

Custom-engineered chimeric foot-and-mouth disease vaccine elicits protective immune responses in pigs

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Chimeric foot-and-mouth disease viruses (FMDV) of which the antigenic properties can be readily manipulated is a potentially powerful approach in the control of foot-and-mouth disease (FMD) in sub-Saharan Africa. FMD vaccine application is complicated by the extensive variability of the South African Territories (SAT) type viruses, which exist as distinct genetic and antigenic variants in different geographical regions. A cross-serotype chimeric virus, vKNP/SAT2, was engineered by replacing the external capsid-encoding region (1B-1D/2A) of an infectious cDNA clone of the SAT2 vaccine strain, ZIM/7/83, with that of SAT1 virus KNP/196/91. The vKNP/SAT2 virus exhibited comparable infection kinetics, virion stability and antigenic profiles to the KNP/196/91 parental virus, thus indicating that the functions provided by the capsid can be readily exchanged between serotypes. As these qualities are necessary for vaccine manufacturing, high titres of stable chimeric virus were obtained. Chemically inactivated vaccines, formulated as double-oil-in-water emulsions, were produced from intact 146S virion particles of both the chimeric and parental viruses. Inoculation of guinea pigs with the respective vaccines induced similar antibody responses. In order to show compliance with commercial vaccine requirements, the vaccines were evaluated in a full potency test. Pigs vaccinated with the chimeric vaccine produced neutralizing antibodies and showed protection against homologous FMDV challenge, albeit not to the same extent as for the vaccine prepared from the parental virus. These results provide support that chimeric vaccines containing the external capsid of field isolates can be successfully produced and that they induce protective immune responses in FMD host species.

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INTRODUCTION

Most of Europe, North America and some countries in South America have eradicated foot-and-mouth disease (FMD) through the administration of inactivated-FMD vaccines since the 1960s (Ward *et al.*, 2007). Despite international regulations limiting routine vaccination in many parts of the world, it remains an important strategy for disease control in countries where FMD is endemic. In sub-Saharan Africa, control of FMD is complicated by the African buffalo that serves as a reservoir of FMD virus (FMDV) and contributes to the spread of FMD to other wildlife species and livestock. Moreover, genetic and antigenic characterization of FMDV field isolates have revealed that these viruses evolve rapidly in different

geographical areas (Esterhuysen, 1994; Vosloo *et al.*, 1995; Bastos *et al.*, 2001, 2003). This has led to the available vaccines being unable to adequately cover the extent of antigenic variation within the South African Territories (SAT) types of FMDV (Reeve *et al.*, 2010). Thus, for vaccination to be effective in sub-Saharan Africa, it requires the incorporation of vaccine strains representative of viruses circulating in that geographical area and that such strains should be available for specific regions. However, adaptation of wild-type SAT viruses in cell culture to produce high yields of stable antigen is an intricate and time-consuming process that is often associated with a low success rate.

Infectious cDNA clone technology (Zibert *et al.*, 1990; Rieder *et al.*, 1993, 1994) for FMDV SAT viruses has

provided a valuable tool for genetic manipulation and biological characterization of field and laboratory strains (van Rensburg *et al.*, 2004; Storey *et al.*, 2007; Maree *et al.*, 2010). Chimeras, containing the external capsid-encoding region (1B-1D/2A) of another FMDV, retain the replication machinery of the backbone as the P1, 2A and 3C proteins are required for FMD viral capsid assembly (Beard *et al.*, 1999) whilst acquiring the antigenic properties of the parental virus, including its ability to bind to cellular receptors that allow host-cell internalization (Maree *et al.*, 2010). This technology may therefore be utilized towards the production of custom-made vaccines specific to geographical regions or in cases of sudden FMDV outbreak. For such a chimera-derived vaccine to be a viable alternative to currently used vaccines, it should have high titre replication in cell culture, stability of the virus particle and antigen, recovery of high 146S antigen mass following chemical inactivation, appropriate immunological specificity and the ability to elicit a protective immune response in animals (Rweyemamu, 1978; Doel, 2003).

In this study, the feasibility of using a chimeric vaccine was assessed by determining its immunogenicity and protective ability following the immunization of pigs. The vaccine was prepared from a chimeric virus containing the external capsid-encoding region of a rapidly replicating, cell-adapted SAT1 vaccine strain, KNP/196/91, that was exchanged into the genetic background of an SAT2 infectious clone. The recovered chimeric virus exhibited comparable plaque morphologies, infection kinetics, virion stability and antigenic profiles to the KNP/196/91 parental virus. In a full potency test of the chimeric vaccine in pigs, good humoral immune responses were elicited and the majority of the animals were protected against homologous FMDV challenge and passed the 50% protective dose (PD₅₀) requirement of the Office International des Epizooties for emergency vaccine use. These results suggest that custom-engineered chimeric FMD vaccines can be produced and applied in a fashion similar to the current inactivated vaccines.

RESULTS

Parental viral properties are retained in the recovered vKNP/SAT2

The FMDV SAT1 isolate KNP/196/91 has been proven to be an efficient vaccine strain displaying the required qualities for a good vaccine candidate (see Introduction). Hence, KNP/196/91 virus that displayed broad antigenic coverage (Reeve *et al.*, 2010) was utilized to investigate the prospect of engineering chimeric vaccines to provide protection against FMD for specific Southern African regions affected by circulating SAT1 viruses.

Plaque morphologies for vKNP/SAT2, KNP/196/91 and vSAT2 viruses were compared on BHK-21, IB-RS-2 and CHO-K1 cell lines (Fig. 1a). As shown in Fig. 1(a) vKNP/

SAT2 and KNP/196/91 viruses formed micro (<1 mm) to medium (3–5 mm) or small (1–2 mm) to large (6–7 mm) plaques on BHK-21 cells, respectively. On IB-RS-2 cells the plaques were micro to small and micro to medium for the vKNP/SAT2 and KNP/196/91 viruses, respectively. vKNP/SAT2, KNP/196/91 and vSAT2 viruses each formed clear plaques on CHO-K1 cells, which is characteristic of viruses that use heparan sulphate proteoglycans (HSPG) as receptors. The comparable ability to infect cultured cells originating from different species is indicative that the vKNP/SAT2 capsid retained the characteristics of the KNP/196/91 virus. The deduced amino acid sequences of the external capsid-encoding region for vKNP/SAT2 and KNP/196/91 viruses were identical following their transfection and recovery in BHK-21 cells (data not shown).

In growth curves performed in BHK-21 cells (Fig. 1b), vKNP/SAT2 displayed similar replicative ability in BHK-21 cells to KNP/196/91 up to 6 h post-infection. At 12 h post-infection KNP/196/91 and vKNP/SAT2 had peak infectivity titres of 8.9×10^8 and 8.2×10^8 p.f.u. ml⁻¹, respectively. For vaccine purposes the optimal harvest time for both vKNP/SAT2 and KNP/196/91 is 12 h post-infection. This compares favourably to the time balance for stable particle survival resulting from the processes of virus assembly, rate of inactivation and the effect of proteolytic enzymes released into virus-infected cultures (Doel & Collen, 1983). The rate of production of infectious vSAT2 particles was lower than for the SAT1 viruses from 4 to 12 h.

To determine the antigenic profiles of vKNP/SAT2 and KNP/196/91, a virus neutralization test (VNT) was performed against a panel of SAT1 and SAT2 pig and cattle antisera (Fig. 2). The data showed similar neutralization values for the wild-type (passage history: PK₁RS₄) and cell culture-adapted (passage history: B₁BHK₉) isolates indicating that the amino acid variation with adaptation to BHK-21 cells did not significantly influence the major antigenic determinants. As expected, SAT2 antisera showed no cross neutralization with either of the SAT1 isolates (Fig. 2).

Antigen yield is determined by the biophysical properties of FMDV

Biophysical stability of the infectious virus or antigen has been correlated with the protective nature of FMD vaccines (Doel & Baccarini, 1981). With this in mind, vKNP/SAT2 and KNP/196/91 were compared in terms of their biophysical properties following treatment at different temperatures, pH values and salt concentrations (Fig. 3). Following virus exposure to temperatures of 25, 37, 45 and 55 °C (Fig. 3a), viable virus particles were detected at decreased concentrations for vKNP/SAT2, similar to those of KNP/196/91. After 30 min at 55 °C the infectious particles of vKNP/SAT2 and KNP/196/91 had been completely inactivated. After extended incubation at 4 °C both viruses were present up to 28 days (Fig. 3b) and the infectious titres observed for vKNP/SAT2 and KNP/196/91

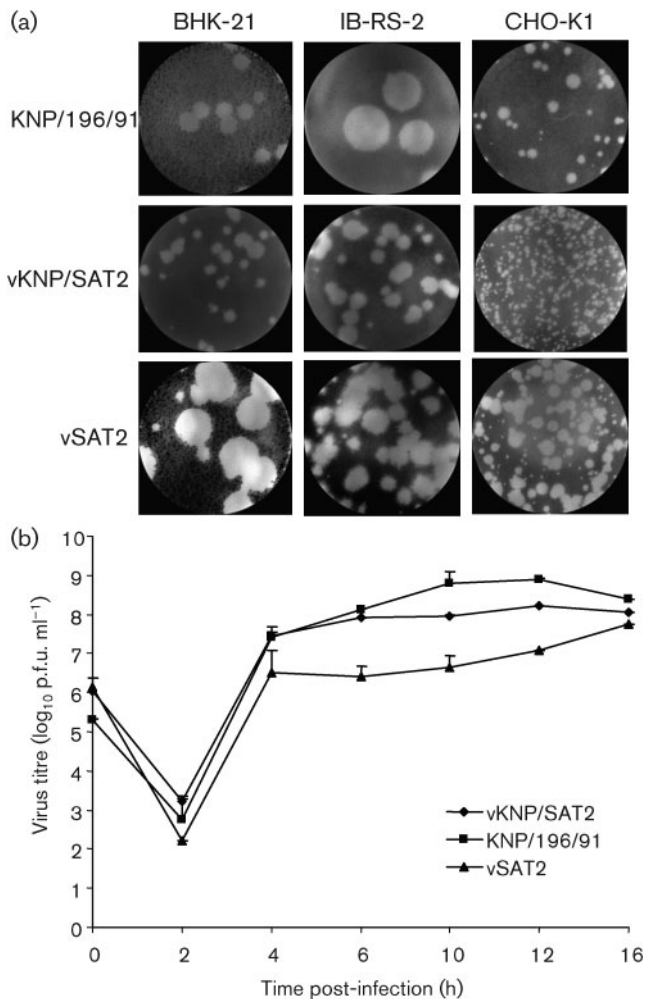


Fig. 1. (a) FMDV replication in BHK-21, IB-RS-2 and CHO-K1 cell cultures expressing diverse cellular receptors utilized by FMDV for cell entry. Morphology of plaques for the KNP/196/91 parental virus, vKNP/SAT2 chimera and vSAT2 were determined by plaque assay at 48 h post-infection. (b) Growth curves for the KNP/196/91 parental virus, vKNP/SAT2 chimera and vSAT2. BHK-21 cells were infected at an m.o.i. of 2 for 1 h and subsequently washed with MES-buffered saline (pH 5.5). Samples were frozen at selected time intervals and titrated on BHK-21 cells. Each data point represents the mean (\pm SD) of at least two wells.

were 2.6×10^3 and 5.7×10^3 p.f.u. ml⁻¹, respectively, under these conditions. Moreover, no significant variation in thermal stability was observed between the two viruses when they were incubated at 25 °C for 14 days (Fig. 3b). However, no infectious particles were detected at 28 days under these conditions.

The stability of the viruses was tested at pH values ranging from 6.5 to 9.0 (Fig. 3c). Treatment of vKNP/SAT2 and KNP/196/91 at pH 6.5 yielded a slight decrease in the titre of infectious particles after 30 min. The titre of infectious particles for both viruses was similar following treatment with buffers at alkaline pH for 30 min (Fig. 3c). The capsid

stability of vKNP/SAT2 was comparable to that of KNP/196/91 at various NaCl concentrations ranging from 50 mM to 1.5 M (Fig. 3d). Taken together, these results did not indicate any differences in the biophysical properties of the parental and chimeric viruses.

Guinea pig antibody titres in relation to vaccine dose

Two separate formulations incorporating inactivated 146S antigens of vKNP/SAT2 and KNP/196/91 were used to assess the antibody response to immunization in guinea pigs (Fig. 4). Following animal immunization, serum samples were collected at weekly intervals and tested by using a sandwich ELISA specific for KNP/196/91. Seroconversion occurred at approximately 7 days post-vaccination (d.p.v.) and serological responses varied with time ($P < 0.001$), dose ($P < 0.001$) and vaccine ($P = 0.001$; Fig. 4). High antibody titres were obtained for guinea pigs vaccinated with all three of the vKNP/SAT2 vaccine doses and titres peaked between 21 and 28 d.p.v. (Fig. 4a). In comparison, the antibody responses elicited by the KNP/196/91 vaccine were similar to those of the same dose of the vKNP/SAT2 vaccine and peaked at 28 d.p.v. (Fig. 4b).

FMDV-specific antibody responses induced by vKNP/SAT2 and KNP/196/91 vaccines in pigs

To investigate vKNP/SAT2 as a potential chimeric vaccine, a 6 μ g dose was taken as the full dose in a decreasing dose potency trial in pigs. Two separate double oil emulsions incorporating inactivated 146S antigens of vKNP/SAT2 and KNP/196/91 were prepared and used for vaccination in a full potency trial (European Pharmacopoeia, 2006; Office International des Epizooties, 2009). The antibody response elicited in pigs varied with time ($P < 0.001$), dose (full, 6.0 μ g; quarter, 1.5 μ g; one-sixteenth, 0.375 μ g; $P = 0.003$) and vaccine ($P = 0.011$) when monitored at weekly intervals by using a KNP/196/91-specific solid phase competition ELISA (SPCE) (Fig. 5). The full and quarter doses of the vKNP/SAT2 vaccine (Fig. 5a, b) elicited an antibody response comparable to the equivalent doses of the KNP/196/91 vaccine (Fig. 5d, e) up to 21 d.p.v., whereafter the titres for vKNP/SAT2 remained similar until the day of challenge. Most animals vaccinated with the vKNP/SAT2 one-sixteenth dose (Fig. 5c) were borderline positive at the time of challenge. However, the results from the SPCE indicated that the KNP/196/91 vaccine elicited positive antibody responses for all vaccine doses (Fig. 5f).

Comparison of neutralization titres and protection

Serum neutralizing-antibody responses were measured by a VNT at the day of challenge for the vaccinated and control animals (Table 1). All of the pigs were negative for FMDV-specific neutralizing antibody at the start of the study (data not shown). Positive neutralizing-antibody titres were

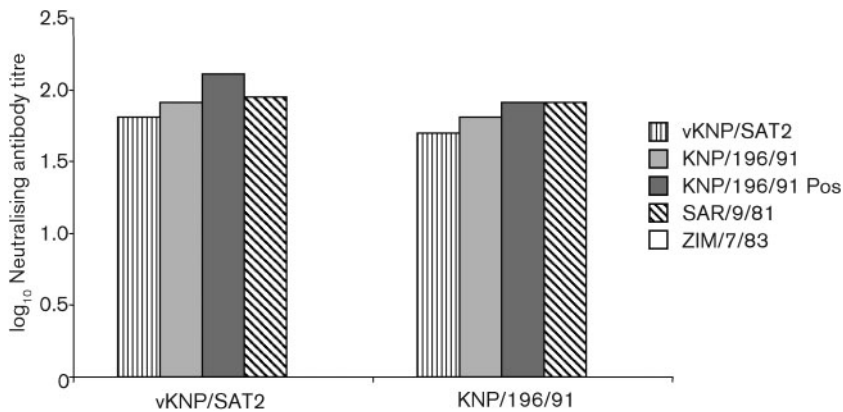


Fig. 2. Antigenic profiles of FMD viruses vKNP/SAT2 (B₁BHK₉) and KNP/196/91 (PK₁RS₄), which has not been cell-culture adapted in BHK-21 cells, tested against FMDV SAT1 antisera (vKNP/SAT2 chimera, KNP/196/91 parental and SAR/9/81) and SAT2 antisera (ZIM/7/83) in a VNT. Pig antisera vKNP/SAT2 and KNP/196/91 were from the vaccinated animals in this potency trial, whereas all other antisera originated from cattle. A positive-control serum of high titre (KNP/196/91 Pos) was included. Serum titres ≥ 1.7 were regarded as positive.

induced for the full doses of both vaccines. For the quarter dose of the vKNP/SAT2 and KNP/196/91 vaccines, three and four animals, respectively, were positive for neutralization of the KNP/196/91 virus. Whilst four pigs

vaccinated with the KNP/196/91 one-sixteenth dose had positive neutralizing antibody titres, the entire vKNP/SAT2 one-sixteenth group had no detectable neutralizing virus titre. Statistical analysis indicated that a significant

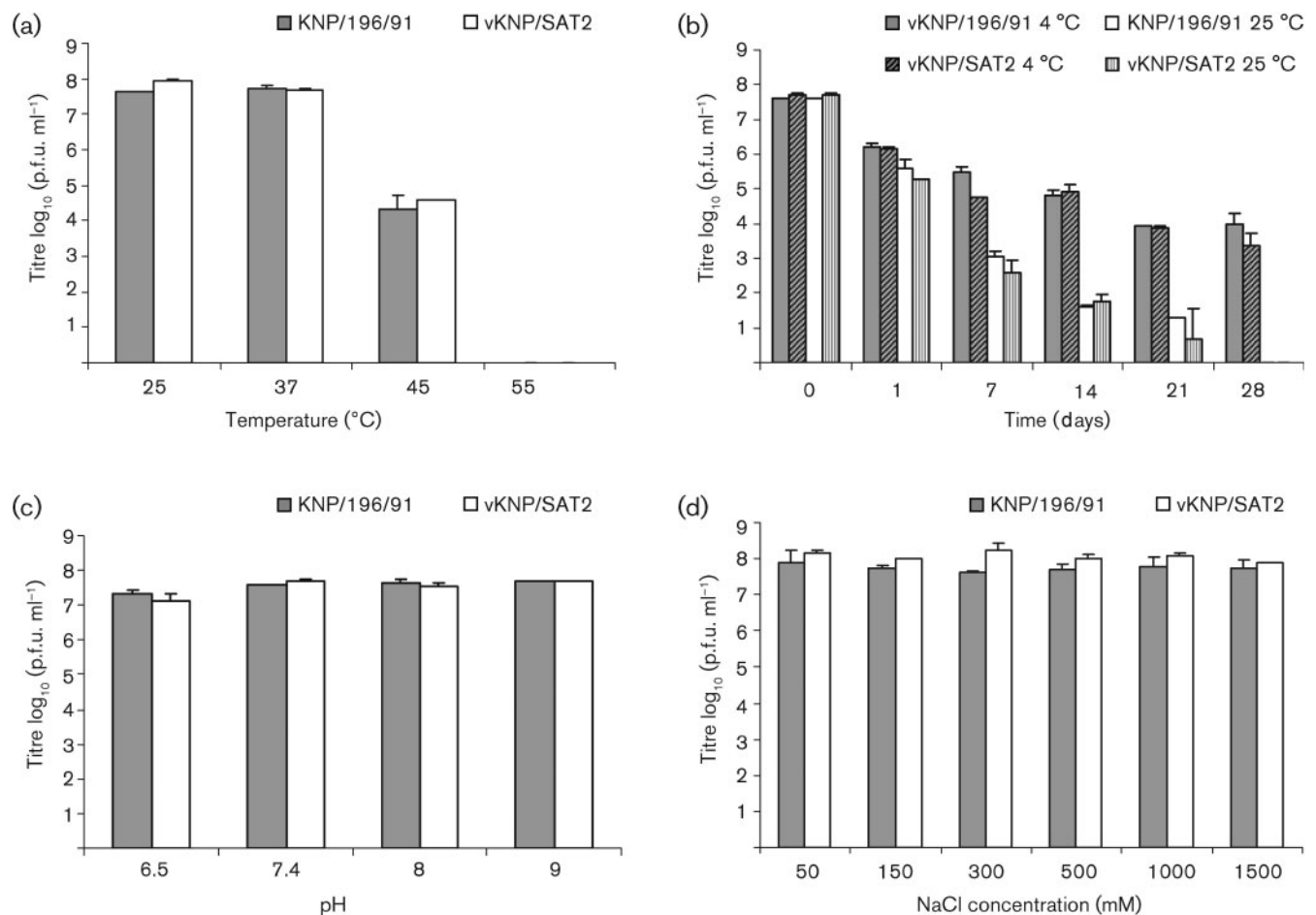


Fig. 3. Biophysical stability of 146S virion particles of KNP/196/91 and the recovered vKNP/SAT2 chimera at different temperatures (a, b), pH (c) and NaCl concentrations (d). Purified virion particles were tested for temperature stability by incubation at the indicated temperatures for 30 min (a), and at 4 °C and 25 °C over a period of 4 weeks (b). All samples were titrated on BHK-21 cells.

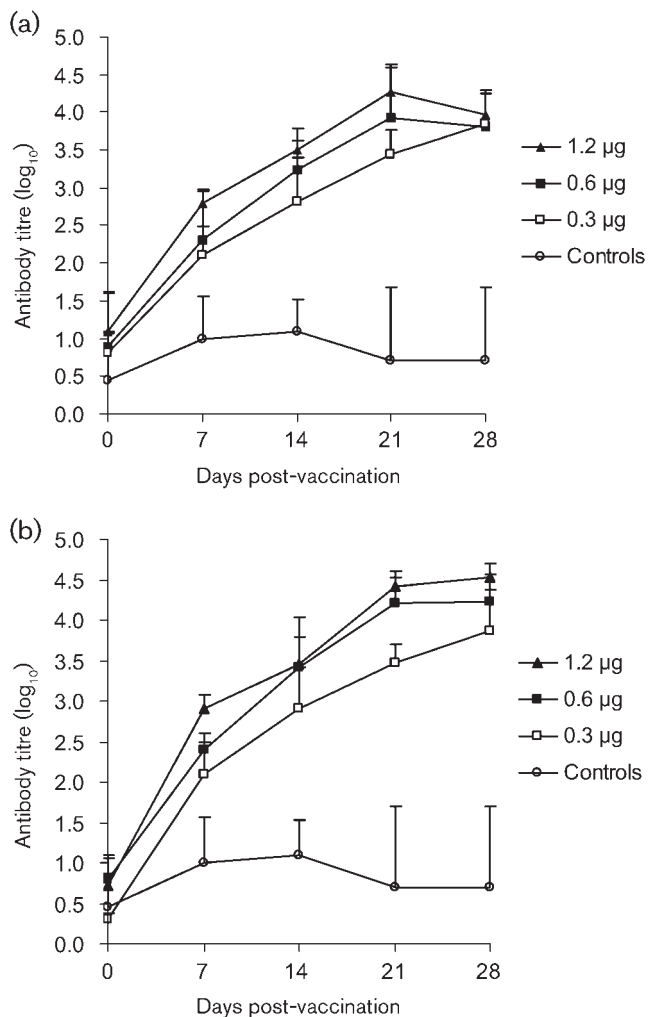


Fig. 4. Antibody responses elicited in guinea pigs for varying antigen payloads (0.3, 0.6 and 1.2 µg) of the vKNP/SAT2 (a) and KNP/196/91 (b) vaccines. Serum samples were tested by using a sandwich ELISA specific for KNP/196/91 and mean titres per group of 10 animals for each vaccine dose were determined at weekly intervals. Bars indicate the mean (\pm SD) of each group.

difference in neutralizing antibody response was induced by the chimeric and parental vaccines for the one-sixteenth dose ($P=0.02$), but not for the full and quarter doses. Comparisons between the immune responses for the three doses of the respective vaccines indicated no difference for KNP/196/91, suggesting that the one-sixteenth dose for the parental vaccine already gives a protective response and that it appears to be as efficient as the full dose under these conditions. For vKNP/SAT2 the neutralizing antibody response conferred by the full and quarter dose was not different. The response against the one-sixteenth dose was significantly lower compared with the full ($P<0.02$) and the quarter dose ($P=0.03$). Similar antibody response profiles at day 28 were observed using both the SPCE (Fig. 5) and VNT (Table 1) for animals from all the groups and vaccinated with both antigens.

The mean neutralizing-antibody titres for the animals protected by the vKNP/SAT2 full and quarter dose were 1.98 (SD=0.09) and 1.97 (SD=0.19) log titre, respectively, and ranged between 1.7 and 2.1 for individual pigs. Despite the fact that a mean 1.8 (SD=0.06) log titre for the two unprotected animals in the vKNP/SAT2 full-dose group was similar to titres observed for protected animals and that it is considered positive for SAT types in the VNT, these animals developed FMD lesions. The three animals of the vKNP/SAT2 one-sixteenth dose that were protected had a lower mean titre of 1.09 (SD=0.25) compared with the unprotected animals in the quarter dose, which had mean antibody levels of 1.49 (SD=0.06) log titre. Following challenge, 60% of the animals receiving the chimeric vaccine were fully protected against disease (Table 1). The onset of FMD lesions in animals with clinical disease was delayed and restricted in distribution compared with the control animals, indicative of partial protection in these animals. For most of the vaccinated pigs increased body temperatures were observed for 1 day only, whereafter temperatures decreased (data not shown). In comparison, the KNP/196/91 vaccine elicited mean titres ranging from 2.21 (SD=0.39) for the full dose, 1.9 (SD=0.27) for the quarter dose and 1.81 (SD, 0.34) for the one-sixteenth dose. Notwithstanding the absence of measurable positive neutralizing-antibody titres for two animals that received the KNP/196/91 vaccine, all animals were protected against challenge. The PD_{50} for the vKNP/SAT2 and parental vaccine were >6.4 and >39.4 , respectively, as calculated by the Kärber method (Kärber, 1931). The vaccine potency is expressed as the number at which 50% of the animals used for the challenge experiments were protected.

DISCUSSION

FMD vaccine candidates should be closely related to field strains (Doel, 1996) and induce an immune response with a broad immunological cross-reactivity (Esterhuysen *et al.*, 1988) for appropriate protection against subtypes (Pay, 1983). This is especially relevant in sub-Saharan Africa where the high degree of antigenic variability in FMDV and the presence of several serotypes, including several subtypes, have important implications for vaccine strain selection. In the present study, by engineering a chimeric virus, a possible alternative to the conventional inactivated-FMD vaccine production for the SAT type viruses was investigated for the development of custom-engineered inactivated-FMD vaccines.

A cross-serotype chimeric virus, vKNP/SAT2, replicated stably in both FMD host- and non-host-species-derived cell lines and the ability to produce plaques was similar for vKNP/SAT2 and KNP/196/91. Although BHK-21 cells are derived from a non-host species, the integrin cellular receptors expressed by these cells while growing in monolayers are recognized by FMDV in an RGD (arginine-glycine-aspartic acid)-dependent manner (Fox *et al.*, 1989; Mason *et al.*, 1994; DiCara *et al.*, 2008 and

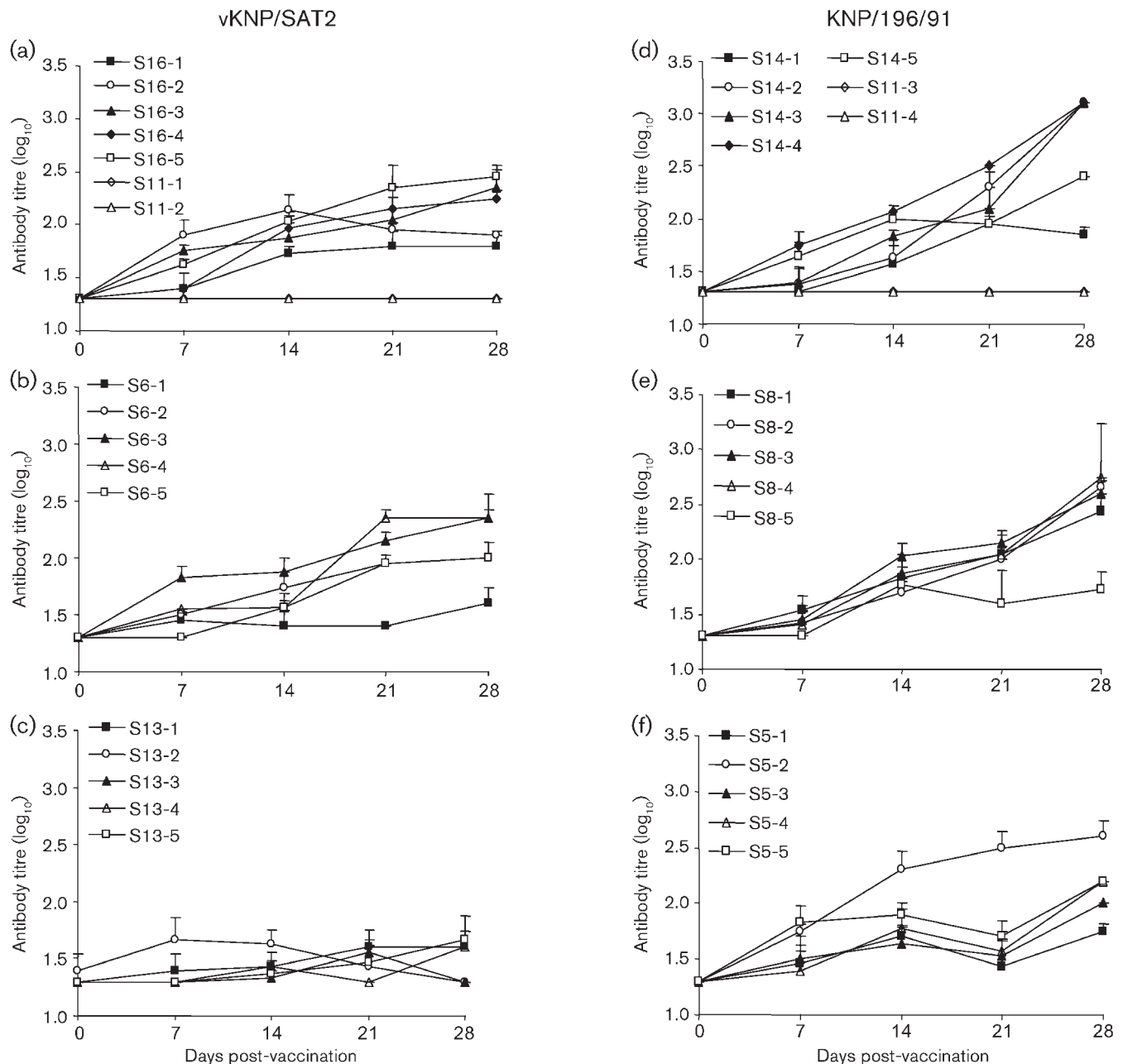


Fig. 5. Antibody responses elicited in pigs by full (6.0 µg), quarter (1.5 µg) and one-sixteenth (0.375 µg) doses of vKNP/SAT2 (a–c) and KNP/196/91 (d–f) vaccines, respectively. Two separate double oil emulsions incorporating inactivated 146S antigens of vKNP/SAT2 and KNP/196/91 were prepared and used for vaccination. Serum samples collected were tested by using a KNP/196/91-specific SPCE and mean titres per group of five animals for each vaccine dose were determined at weekly intervals. Means were determined from at least two replicates. Animals of the control group (S11 animals) were inoculated with a placebo formulation without antigen.

references therein). The porcine cell line IB-RS-2 was utilized for virus isolation and the VNT, and expresses mainly $\alpha v \beta 8$ on the cell surface (Burman *et al.*, 2006). In contrast, CHO-K1 cells lack the integrin cellular receptors used by FMDV for cell entry and express HSPG, which is used as an alternative receptor by cell culture-adapted FMDV (reviewed by Jackson *et al.*, 2003). Notably, the

presence of the external capsid proteins of an SAT1 virus in the genetic background of an SAT2 virus did not alter the biological properties of the chimera markedly, suggesting that it is a method that leads to the design of good vaccine candidates. In fact, vKNP/SAT2 retained the rapid infection kinetics of KNP/196/91 in BHK-21 cells that is imperative for production of a high yield of inactivated

Table 1. Neutralizing antibody responses and post-challenge clinical data for pigs vaccinated with vKNP/SAT2 and KNP/196/91

Vaccines were administered as a single inoculation at day 0. Neutralizing antibody titres were determined on day 28 post-vaccination and are the mean values of three replicates of the VNT. Titres of $\geq \log_{10} 1.7$ were regarded as positive. The maximum temperature observed for each animal after challenge is shown. Temperatures of ≥ 39.6 °C were regarded as positive for fever. The degree of disease post-challenge was defined as: none, no lesions on any feet; mild, lesions on only one foot; severe, lesions on two to three feet. Clinical signs of FMD were regarded as lesions on any foot, other than the limb used for challenge, and elevated body temperatures of ≥ 39.6 °C. Total protection was considered as a complete absence of lesions.

Vaccine	Animal	Neutralizing antibody titre	Post-challenge clinical data	
			Temperature (°C)	Degree of disease
vKNP/SAT2				
6 µg	16-1	1.7	39.9	Mild
	16-2	1.8	39.7	Mild
	16-3	1.9	39.2	None
	16-4	2.0	39.8	None
	16-5	2.0	39.2	None
1.5 µg	6-1	1.4	39.8	Mild
	6-2	1.5	39.6	Mild
	6-3	2.1	39.3	None
	6-4	2.1	39.3	None
	6-5	1.7	39.2	None
0.375 µg	13-1	0.8	40.4	None
	13-2	0.4	40.6	Severe
	13-3	0	41.0	Mild
	13-4	1.2	39.8	None
	13-5	1.2	39.9	None
KNP/196/91				
6 µg	14-1	1.7	39.2	None
	14-2	2.5	39.9	None
	14-3	2.5	39.3	None
	14-4	2.5	39.0	None
	14-5	1.9	39.6	None
1.5 µg	8-1	2.0	39.3	None
	8-2	2.2	39.0	None
	8-3	1.9	39.1	None
	8-4	1.8	39.1	None
	8-5	1.5	39.4	None
0.375 µg	5-1	1.3	39.9	None
	5-2	2.2	39.5	None
	5-3	1.7	39.6	None
	5-4	1.9	39.4	None
	5-5	1.8	39.4	None
Controls				
Placebo*	11-1	0	39.9	Severe
	11-2	0	40.1	Severe
	11-3	0	41.0	Severe
	11-4	0	40.2	Severe

*Control animals received a placebo formulation containing Tris/KCl buffer.

146S particles to be used as FMD vaccine. Both vKNP/SAT2 and KNP/196/91 displayed the cell culture-adaptation phenotype (Maree *et al.*, 2010), possibly because of interactions with HSPG receptors (Jackson *et al.*, 1996, 2003; Sa-Carvalho *et al.*, 1997; Fry *et al.*, 1999). Such alteration in FMD viruses' ability to utilize HSPG is

important as it is associated with more rapid replication in BHK-21 cells, a change in cell tropism, a more effective cell killing capacity and could contribute to improved production of FMD vaccines in suspension cultures (Amadori *et al.*, 1994; Sevilla *et al.*, 1996; Baranowski *et al.*, 1998).

The potential of vKNP/SAT2 as a vaccine strain producing high yields of stable antigen was emphasized by its comparable biophysical properties to those of KNP/196/91. Virion stability is of importance during the vaccine manufacturing process as the maintenance of intact 146S particles is relevant to the amount of immunogenicity induced by antigens and to vaccine efficacy (Doel & Baccarini, 1981; Doel, 2003). In the present study, a comparable decrease in infectivity was observed for the respective viruses at room temperature and above. The similar capsid stabilities of vKNP/SAT2 and KNP/196/91 observed at various NaCl concentrations is applicable to the range of ionic strengths that might exist during the purification of 146S particles in the vaccine production process. Unlike most other picornaviruses, FMDV is susceptible to low-pH-induced capsid disassembly, a characteristic of great importance to its pathogenicity (King, 2000). Both KNP/196/91 virus and its derivative were stable throughout a range of acidic to basic pH treatments. In addition to the above-mentioned qualities necessary for successful vaccine manufacturing, KNP/196/91 also displays good *r* values, which are an indication of the probability of protection for vaccinated animals against circulating variant viruses in the field (Esterhuysen, 1994; Reeve *et al.*, 2010). Moreover, comparable neutralization profiles were obtained for the respective viruses tested against other SAT1 and 2 antisera, confirming that vKNP/SAT2 retained the antigenic properties of KNP/196/91.

The advantages of using oil formulations for FMD vaccines have been well established and the Montanide ISA 206B (water/oil/water) has proven to be an efficient adjuvant (Barnett *et al.*, 1996) for the SAT types. Long-lasting immune responses were observed for cattle following immunization with vaccine containing KNP/196/91 antigen formulated in Montanide ISA 206B oil-based adjuvant (Hunter, 1996; Cloete *et al.*, 2008). The vKNP/SAT2 and KNP/196/91 vaccines elicited good humoral immune responses in guinea pigs and pigs. The majority of the pigs vaccinated with vKNP/SAT2 were protected against live-virus challenge. In addition, the onset of disease was delayed for most of the vKNP/SAT2-vaccinated pigs when compared with the control animals, and the clinical signs were less severe. This is indeed promising as antigen doses of 6 µg correlate with emergency FMD vaccines ($PD_{50} \geq 6$) that induce rapid protective immune responses (Barnett & Carabin, 2002).

Similar neutralizing antibody titres were elicited in pigs vaccinated with the vKNP/SAT2 and KNP/196/91 vaccines, as were observed for SAT type vaccines that conferred protection *in vivo* by Barnett *et al.* (2003). However, for some of the animals it is difficult to find a correlation between protection and neutralizing-antibody response. Protection was observed for three animals of the vKNP/SAT2 one-sixteenth group, which showed no detectable titre in the VNT. Although higher antigen doses elicited more neutralizing antibodies for most animals vaccinated

with vKNP/SAT2 and KNP/196/91, even at low antigen concentrations protective immunity was induced. This phenomenon may be explained by additional neutralizing mechanisms that exist in the host, such as complement-enhanced neutralization. Another explanation might be the presence of a cell-mediated immunity (CMI) component contributing to the extent of protection, which could have a different response depending on the difference in non-capsid proteins in the virion. CMI parameters were not measured in this study. Previous studies have indicated that even though antibodies were non-neutralizing *in vitro* and/or at low concentrations, these were in fact protective *in vivo* (Anderson *et al.*, 1971; McCullough *et al.*, 1992; Vianna Filho *et al.*, 1993; Brehm *et al.*, 2008).

The antigen payload, the integrity of the FMDV particle and the conformation of the viral epitopes, are important factors to consider for FMD vaccine efficacy. The reason for the lesser extent of protection in pigs conferred by the vKNP/SAT2 chimeric vaccine is not clear. This matter could be addressed by characterization with well defined mAbs directed against SAT1 FMDV or revaccination of animals to determine the stability of the formulated antigen. To refine the construction of SAT type chimeras, the external capsid-encoding region (1B-1D/2A) can be further manipulated to make such viruses more specific in their ability to infect and replicate in the cells most commonly used for vaccine production. It would also be possible to design SAT type FMD vaccines where regions of the genome are engineered to optimize epitope representation. An additional benefit of using this recombinant DNA technology is that reverse genetics allows for modifications that could be incorporated to support serological differentiation of infected from vaccinated animals for surveillance of FMD in sub-Saharan Africa. The potential now exists to generate more effective new-generation chemically inactivated-FMD vaccines for this highly infectious and economically important disease, which are custom-engineered and specifically produced for geographical areas.

METHODS

Cell lines, viruses and plasmids. Baby hamster kidney-21 cells, clone 13 (BHK-21, ATCC CCL-10), Instituto Biologico Renal Suino-2 cells (IB-RS-2) and Chinese hamster ovary cells strain K1 (CHO-K1, ATCC CCL-61) were propagated as described previously (Storey *et al.*, 2007). Primary pig kidney cells (PK) were maintained in RPMI medium (Sigma-Aldrich) supplemented with 10% FCS (Delta Bioproducts). The SAT1 virus KNP/196/91 (passage history: PK₁RS₄, subscript indicates the number of passages) was originally isolated from a buffalo in the Kruger National Park, South Africa, and was passaged in cattle (indicated as B) and BHK-21 cells (passage history: PK₁RS₄B₁BHK₄). Homologous challenge virus was prepared by three passages of KNP/196/91 in pigs (passage history: PK₁RS₄B₁BHK₄P₃). The external capsid-encoding region (1B-1D/2A) of plasmid pSAT2, a genome-length infectious cDNA clone of SAT2/ZIM/7/83 (van Rensburg *et al.*, 2004), was replaced with that of KNP/196/91 to yield plasmid pKNP/SAT2 (constructed by H.G. van Rensburg). In constructing the recombinant clone, pSAT was digested

with endonucleases *SspI* and *XmaI* to excise the approximately 2 kb external capsid-encoding region from the pSAT2 clone to facilitate cloning of the corresponding KNP/196/91 amplicon containing the same restriction endonuclease sites.

RNA synthesis, transfection and virus recovery. For RNA synthesis, plasmids pKNP/SAT2 and pSAT2 were linearized with *SwaI* and *NotI*, respectively, and *in vitro* transcribed using a MEGAscript T7 kit (Ambion). *In vitro*-transcribed RNA was transfected into BHK-21 cells using Lipofectamine 2000 reagent (Invitrogen). The cells were maintained at 37 °C for 48 h in virus growth medium (VGM; Eagle's basal medium supplemented with 1% FCS and 1% HEPES) and passaged as described previously (van Rensburg *et al.*, 2004). The viruses derived from the genome-length cDNA, designated vKNP/SAT2 and vSAT2, were confirmed by nucleotide sequencing and used in all subsequent experiments. Viral amplification was performed in BHK-21 cell monolayers for chimeric (vKNP/SAT2), parental (KNP/196/91) and vSAT2 viruses. Viruses were harvested at 90% cytopathic effect at passage five.

Plaque and growth-kinetics assays. BHK-21 cell monolayers were infected with KNP/196/91, vKNP/SAT2 and vSAT2 at an m.o.i. of 2. After 1 h of adsorption, cells were washed with MBS (MES-buffered saline, pH 5.5) and then incubated with VGM at 37 °C. At several time points post-infection, cells and culture medium were frozen at -80 °C. Virus titres were determined by plaque assays (Rieder *et al.*, 1993). Briefly, cells were infected with virus for 1 h, followed by the addition of 2 ml tragacanth overlay and incubation for 48 h. The cells were then stained with 1% (w/v) methylene blue in 10% ethanol and 10% formaldehyde, prepared with PBS (pH 7.4).

Virus purification and stability. vKNP/SAT2 and KNP/196/91 viruses were concentrated using 8% PEG-8000 (Sigma-Aldrich) and purified on sucrose density gradients (10–50%), prepared in TNE buffer [50 mM Tris (pH 7.4), 10 mM EDTA, 150 mM NaCl], as described by Knipe *et al.* (1997). Following fractionation, peak fractions corresponding to 146S virion particles [extinction coefficient $E_{259}^{1\%} = 79.9$; Doel & Mowat, 1985] were pooled, the amount of antigen calculated from the peak area, analysed by SDS-PAGE and titrated on BHK-21 cells. The stability of the purified virion particles at different temperatures was determined by incubating the virion particles, diluted 1:50 in 2× TNE buffer [100 mM Tris (pH 7.4), 10 mM EDTA, 150 mM NaCl], at 25, 37, 45 and 55 °C for 30 min, and at 4 and 25 °C over a period of 4 weeks. Following cooling on ice, the viruses were titrated on BHK-21 cells. The stabilities of the purified virion particles at different pH (pH 6.5–9.0) and NaCl concentrations (50–1500 mM) were determined by diluting the virion particles 1:50 in TNE buffer and incubating at room temperature for 30 min. After pH neutralization with buffer [1M Tris (pH 7.4), 150 mM NaCl], the viruses were titrated on BHK-21 cells.

Antigen preparation and vaccine formulation. The vKNP/SAT2 and KNP/196/91 viruses harvested from infected BHK-21 cell monolayers were inactivated with 5 mM binary ethyleneimine for 26 h at 26 °C, concentrated and purified as described above. Two separate vaccine formulations, incorporating vKNP/SAT2 and KNP/196/91 inactivated 146S antigens as double oil emulsions with Montanide ISA 206B (Seppic), were prepared. The antigen was diluted in Tris/KCl buffer (0.1 M Tris, 0.3 M KCl, pH 7.5) to the required concentration. The oil adjuvant was subsequently mixed into the aqueous antigen phase (1:1) at 30 °C for 15 min and stored at 4 °C for 24 h. This was followed by a second brief mixing cycle for 5 min. A placebo vaccine was formulated that contained all the components except antigen.

Immunization of guinea pigs. The vaccines, formulated as described above, were initially tested in guinea pigs to determine

the immunogenicity of the respective antigens. Six groups of 20 female guinea pigs (400–800 g each) were immunized intramuscularly with vaccines containing 0.3, 0.6 and 1.2 µg of the inactivated vKNP/SAT2 or KNP/196/91 antigen. Control animals received the placebo vaccine formulation. Ten animals from each group were bled alternately on 0, 7, 14, 21 and 28 d.p.v. Animals were anaesthetized intra-muscularly with a combination of xylazine and ketamine.

Vaccination and viral challenge of pigs. Thirty-four FMD-seronegative pigs (3–4 months of age and weighing 25–30 kg) were divided randomly into six groups of five animals each, and one control group of four animals. Each group was housed in a separate room in the high-containment animal facility of the Onderstepoort Veterinary Institute (ARC-OVI). Subsequent to an initial acclimatization period, the pigs were vaccinated by the intramuscular route immediately caudal to the ear with 2 ml (full dose), 0.5 ml (quarter dose) and 0.125 ml (one-sixteenth dose) of 3 µg ml⁻¹ of either vKNP/SAT2 or KNP/196/91 vaccine. Control animals were vaccinated with a placebo vaccine formulation that lacked viral antigen. Serum samples were collected at 0, 7, 14, 21 and 28 d.p.v. for serological assays. At 28 d.p.v. the pigs were inoculated intra-epidermally in the coronary band of the left hind heel bulb with 10⁴ TCID₅₀ KNP/196/91 challenge virus. During each of these procedures the pigs were sedated using azaperone. The animals were examined daily for fever and clinical signs. Body temperatures of ≥39.6 and ≥40 °C were considered as mild and severe fever, respectively. Upon observation of generalization of clinical lesions to other sites (e.g. to the snout and other legs), pigs were removed from the experiment and euthanized. At day 10 post-infection, the remainder of the animals were euthanized.

Antibody detection in guinea pigs. A SAT1 KNP/196/91-specific sandwich ELISA was performed on guinea pig sera to measure the amount of anti-KNP/196/91 antibodies present in each sample. The KNP/196/91 virus was added to a 96-well microplate (Nunc Maxisorp) coated with rabbit anti-KNP/196/91 antiserum. After incubation overnight at 4 °C the plates were washed with 1× PBS containing 0.05% Tween 80 (PBS-T80). One hundred microlitres of each sample was added to normal bovine serum followed by diluting 1:1000 in 1× PBS containing 0.5% (w/v) casein (PBS-casein). The dilution was added to the plates in triplicate and diluted 1:1 in PBS-casein. The plates were then incubated for 1 h at 37 °C and washed with PBS-T80. HRP-conjugated rabbit anti-guinea pig IgG (Sigma-Aldrich), diluted 1:100 in PBS-casein, was added and the plates incubated for 1 h at 37 °C. The plates were washed with PBS-T80, followed by addition of the substrate solution consisting of 1 ml (1% w/v) 3,3',5,5' tetramethylbenzidine, sodium phosphate/citric acid buffer and 30 ml (30% w/v) H₂O₂. After incubating for 15 min at room temperature, the reactions were stopped with 1 M H₂SO₄ and OD₄₉₂ was measured with a LabSystems Multiscan Plus Photometer. The titre was determined from the log₁₀ reciprocal antibody dilution giving 1.0 OD₄₉₂ unit. Sera were considered positive when the titre was equal or greater than twice the value of the negative control sera.

Antibody detection in pigs. Antibodies in pig sera against KNP/196/91 were detected by an SAT1 KNP/196/91-specific SPCE. The SPCE was essentially carried out as described above. Briefly, the KNP/196/91 virus was trapped by rabbit anti-KNP/196/91 antiserum. After incubation overnight at 4 °C the plates were washed with PBS-T80. One hundred microlitres of a 1:20 dilution in PBS-casein of each sample was added in triplicate and diluted 1:1 in 50 µl of PBS-casein across the plate. Guinea pig anti-KNP/196/91 antiserum diluted 1:6000 in PBS-casein was added to the wells then incubated and washed. The addition of anti-species conjugate and the subsequent detection steps were as described above. Antibody titres were determined at the dilution where 50% inhibition was observed

between the pig sera and the guinea pig anti-KNP/196/91 antisera. A cut-off value of \log_{10} 1.7 was considered positive.

VNT. Neutralizing antibodies against SAT1 KNP/196/91 in serum samples collected at 28 d.p.v. from pigs were measured with a VNT, according to the method described in the *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* by Office International des Epizooties (2009) using IB-RS-2 cells in microtitre plates. The antibody titres were calculated as \log_{10} of the reciprocal of the final serum dilution that neutralized 100 TCID₅₀ of virus in 50% of the wells.

Data analysis. Antibody titres were presented on a \log_{10} scale as means and SD. Repeated measures ANOVA analyses were performed to estimate the effects of vaccination strain and dose on ELISA antibody titres over time. VNT titres were compared using Student's *t*-test with Bonferroni adjustment for multiple comparisons. Statistical analyses were performed using commercially available software (SPSS version 17.0 for Windows; SPSS) and results interpreted at the 5% level of significance.

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