 viruses were observed in the conventional FMDV vaccinated animals.

Table 1. Induction of virus neutralizing antibodies in guinea pigs by DNA vaccines

Vaccine	Antibody titer ($\log_{10}$) Animal No.					
	1	2	3	4	5	6
"Empty" DNA vaccine	0.3	0.6	0.9	0.6	0.6	0.3
Homologous DNA vaccine	1.5	1.5	1.5	1.2	1.5	1.5
Chimeric DNA vaccine	1.2	1.5	1.5	1.2	1.5	1.2
Conventional vaccine:						
Serotype O antibodies	1.5	1.8	1.8	1.8	1.2	1.5
Serotype Asia 1 antibodies	1.5	1.8	1.8	1.8	1.8	1.5
Negative control	<0.3					

Protective effect of DNA vaccines

The conventional vaccine- and PBS-immunized animals showed the protection or expression of the disease, respectively, upon the challenge (Table 2). The PBS-immunized animals that were not protected developed vesicles on both feet by 48 hrs. In conventional vaccine-immunized animals, 4 of 6 animals were fully protected without any signs of disease. Upon immunization with the homologous DNA construct (pcFAs), 3 animals were fully protected and the remaining animals showed lesions at the site of inoculation. In the chimeric DNA construct (pcFAsOP)-vaccinated animals, 2

animals were fully protected and the remainder was partially protected. None of the empty DNA (pcFAsXP)-vaccinated animals was protected.

Table 2. Protection of guinea pigs by DNA vaccines against the challenge with virulent FMDV

	Animal No.					
	1	2	3	4	5	6
"Empty" DNA vaccine[1]	None					
Homologous DNA vaccine[1]	++	++	+	+	++	+
Chimeric DNA vaccine[2]	+	+	++	+	–	++
Conventional vaccine[2]	++	++	+	++	+	++
Negative control[2]	None					

Full (++), partial (+), and no (–) protection. [1]Serotype Asia 1 or [2]serotype O was used for the challenge.

Discussion

FMD is controlled in developed countries through the use of vaccine containing inactivated FMDV, but the limitations and concerns about the use of conventional vaccines has led to the development of a new generation of vaccines using recombinant DNA technology. The DNA vaccine constructs that produced empty capsids from the polystructural protein (P1) with co-expression of the processing enzyme 3C showed that level of immunity and immunological memory were still lower than with the infectious virus. The DNA vaccine required other non-structural proteins for achieving a better immune response in the vaccinated animals (Chinsangaram *et al.*, 1998; Cedillo-Barron *et al.*, 2001; Guo *et al.*, 2005). Nevertheless, the genome-length cDNA copy of FMDV provides all the virus-specific epitopes to induce stronger immune response in the vaccinated animal.

In this study, a chimeric cDNA construct (pcFAsOP) containing the structural gene of serotype O in the genome backbone of serotype Asia 1 was constructed to produce chimeric FMDV that was used as a vaccine. The genome-length cDNA of Asia 1 virus (IND 63/72) constructed downstream of T7 promoter (pFAs) did not produce infectious virus particles, when *in vitro* transcribed RNA molecules were transfected into BHK-21 cells (unpublished data). The chimeric cDNA construct (pcFAsOP) downstream of HCMV immediate early gene promoter also failed to produce infectious virus particles. The non-infectious cDNAs had been reported earlier for Japanese encephalitis virus (Sumiyoshi *et al.*, 1992) and Dengue virus type 4 (Lai *et al.*, 1991), where the genome-length cDNA clones were successfully obtained, but none of the corresponding RNA transcripts was infectious. The sequence of constructed genome-length Asia 1 cDNA (Genbank Acc. No. AY304994) was compared with the other Asia 1 serotype sequences. Some nucleotide changes were found in the UTR, which might have resulted in the production of non-infectious particles. No difference in the nucleotide sequences were observed between the chimeric DNA construct (pcFAsOP) genome derived from the genome-length Asia 1 cDNA (pFAs). The expression analysis of the genome-length homologous (pcFAs) and heterologous (pcFAsOP) DNA constructs in transfected BHK-21 cell immunoprecipitated with serotype-specific antibodies showed the presence of FMDV specific proteins. Thus, HCMV immediate early gene promoter was confirmed as suitable one for the transcription of cDNA and translation to the corresponding viral proteins indicating that the viral sequences in the construct were in the correct open reading frame.

Neutralizing antibodies to FMDV were observed in guinea pigs inoculated with the plasmid DNA containing HCMV immediate early gene promoter driven genome-length FMDV type A_{12} cDNA (Ward *et al.*, 1997). In the present study, the genome-length serotype Asia 1 genome backbone containing the structural protein genes of FMDV Asia 1 (pcFAs) or O (pcFAsOP) DNA constructs were used as a DNA vaccine candidate and tested for their ability to elicit immune response and protection in guinea pigs. The animals inoculated with the chimeric heterologous DNA construct (pcFAsOP) showed a total antibody titer of $\log_{10}2 \pm 0.06$ against the serotype O and $\log_{10}1.45 \pm 0.06$ against serotype Asia 1. Because the genome backbone of the heterologous DNA construct was serotype Asia 1, it was expected that the polyclonal antibodies elicited against the chimeric virus will crossreact, due to the presence of antibodies against epitopes present in the non-structural protein coding genes of Asia 1 virus. The genome-length Asia 1 homologous DNA construct (pcFAs) vaccinated animals showed an ELISA titer of $\log_{10}1.95 \pm 0.07$ and for the empty DNA construct (pcFAsXP) without the structural gene it was $\log_{10}1.3 \pm 0.06$, what further confirmed the notion that antibody was elicited by the epitope present in the non-structural protein genes. Earlier studies have identified linear and conformational B and T cell epitope regions present on both the structural and non-structural regions of FMDV (Lea *et al.*, 1994; Blanco *et al.*, 2001). The epitopes that are present in the non-structural protein coding region are common for all the serotypes and only the structural protein gene coding region determines the serotype-specific neutralizing antibody. The VNT titers for both the homologous and heterologous genome-length DNA constructs were $\log_{10}1.5$ for five animals and $\log_{10}1.2$ for the other animal against Asia 1 and $\log_{10}1.5$ and $\log_{10}1.2$ for three animals each for O serotype viruses. The empty DNA construct (pcFAsXP) lacking structural gene elicited less neutralizing antibody confirmed that the epitope present in the structural gene (P1) was essential for the production

of serotype specific neutralizing antibodies. These results confirmed that the constructed genome-length cDNAs were functional and able to induce neutralizing antibodies. Custom-made conventional FMDV vaccines were produced by replacing the antigenic determinants of an infectious genome-length cDNA copy of the vaccine strain SAT2 serotype with the antigenic determinants of the appropriate field strain and such a vaccine elicited serotype-specific immune response (van Rensburg and Mason, 2002; van Rensburg *et al.*, 2004). The conventional FMDV polyvalent vaccine-inoculated animals demonstrated higher levels of total and neutralizing antibodies against both serotypes Asia 1 and O than the DNA-inoculated animals.

The protective efficacy of the DNA vaccine constructs was evaluated in guinea pigs. The vaccinated animals were challenged with the virulent FMDV five weeks after booster vaccination. Two animals in heterologous DNA construct (pcFAsOP) and three animals in homologous DNA construct (pcFAs) vaccinated animals were completely protected from the virulent challenge. In the case of conventional FMDV vaccine, four animals were completely protected. The challenge results showed that the DNA vaccine constructs were not as effective as the conventional vaccine in protecting the animals against disease, but the neutralizing antibody response was consistent with the observed protection in animals.

Further studies are aimed at the detailed characterization of the genome-length homologous and chimeric heterologous cDNA constructs for the production of infectious virus particles, preparation of chimeric inactivated vaccine, and evaluation of its humoral and cellular immune response.

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