Custom-Engineered Chimeric Foot-and-Mouth Disease Vaccine Elicits Protective Immune Responses in Pigs

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SUMMARY

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-coding region (1B-1D/2A) of an infectious cDNA clone of the SAT2 vaccine strain, ZIM/7/83, with that of the 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. With these qualities necessary for vaccine manufacturing, high titres of stable chimera 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 to commercial vaccine requirements the vaccines were evaluated in a full potency test. Pigs vaccinated with the chimeric vaccine produced neutralising 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 induces protective immune responses in FMD host species.

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 the FMD virus (FMDV) and contributes to the spread of FMD to other wildlife species and livestock. Moreover, genetic and antigenic characterisation 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), which leads to the inability of the available vaccines to adequately cover the extent of antigenic variation within the South African Territories (SAT) types of FMDV (Maree *et al*., 2008). Thus, for vaccination to be effective in the sub-Saharan African region, it requires the incorporation of vaccine strains representative of viruses circulating in that geographical area and 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 characterisation of field and laboratory strains (van Rensburg *et al*., 2004; Storey *et al*., 2007; Maree *et al*., 2010). Chimeras, containing the external capsid-coding region (1B-1D/2A) of another FMDV, retain the replication machinery of the backbone as the P1, 2A and 3C 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 allowing host-cell internalisation (Maree *et al.*, 2010). This technology may therefore be utilised towards the production of custom-made vaccines specific to geographical regions or in cases of sudden FMDV outbreaks. 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 protecting immune response in animals (Rweyemamu, 1978; Doel, 2003).

In this study, the feasibility of a chimera vaccine was assessed by determining the immunogenicity and protective ability following immunisation of pigs. The vaccine was prepared from a chimeric virus containing the external capsid-coding region of a rapidly replicating, cell-adapted SAT1 vaccine strain, KNP/196/91, exchanged in the genetic background of a 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 chimera vaccine in pigs, good humoral immune responses were elicited and the majority of the animals were protected against homologous FMDV challenge and passed the PD50 ≥ 6 requirement of the Office International des Epizooties (OIE) 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 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, the KNP/196/91 virus that displayed broad antigenic coverage (Maree *et al.*, 2008) was utilised 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 the 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. 1a the 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. Each of the vKNP/SAT2, KNP/196/91 and vSAT2 viruses formed clear plaques on CHO-K1 cells, characteristic of viruses that use heparan sulfate proteoglycans (HSPG) as receptors. The comparable abilities 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-coding region for the vKNP/SAT2 and KNP/196/91 viruses were identical following transfection and recovery in BHK-21 cells (data not shown).

In growth curves performed in BHK-21 cells (Fig. 1b), vKNP/SAT2 displayed similar replication ability in BHK-21 cells as 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 x 10^8 and 8.2 x

10^8 pfu ml^{-1}, respectively. For vaccine purposes the optimal harvest time for both vKNP/SAT2 and KNP/196/91 is 12 h post-infection, which compares favourably to the time balance of stable particle survival resulting from the processes of virus assembly, rate of inactivation and the effect of proteolytic enzymes released during virus-infected cultures (Doel and Collen, 1984). 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 the virus neutralisation assay was performed against a panel of SAT1 and SAT2 pig and cattle antisera (Fig. 2). The data showed similar neutralisation values for the wild-type (PK$_1$RS$_4$) and cell culture adapted (B$_1$BHK$_9$) 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 neutralisation with either of the SAT1 isolates (Fig. 2).

Antigen yield is determined by bio-physical properties of FMDV

Bio-physical stability of the infectious virus or antigen has been correlated to the protective nature of FMD vaccines (Doel and Baccarini, 1981). With this in mind, the vKNP/SAT2 and KNP/196/91 were compared in terms of their bio-physical properties following treatment at different temperatures, acidic and alkaline conditions 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 that of the 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 particles observed for vKNP/SAT2 and KNP/196/91 were 2.6 x 10^3 pfu ml^{-1} and 5.7 x 10^3 pfu 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.

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 rendered a slight decrease in infectious particles after 30 min. The concentration 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 indicated no differences in bio-physical 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 asses the antibody response to immunisation in guinea pigs (Fig. 4). Following animal immunisation, serum samples were collected at weekly intervals and tested in a sandwich ELISA specific for KNP/196/91. Sero-conversion occurred at approximately 7 days post-vaccination (dpv) and serological responses varied over 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 the vKNP/SAT2 vaccine doses and titres peaked between 21 and 28 dpv (Fig. 4a). In comparison, the antibody responses elicited by the KNP/196/91 vaccine were similar to that of the same doses of the vKNP/SAT2 vaccine and peaked at 28 dpv (see 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 µg 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 the vKNP/SAT2 and KNP/196/91 were prepared and used for vaccination in a full potency trial (European Pharmacopoeia, 2006; OIE Manual of Standards, 2009). The antibody response elicited in pigs varied by time (P < 0.001), dose (full, 6.0 µg; quarter, 1.5 µg; one-sixteenth, 0.375 µg; P = 0.003), and vaccine (P = 0.011) when monitored at weekly intervals using a KNP/196/91-specific SPCE (Fig. 5). The full and quarter doses of the vKNP/SAT2 vaccine (Fig. 5a and b) elicited an antibody response comparable to equivalent doses of the KNP/196/91 vaccine (Fig. 5d and e) up to 21 dpv, where after the titres for the vKNP/SAT2 remained similar until the day of challenge. Most animals vaccinated with the vKNP/SAT2 one-sixteenth dose (Fig. 5c) were border-line positive at the time of challenge. However, the results from the ELISA indicated that the KNP/196/91 vaccine elicited positive antibody responses for all vaccine doses (Fig. 5f).

Comparison of neutralisation titres and protection

Serum neutralising antibody responses were measured by the VNT at the day of challenge for the vaccinated and control animals (Table 1). All of the pigs were negative for FMDV-specific neutralising antibody at the onset of the study (results not shown). Positive neutralising antibody titres were 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 neutralisation of the KNP/196/91 virus. Whilst four pigs vaccinated with the KNP/196/91 one-sixteenth dose had positive neutralising antibody titres, the entire vKNP/SAT2 one-sixteenth group had no detectable VN titre. Statistical analysis indicated

that a significant difference in neutralising antibody response was induced by the chimera and parental vaccines for the one-sixteenth dose (P = 0.02), but not for the full and quarter doses. Comparison 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 is already giving a protective response and appears to be as efficient as the full dose under these conditions. In contrast, for vKNP/SAT2 the neutralising antibody response conferred by the full and quarter dose was not different. The response against the one-sixteenth dose was significantly lower compared to the full (P =< 0.02) and the quarter dose (P = 0.03). Similar antibody response profiles at day 28 were observed in both the SPCE (Fig. 5) and VNT (Table 1) for animals from all the groups and vaccinated with both antigens.

The mean neutralising 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 an average 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 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 average titre of 1.09 (SD, 0.25) compared to the unprotected animals in the quarter dose, which had average 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 to the control animals, indicative of partial protection in these animals. For most of the vaccinated pigs increased body temperatures were observed for one day only, whereafter temperatures decreased (not shown). In comparison, the KNP/196/91 vaccine elicited average 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 measureable positive neutralising antibodies titres observed for two animals that received the KNP/196/91 vaccine, all animals were protected against challenge. The 50% protective dose (PD50) for the vKNP/SAT2 and parental vaccine was >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 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 vaccine production of 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 recognised by FMDV in a RGD-dependent manner (Fox *et al*., 1989; Baxt and Becker, 1990; Mason *et al*., 1994; DiCara *et al*., 2008 and references therein). The porcine cell line IB-RS-2 was utilised for virus isolation and the VNT and expresses mainly αvβ8 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 in Jackson *et al.*, 2003). Notably, the presence of the external capsid proteins of a SAT1 virus in the genetic background of a SAT2 virus did not alter the biological properties of the chimera significantly, 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 which is imperative for production of a high yield of inactivated 146S particles to be used as FMD vaccines. Both vKNP/SAT2 and KNP/196/91 displayed the cell culture adaptation phenotype (Maree *et al.*, 2010) possibly due to interactions with HSPG receptors (Jackson *et al.*, 1996; Sa-Carvalho *et al.*, 1997; Fry *et al.*, 1999; Jackson *et al.*, 2003). Such alteration in FMD viruses' ability to utilise 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 emphasised by the comparable bio-physical properties to that of KNP/196/91. Virion stability is of importance during the vaccine manufacturing process as the maintenance of intact 146S particles are relevant for the immunogenicity induced by antigens and vaccine efficacy (Doel and 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 stability of vKNP/SAT2 and KNP/196/91 observed at various NaCl concentrations is applicable to the range of ionic strengths that may 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 the KNP/196/91 virus and its derivative were stable through 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 to protect vaccinated animals against circulating variant viruses in the field (Esterhuysen *et al*., 1994, Maree *et al*., 2008). Moreover, comparable neutralisation 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 (W/O/W) has proven to be an efficient adjuvant (Barnett *et al*., 1996) for the SAT types. Long-lasting immune responses were observed for cattle following immunisation 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 the 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 to the control animals, and the clinical signs were less severe. This is indeed promising as antigen doses of 6 µg correlate to emergency FMD vaccines (PD50 ≥ 6) that induce rapid protective immune responses (Barnett and Carabin, 2002).

Similar neutralising antibody titres were elicited in pigs vaccinated with the vKNP/SAT2 and KNP/196/91 vaccines as was observed for SAT type vaccines that conferred protection *in*

vivo by Barnett and co-workers (2003). However, for some of the animals it is difficult to find a correlation between protection and neutralising 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 neutralising 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 neutralising mechanisms that do exist in the host, like complement enhanced neutralisation. 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-neutralising *in vitro* and/or at low concentrations, these were in fact protective *in vivo* (Anderson *et al*., 1971; McCullough *et al*., 1992; Filho *et al*., 1993; Brehm *et al*., 2008).

The antigen payload, as well as the integrity of the FMDV particle and conformation of the viral epitopes, is important factors to consider for FMD vaccine efficacy. The reason for the lesser extent of protection in pigs conferred by the vKNP/SAT2 chimera vaccine is not clear. This matter could be addressed by characterisation with well defined monoclonal antibodies directed against SAT1 FMDV or re-vaccination of animals to determine the stability of the formulated antigen. To refine the construction of SAT type chimeras, the external capsid-coding region (1B-1D/2A) can be further manipulated to make such viruses more specific for its ability to infect and replicate in cells most commonly used for vaccine production and it would be possible to design SAT type FMD vaccines where regions on the genome are engineered to optimise epitope representation. An additional benefit of using this

recombinant DNA technology is that the reverse genetics system allows for modifications which could be incorporated to support DIVA applications 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 geographic 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% fetal calf serum (FCS; Delta Bioproducts). The SAT1 virus KNP/196/91 was originally isolated (PK_1RS_4; subscript indicates the number of passages) from a buffalo in the Kruger National Park, South Africa, and was passaged in cattle (indicated as B) and BHK-21 cells (passage history: $PK_1RS_4B_1BHK_4$). Homologous challenge virus was prepared by three passages of KNP/196/91 in pigs (passage history: $PK_1RS_4B_1BHK_4P_3$). The external capsid-coding 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). Towards construction of the recombinant clone, pSAT was digested with endonucleases *Ssp*I and *Xma*I to excise the *ca.* 2-kb external capsid-coding 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 the 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 (BME) supplemented with 1% calf serum, 1% HEPES) and passaged as described previously (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 the chimera (vKNP/SAT2), parental (KNP/196/91), and vSAT2 viruses. Viruses were harvested at 90% cytopathic effect (CPE) at passage five.

Plaque and growth kinetics assays.

BHK-21 cell monolayers were infected with KNP/196/91, vKNP/SAT2 and vSAT2 at a 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 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.

The 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 $E_{259nm}(1\%) = 79.9$; Doel & Mowat, 1985) were pooled, the amount of antigen (μg) 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 x 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 four weeks. Following cooling on ice, the viruses were titrated on BHK-21 cells. The stability of the purified virion particles at different pHs (6.5 to 9.0) and salt concentrations (NaCl; 50 mM to 1.5 M) was determined by diluting the virion particles 1:50 in TNE buffer and incubation at room temperature for 30 min. After pH neutralisation 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 (BEI) 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, Paris) 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 (50:50) 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.

Immunisation of guinea pigs.

The above formulated vaccines 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 immunised 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 dpv. Animals were anaesthetised 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 vaccines. Control animals were vaccinated with a placebo vaccine formulation that lacked viral antigen. Serum samples were collected at 0, 7, 14, 21 and 28 dpv for serological assays. At 28 dpv the pigs were inoculated intra-epidermally in the coronary band of the left hind heel bulb with 10^4 TCID$_{50}$ 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 $\geq$39.6 °C and $\geq$40 °C were considered as mild and severe fever, respectively. Upon observation of generalisation of clinical lesions to other sites (e.g. to snout, other legs), pigs were removed from the experiment and euthanised. At day 10 post-infection the remainder of the animals were euthanised.

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 4ºC the plates were washed with 1 x PBS containing 0.05% Tween-80 (PBS-T80). Of each sample, 100 µl was added to normal bovine serum followed by a 1/1000 dilution in 1 x 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, incubated for 1 h at 37 °C and washed. Horseradish peroxidase-conjugated rabbit anti-guinea pig IgG (Sigma-Aldrich), diluted 1:100 in PBS-casein, was added for 1 h at 37 °C. The plates were washed with PBS-T80, followed by addition of the substrate solution 3,3'.5,5' tetramethylbenzidine (TMB) sodium phosphate/citric acid buffer and H_2O_2. After incubation for 15 min at room temperature, the reactions were stopped with 1 M H_2SO_4 and the optical density (OD) at 492 nm 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 a SAT1 KNP/196/91-specific solid-phase competition ELISA (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 ºC the plates were washed with PBS-T80. Of each sample, 100 µl of a 1/20 dilution 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 incubated and washed. The addition of antispecies conjugate and subsequent

detection steps were as described before. 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.

Virus neutralisation assay.

Neutralising antibodies against SAT1 KNP/196/91 in serum samples collected at 28 dpv from pigs were measured with the virus neutralisation test (VNT), according to the method described in the OIE Manual of Standards (2009) using IB-RS-2 cells in micro-titre plates. The antibody titres were calculated as the $\log_{10}$ of the reciprocal of the final serum dilution that neutralised 100 $TCID_{50}$ of virus in 50% of the wells.

Data Analysis.

Antibody titres were presented in a $\log_{10}$ scale as means and standard deviations. 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 Inc., Chicago, IL, USA) and results interpreted at the 5% level of significance.

with additional statistical analysis.

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FIGURE LEGENDS

Fig. 1a. FMDV replication in BHK-21, IB-RS-2 and CHO-K1 cell cultures expressing diverse cellular receptors utilised by FMDV for cell entry. The morphology of plaques for the KNP/196/91 parental virus, derived vKNP/SAT2 chimera and vSAT2 were determined by plaque assay at 48 h post-infection.

Fig. 1b. Growth curves of the KNP/196/91 parental virus, vKNP/SAT2 chimera and vSAT2. BHK-21 cells were infected at a 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 (± SD) of at least two wells. Fig. 2. Antigenic profiles of FMD viruses vKNP/SAT2 (B$_1$BHK$_9$) and KNP/196/91 (PK$_1$RS$_4$), that has not been adapted in BHK-21 cells, tested against FMDV SAT1 antisera (vKNP/SAT2 chimera, KNP/196/91 parental, SAR/9/81); SAT2 antisera (ZIM/7/83) in the VNT. Pig antisera vKNP/SAT2 and KNP/196/91 were from the vaccinated animals in this

potency trial, whereas all other antisera were from cattle origin. A positive control serum of high titre was included. Serum titres of ≥ 1.7 were regarded as positive.

Fig. 3. Bio-physical 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 four 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 in a sandwich ELISA specific for KNP/196/91 and the average titres per group of 10 animals for each vaccine dose were determined at weekly intervals. Bars indicate the mean (± SD) of each group.

Fig. 5. Antibody responses elicited in pigs by full (6.0 μg), quarter (1.5 μg) and one-sixteenth (0.375 μg) doses of the 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 in a KNP/196/91-specific solid phase competition ELISA (SPCE) and the average titres per group of five animals for each vaccine dose were determined at weekly intervals. Averages were determined from at least two repeats. The control group (animals S11) was inoculated with a placebo formulation without antigen.

Table 1. Neutralising antibody responses and post-challenge clinical data for pigs vaccinated with vKNP/SAT2 and KNP/196/91
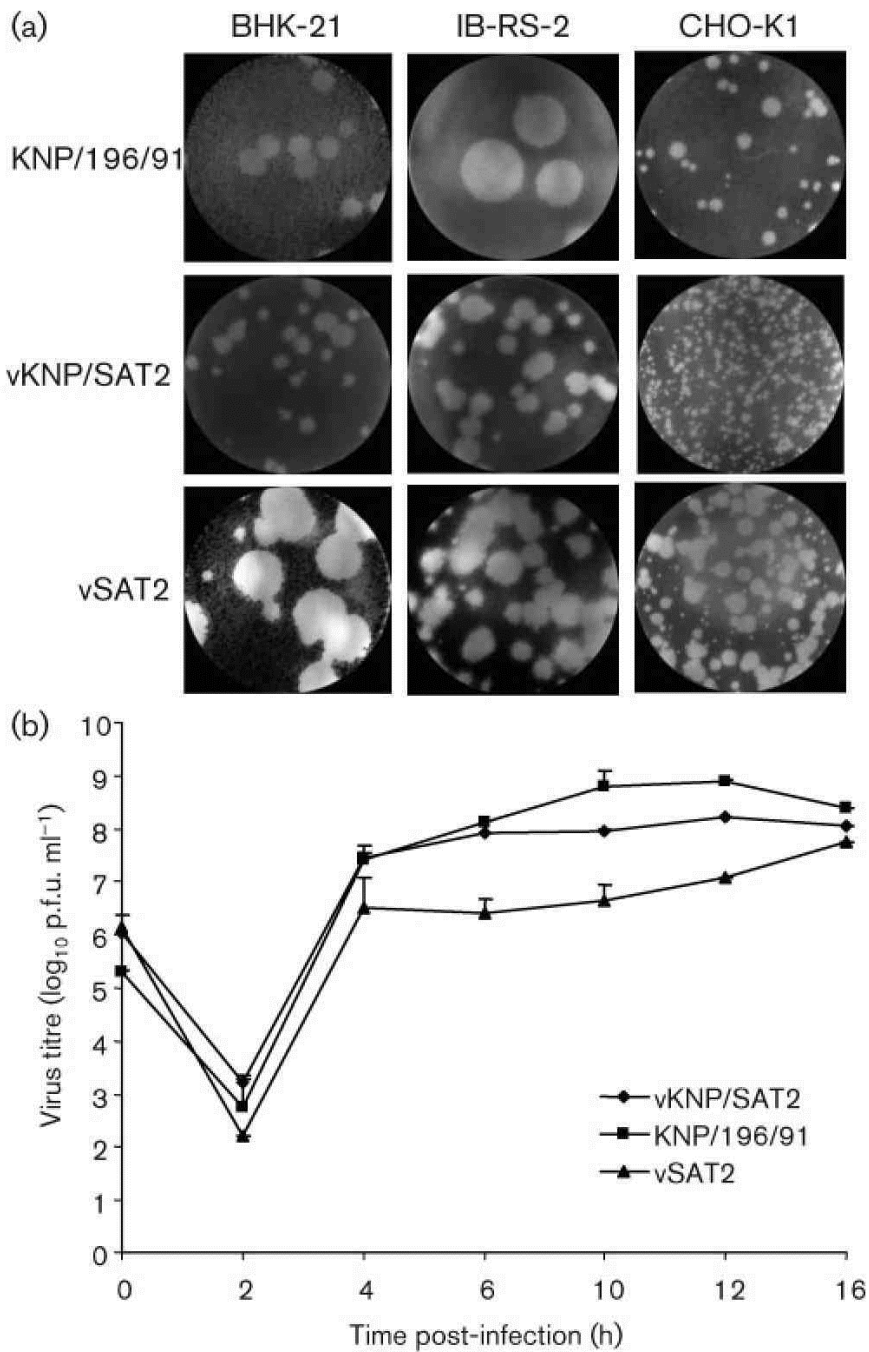
Fig. 1. (a) FMDV replication in BHK-21, IB-RS-2 and CHO-K1 cell cultures expressing diverse cellular receptors utilized by FMDV for cell entry. Morphology of plaques for the KNP/196/91 parental virus, vKNP/SAT2 chimera and vSAT2 were determined by plaque assay at 48 h post-infection. (b) Growth curves 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.
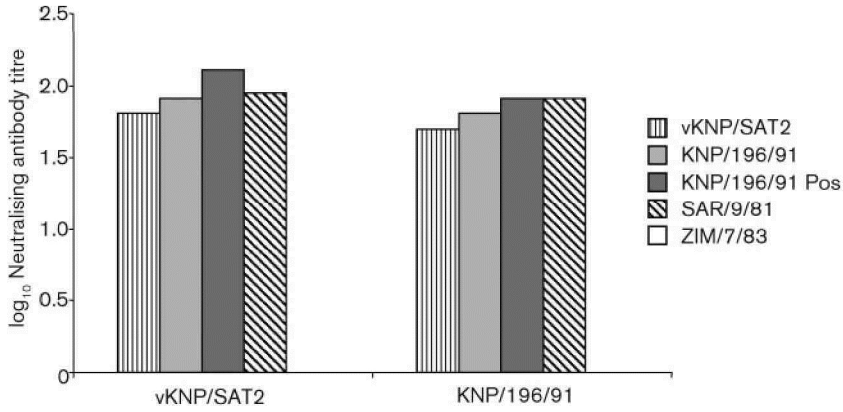
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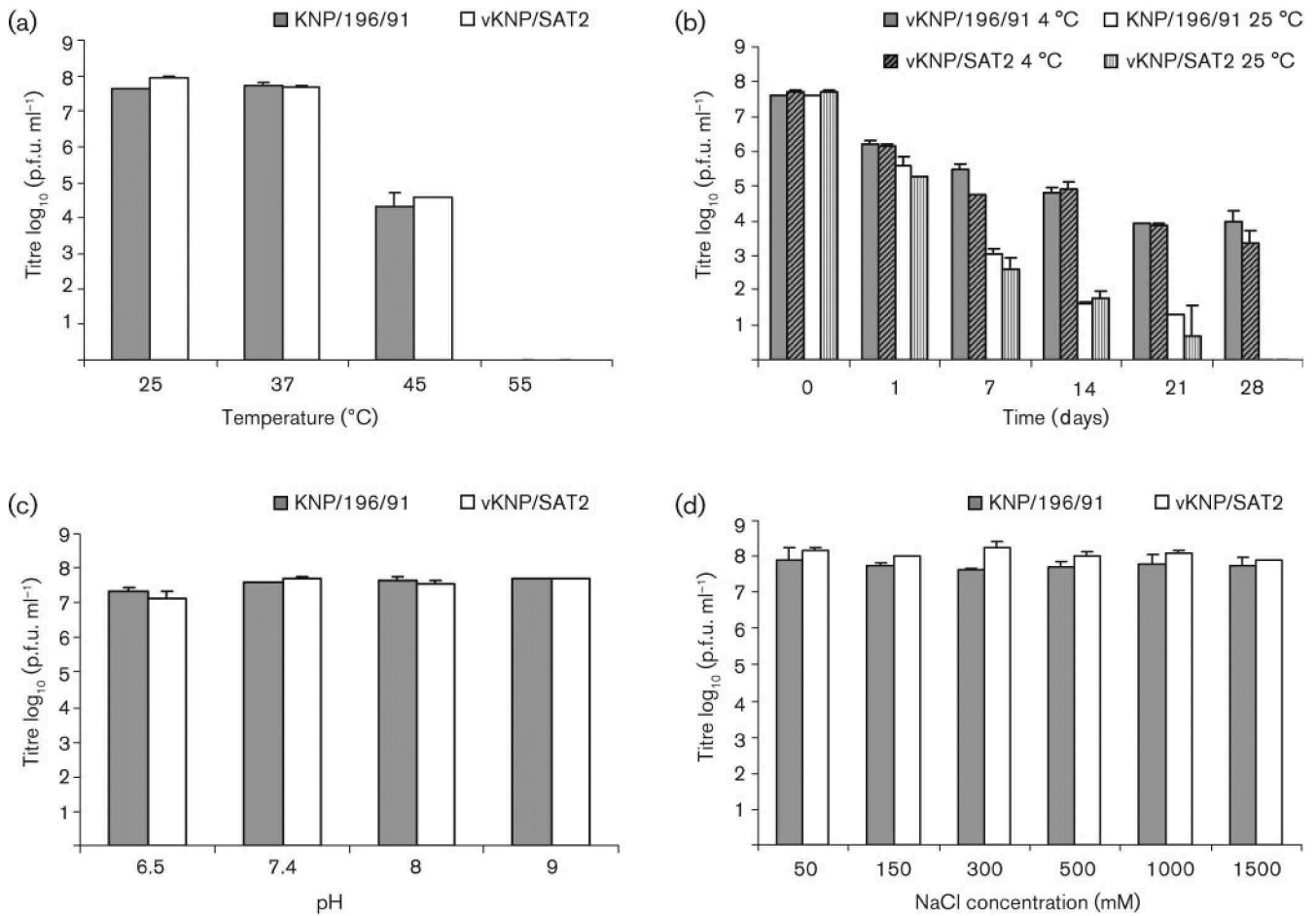
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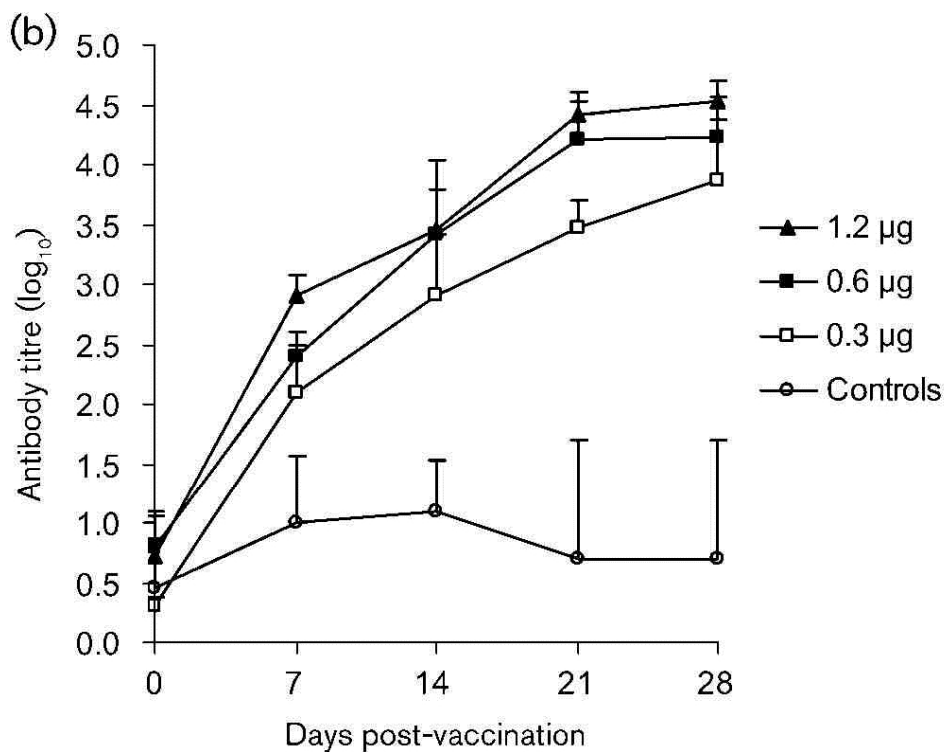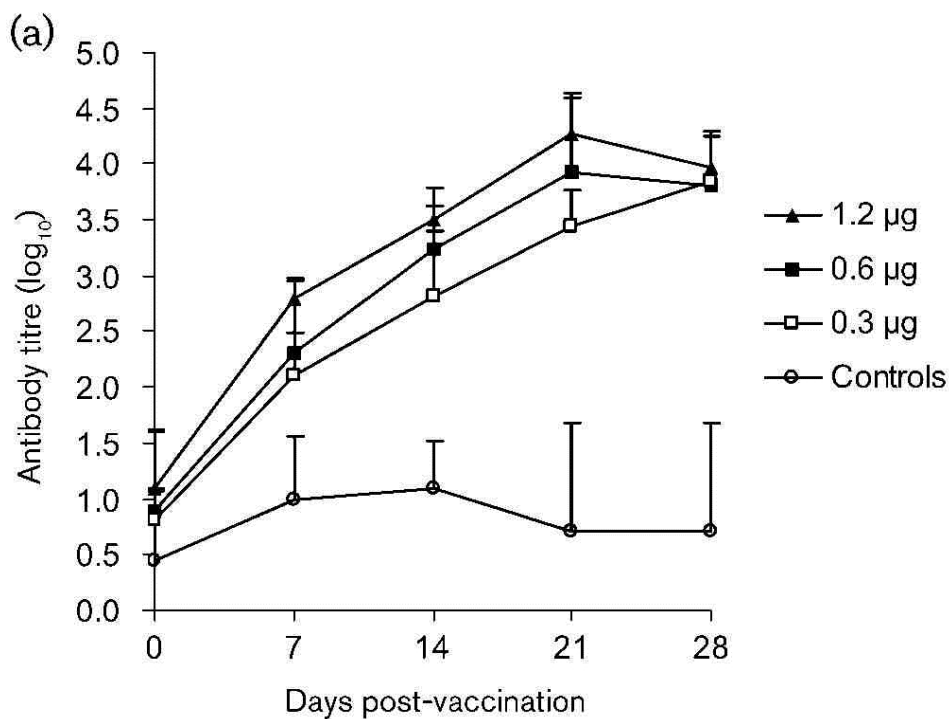
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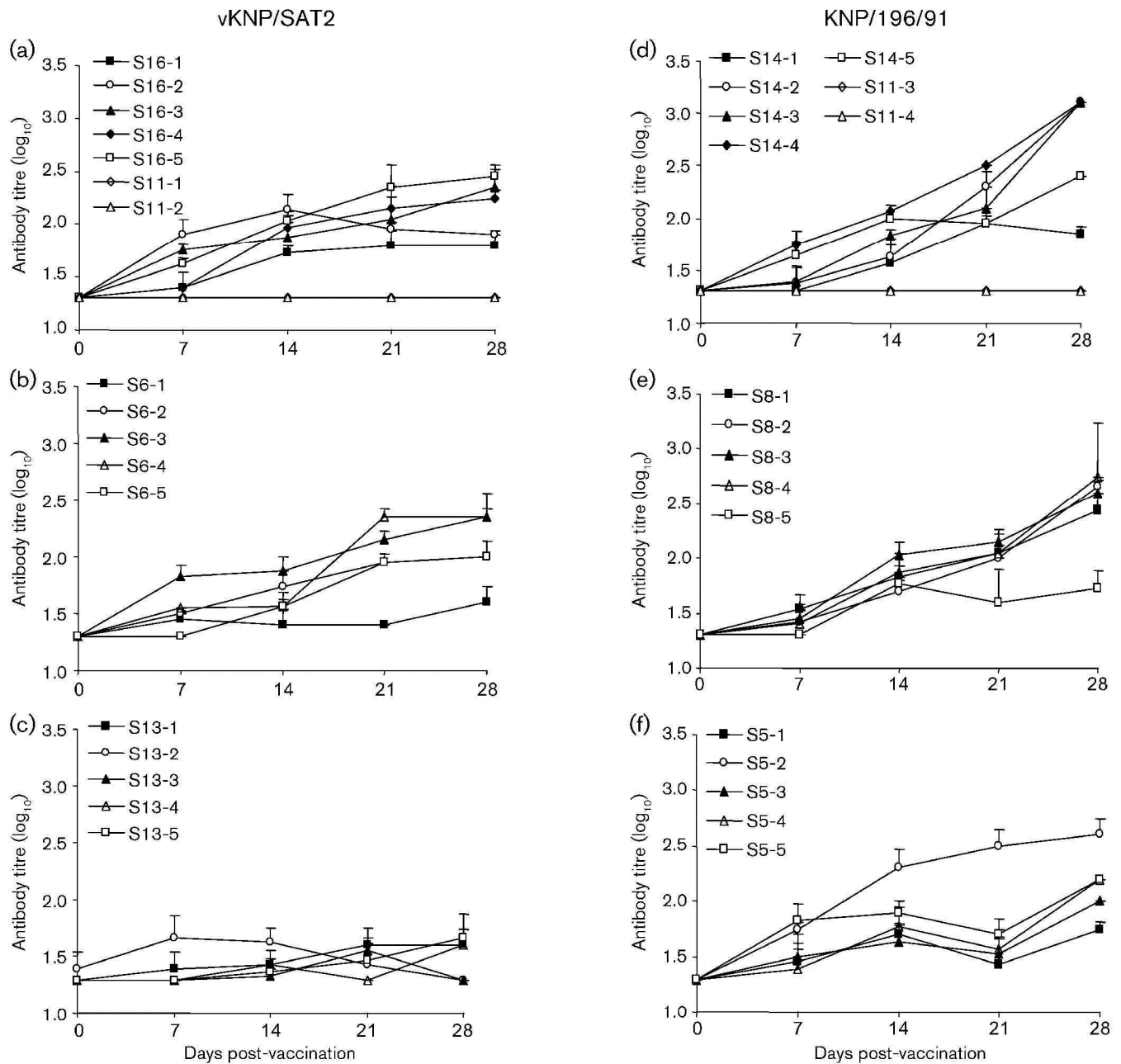
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Table 1. Neutralising antibody responses and post-challenge clinical data for pigs vaccinated with vKNP/SAT2 and KNP/196/91

Vaccine[*]	Animal	Neutralising 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

[*] Vaccines were administered as a single inoculation at day 0.
[†] Neutralising antibody titres were determined on day 28 post-vaccination and are the average values of three repeats of the VNT. Titres of $\geq \log_{10} 1.7$ were regarded as positive.
[‡] The maximum temperature observed for each animal after challenge is indicated. 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 that

the limb used for challenge, and elevated body temperatures of ≥ 39.6°C. Total protection was considered as complete absence of lesions.

‖ Control animals received a placebo formulation containing Tris-KCl buffer.