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PII: S0378-1135(08)00162-4
DOI: doi:10.1016/j.vetmic.2008.04.030
Reference: VETMIC 4025

To appear in: *VETMIC*

Received date: 7-12-2007
Revised date: 10-4-2008
Accepted date: 23-4-2008

Please cite this article as: Harmsen, M.M., Fijten, H.P.D., Dekker, A., Eblé, P.L., Passive immunization of pigs with bispecific llama single-domain antibody fragments against foot-and-mouth disease and porcine immunoglobulin, *Veterinary Microbiology* (2007), doi:10.1016/j.vetmic.2008.04.030

Passive immunization of pigs with bispecific llama single-domain antibody fragments against foot-and-mouth disease and porcine immunoglobulin.

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Abstract

Foot-and-mouth disease (FMD) is a contagious viral disease of cloven-hoofed animals that occasionally causes outbreaks in Europe. We aim to develop an immunotherapy that confers rapid protection against FMD in outbreak situations. For this purpose we previously isolated llama single-domain antibody fragments (VHHs) binding to FMDV or porcine immunoglobulin (pIg). The pIg binding VHHs can be genetically fused to other VHHs, resulting in so-called VHH2s. As compared to non-pIg binding VHHs such VHH2s have a 100-fold increased serum half-life which is essential for effective immunotherapy. We have now produced three bispecific VHH2s by fusion of three FMDV binding VHHs (clones M3, M8 and M23) to a pIg binding VHH (VI-4). The resulting yeast-produced VHH2s bound FMDV and pIg with high affinity (K_D about 1 nM) and neutralized FMDV in vitro as efficiently as their monovalent counterparts. To evaluate their therapeutic potential all three VHH2s were intramuscularly injected into pigs that were challenge infected with FMDV 24 h later. Administration of one of these VHH2s (M23ggsVI-4) reduced the viremia significantly (P=0.0034) and reduced viral shedding almost significantly (P=0.11). However, it did not prevent development of clinical signs or transmission of FMDV. These results suggest that immunotherapy using bispecific VHH2s binding to FMDV and pIg is possible in principle, but should be improved by increasing VHH2 dosage or using more potent VHH2s.

Keywords: Foot-and-mouth disease; Passive vaccination; Single-domain antibody; Nanobody

1. Introduction

Foot-and-mouth disease (FMD) is a contagious disease of cloven-hoofed animals caused by a virus of the *Picornaviridae* family. In Europe, livestock is highly susceptible to FMD due to the non-prophylactic-vaccination policy that is in force in the European Union (EU). Outbreaks of FMD can have severe socio-economic consequences, as was exemplified by the outbreaks in Europe in 2001 (Thompson et al., 2002). EU legislation (2003/85/EC) now favours emergency vaccination to control FMD outbreaks. However, it takes at least four days until vaccination prevents FMDV infection of pigs (Salt et al., 1998). Furthermore, although pigs vaccinated four days prior to challenge are protected against clinical disease, they can transmit FMD virus to contact susceptible animals, which then develop clinical FMD (Salt et al., 1998). Similarly it was observed that vaccination of pigs significantly reduces FMDV transmission, so that the epidemic comes to an end, when challenge infected 2 weeks post vaccination, but not when challenged 1 week post vaccination (Eblé et al., 2004; Eblé et al., 2008). Therefore, there is a need for therapies that provide rapid protection against FMD (Goris et al., 2008). Such rapid protection can be achieved by passive transfer of neutralising monoclonal antibodies (mAbs) or hyperimmune serum directed against FMDV (Blancou, 2002; Davidson, 1997; Dunn et al., 1998; McCullough et al., 1986).

We aim to develop such passive immunotherapy using recombinant single-domain antibody fragments (VHHs) derived from camelid heavy-chain antibodies. The use of VHHs has several advantages (Harmsen and De Haard, 2007). They are well-expressed in microorganisms (Frenken et al., 2000), have a high stability (Van der Linden et al., 1999) and are well-suited for construction of larger molecules such as genetic fusions of two VHH domains (Conrath et al., 2001). We previously isolated both neutralizing and non-neutralizing FMDV binding VHHs, recognizing four independent antigenic sites (Harmsen et al., 2007). A

disadvantage of VHHs is their rapid renal clearance due to their small size (Cortez-Retamozo et al., 2002). To enable therapeutic use of VHHs in pigs we previously also isolated several porcine immunoglobulin (pIg) binding VHHs that can be genetically fused to other VHHs. The resulting VHH2s have an 100-fold increased serum half-life (Harmsen et al., 2005a), presumably because the complex of the VHH2 and pIg is sufficiently large in order not to pass the renal filter.

In the present study we have assessed the therapeutic use of FMDV binding VHH2s in pigs. We generated three VHH2s having different FMDV binding VHHs (clones M3, M8 and M23) and an identical pIg binding VHH. We used a pIg binding VHH (clone VI-4) that was specific for the light chain (Harmsen et al., 2005a) since this is not expected to interfere with the Ig effector functions encoded by the heavy chain. We assessed the effect of passive immunisation with the resulting VHH2s on FMD challenge infection and transmission in pigs.

2. Materials and Methods

2.1. Cells and viruses

The O_1 Manisa/Turkey/69 FMDV isolate was used for both the preparation of viral antigen for use in ELISA and as challenge-virus in pig experiments. For viral antigen production FMDV was cultured using baby hamster kidney (BHK-21) cells grown in suspension as described previously (Harmsen et al., 2007). The challenge virus was prepared by pig passage. Secondary porcine kidney cells were used to determine virus titers as described previously (Eblé et al., 2004) or to determine the in vitro virus neutralizing capacity of VHH2s as described previously (Harmsen et al., 2007).

2.2. Construction of plasmids

Plasmid pRL193 is suitable for construction of genetic fusions of two VHHs tethered together by a linker consisting of the amino acids GGS (Harmsen et al., 2005a). In order to do so, first the N-terminal VHH encoded by pRL193 should be replaced by the VHH of interest using the unique *Pst*I and *Bst*EII sites. Then the C-terminal VHH should be inserted using the *Pvu*II and *Hin*dIII sites, resulting in the introduction of a second *Pst*I and a second *Bst*EII site. We deleted the *Pst*I and *Bst*EII sites of the VI-4 VHH contained at the C-terminal position of plasmid pRL193 (Harmsen et al., 2005a) by site-directed mutagenesis, resulting in plasmid pRL254. The *Bst*EII site was silently deleted whereas deletion of the *Pst*I site resulted in the conservative mutation Q6E. Insertion of the 0.35 kb *Pst*I-*Bst*EII fragments encoding the VHHs M3, M8 and M23 (Harmsen et al., 2007) into pRL254 resulted in plasmids pRL259, 260 and 261 (Table 1). These 2 micron-based plasmids are suitable for small scale yeast

production of the encoded VHH2s. For large scale production we inserted the VHH2 encoding regions into the MIRY-plasmid pRL44 as described previously (Harmsen et al., 2005b), resulting in the plasmids shown in Table 1.

2.3. Production of VHHs

The three VHH2s were produced by 100-L scale fermentation of yeast transformants containing the MIRY plasmids using previously described methods (Harmsen et al., 2005b). They were purified from the concentrated cell-free culture supernatant by IMAC followed by cation exchange chromatography as described previously (Harmsen et al., 2007) but using a SP-sepharose HP column (GE Healthcare, Piscataway, NJ) and only 5 column volumes for VHH2 elution. Furthermore, during buffer change to 20 mM sodium citrate, pH 4.2, prior to cation exchange chromatography of M3ggsVI-4_{Q6E} and M23ggsVI-4_{Q6E}, but not of M8ggsVI-4_{Q6E}, a precipitate was formed, that was removed by 0.45 μm filtration. After purification the buffer was changed to PBS and the concentration of purified VHH2 was determined using the bicinchonic acid assay (Pierce, Rockford, IL). The purified VHH2s were diluted to 40 mg/ml and stored at -70°C.

The small scale yeast production of the control VHHs M3, M8, M23 and VI-4 encoded by pUR4585-derived plasmids, and their purification by a single IMAC step has been described previously (Harmsen et al., 2005a; Harmsen et al., 2007).

2.4. ELISAs

ELISAs to determine whether VHH2s bind to pIg and FMDV were essentially performed as described previously (Harmsen et al., 2005a; Harmsen et al., 2007). Polystyrene

plates were directly coated with either FMDV O1 Manisa preparations (Harmsen et al., 2007) or pIgG (Harmsen et al., 2005a) and subsequently incubated with 2-fold dilution series of VHHs. Bound VHH was subsequently detected using a peroxidase-conjugated monoclonal antibody against the c-myc tag (Harmsen et al., 2007). For detection of bispecific binding to both FMDV and pIg we incubated FMDV-coated plates with dilution series of VHH2s. Bound VHH2 was detected by subsequent incubation with 1 mg/l biotinylated pIgG, peroxidase-conjugated streptavidin and TMB staining (Harmsen et al., 2005a).

2.5. Affinity measurements

The affinity of the bispecific VHH2s for FMDV antigen was determined by ELISA using competition with antigen in solution (Friguet et al., 1985). Thus, 1 nM of VHH2 was preincubated with a dilution series of FMDV antigen at 37 °C and then transferred to a plate coated with FMDV antigen at low density (1 mg/l total protein, corresponding to approximately 0.2 mg/l 146S particles). The VHH2 bound to the coated FMDV was then detected by subsequent incubation with biotinylated pIgG and peroxidase-conjugated streptavidin (see previous section). For determination of the affinity constant by Scatchard analysis we assumed that all antigen was present as 146S particles and that the molecular weight of an intact 146S particle corresponds to 8200 kDa (Vande Woude et al., 1972).

The affinity for pIgG was determined using surface plasmon resonance on a BIAcore instrument as described previously (Harmsen et al., 2005a).

2.6. Animal experiments

2.6.1. Animals

153

154 Conventionally raised large white swine, about 10-weeks-old and weighing
155 approximately 25 kg were used to perform viral challenge. All animals were housed in
156 disease-secure isolation facilities in an FMDV-free area. Animal experiments were performed
157 under the supervision of the Animal Experimental Committee and according to The Dutch
158 Law on Animal Experiments.

159 Two experiments to determine the effect of passive immunisation with VHH2s on FMD
160 infection of pigs were performed. In the first experiment we administered three different
161 VHH2s and measured the development of clinical FMD, viremia and viral shedding after
162 challenge infection. In the second experiment we administered a single VHH2 and measured
163 FMDV transmission in addition to the above mentioned parameters.

164

165 *2.6.2. Experiment 1: swine challenge experiment*

166

167 Twenty pigs were randomly allocated to five groups, each containing four pigs. All
168 groups were housed in separate rooms. Within each group wooden barriers were placed
169 between the individual animals to prevent physical contact. After a five-day acclimatisation
170 period the pigs in groups 1, 2 and 3 received, respectively, VHH2s M3ggsVI-4_{Q6E}, M8ggsVI-
171 4_{Q6E} and M23ggsVI-4_{Q6E} at a dose of 3 mg/kg by intramuscular injection into the hind thigh.
172 The pigs in group 4 received an equimolar mixture of these three VHH2s at a total VHH2
173 dose of 3 mg/kg. The control pigs in group five received PBS. Twenty four hours after VHH2
174 administration all pigs were challenged by intradermal inoculation into the bulb of the heel of
175 the lateral claw of the left-hind foot with 0.1 ml 10^4 PFU/ml FMDV O1 Manisa. After
176 challenge, we collected heparinised blood, serum and oropharyngeal fluid (OPF) samples
177 using cotton mouth swabs. Furthermore, the feet and mouth were inspected for the presence

178 of lesions characteristic of FMD. Pigs were considered protected when they did not develop
179 lesions on other sites than the challenge site. Sampling and inspection for lesions was done
180 daily until 10 days post challenge infection (DPI) and then every other day until 14 DPI. Pigs
181 that developed generalized FMD were euthanized to avoid overexposure of the other pigs to
182 virus excreted by diseased pigs. The experiment was ended at 14 DPI.

183 OPF and heparinized blood samples were assayed for the presence of virus by plaque
184 titration. Serum samples were tested in a commercially available ELISA (Ceditest® FMDV-
185 NS, Cedi-Diagnostics B.V., Lelystad, The Netherlands) for detection of antibodies against
186 non-structural proteins (NSP) of FMDV. This ELISA detects antibodies against NSP 3B and
187 thus detects pigs that seroconvert after FMDV infection.

188

189 *2.6.3. Experiment 2: swine transmission experiment*

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191 A swine transmission experiment was performed using sixteen pigs allocated into eight
192 pairs of two pigs. Each pair was individually housed without barriers for physical contact.
193 Five pairs received 3 mg/kg M23ggsVI-4_{Q6E}. Three control groups received PBS. Twenty
194 four hours after VHH2 administration one of the pigs in each group was removed from the
195 room and the remaining pig was inoculated with FMDV. The contact pigs were returned to
196 their group 24 h after challenge. The pigs were sampled and inspected for the presence of
197 lesions daily until 11 DPI and every other day until 17 DPI. The procedures for VHH2
198 administration, challenge infection and sampling were exactly the same as described in the
199 previous section. Both pigs in a group were euthanized once the contact pig developed
200 generalized FMD. If necessary, pigs with generalized FMD received buprenorfine to reduce
201 pain.

202

2.6.4. Statistical methods

A linear mixed effect model (S-plus 6.2) using maximum likelihood optimisation was used to determine the difference between the day of challenge (0 DPI) and the other days when samples were collected (1-17 DPI) and whether differences existed between the M23ggsVI-4_{Q6E} and PBS (control) groups for both viral load detected in blood and OPF. In the model, group and number of days after vaccination were included as explanatory variables and, additionally, the animal number was included as a random effect variable, to take into account that observations originating from the same animal are not independent. For samples from which no virus was isolated the detection limit of the test was used for statistical analysis. Initially we analysed the combined data of animals from the challenge experiment and the transmission experiment that were directly challenged, *i.e.* excluding the contact pigs. Because apparently not all challenge infections resulted in infectious pigs, a second analysis was performed from which the pigs that did not show clinical FMD signs (pigs 9028, 9029, 9824 and 9830) were excluded.

3. Results

3.1. Production of bispecific VHH2s

We produced three bispecific VHH2s by fusion of three VHHs (M3, M8 and M23) to the VI-4 VHH, that binds to pIg light chain. For facile molecular cloning we deleted a *Pst*I restriction site resulting in the replacement of glutamine at position 6 of VI-4 by glutamic acid (mutation Q6E). Both residues occur frequently at this position in VHHs (Harmsen et al., 2000). Furthermore, two clones (VI-7 and VI-8) with high sequence homology to VI-4, suggesting that they are derived from the same B-cell recombination event, also contain a glutamic acid at position 6 (Harmsen et al., 2005a). The three VHH2s and their monovalent VHH counterparts were produced in yeast, purified by IMAC and analysed by SDS-PAGE (Fig. 1). They all contained predominantly molecules of the expected molecular mass of about 30 kDa for a VHH2 and about 15 kDa for a VHH. All three VHH2 preparations also contained a small amount of molecules migrating at about 28 kDa that could represent VHH2 degradation product. Furthermore all VHHs and VHH2s contained a small amount of molecules with an about 2 kDa increased molecular mass as compared to the main product.

3.2. Antigen binding of bispecific VHH2s

The three VHH2s bound pIg with low nanomolar affinities that were comparable to the monovalent VI-4 VHH (Table 2). The conservative mutation Q6E within the pIg binding moiety of the VHH2s apparently did not affect the affinity for pIg. The VHH2s bound to pIg and FMDV in an ELISA at concentrations that are comparable to their monovalent counterparts (Table 2). Similarly, their in vitro FMDV neutralizing capacity was comparable

to their corresponding monovalent counterparts (Table 2). Thus, antigen binding was not compromised due to fusion of the two VHH domains that form the various VHH2s. When VHH2s were complexed with pIg, by mixing with 50% normal swine serum, the in vitro neutralizing capacity of M8ggsVI-4_{Q6E} and M23ggsVI-4_{Q6E} increased 4- and 30-fold, respectively (Table 2). Furthermore, only the VHH2s were able to bind to FMDV and pIg simultaneously in ELISA (Table 2).

3.3. Effect of VHH2 administration on FMD infection of pigs (experiment 1)

Infected control pigs developed generalized FMD at 2-3 DPI (Table 3). Most of the pigs that received any of the three single VHH2s or a VHH2 mixture 24 h prior to challenge infection developed generalized FMD at the same rate as the control pigs (Table 3). One control pig died already at 2 DPI, probably as a result of the FMDV infection. Two pigs that received M23ggsVI-4_{Q6E} and one pig that received the VHH2 mixture did not develop any lesions, not even at the challenge site, until the end of the experiment at 14 DPI. Furthermore, in samples from these three pigs no viremia nor viral shedding could be detected during the experiment (pigs 9028 and 9029 in Table 4; results not shown for the pig that received the VHH2 mixture). This could indicate that M23ggsVI-4_{Q6E} protected some but not all pigs.

3.4. Effect of M23ggsVI-4_{Q6E} administration on FMD clinical signs and transmission (experiment 2)

We further assessed whether M23ggsVI-4_{Q6E} could protect pigs against FMDV transmission. One of the three piglets that received PBS (control) and were directly challenged with FMDV did not develop any clinical signs of FMD during the experiment

(Table 4, pig 9830). This indicates that the FMD challenge was not effective in each animal. Also one of the five pigs that received M23ggsVI-4_{Q6E} and were directly challenged with FMDV did not develop any clinical FMD signs (Table 4, pig 9824) as was seen before with two piglets that received M23ggsVI-4_{Q6E} in the challenge experiment (Table 4, pigs 9028 and 9029). As a result transmission could only be assessed from two pairs of control pigs and four pairs of pigs that received M23ggsVI-4_{Q6E}. Both groups of pigs transmitted FMD equally fast, within 2 days (Table 4).

We next analysed the virus load in blood (Fig. 2a) and secretion in OPF (Fig. 2b) of all directly inoculated pigs that received M23ggsVI-4_{Q6E} or PBS from both the challenge and transmission experiment. Both the M23ggsVI-4_{Q6E} and PBS immunized groups showed a significant rise in virus titers in blood from 2-3 DPI and in OPF from 2-5 DPI. As compared to the PBS immunized group the M23ggsVI-4_{Q6E} immunized group showed a statistically significant reduced virus load in blood (P=0.0034) and a reduced viral secretion in OPF that was not significant (P=0.11). If only the pigs that showed some clinical FMD signs were used for statistical analysis the reduction in viremia was again significant (P=0.005) whereas the reduction in viral shedding was almost significant (P=0.074) at P=0.05 level.

This reduction cannot be attributed to the neutralization of virus in samples containing M23ggsVI-4_{Q6E} because the level of this VHH2 in blood is insufficient for such neutralization. Assuming that the VHH2 dosage given (3 mg/kg) first enters the circulation, which forms 10% of the body mass, then the maximum M23ggsVI-4_{Q6E} concentration in blood is 30 mg/L. The concentration in OPF is expected to be even lower. In a control experiment we have shown that addition of M23ggsVI-4_{Q6E} at concentrations as high as 200 mg/L did not affect the amount of virus isolated in virus isolations (results not shown).

4. Discussion

We produced three bispecific VHH2s by fusion of three VHHs (M3, M8 and M23), binding to three functionally independent antigenic sites of FMDV (Harmsen et al., 2007), to the VI-4 VHH, that binds to pIg light chain. Al three VHH2s were predominantly produced by recombinant yeast cells as molecules of the expected molecular mass. In addition we found a small amount of molecules with a slightly lower molecular mass. These most likely represent degradation products generated by cleavage of the myc tag, as has also been observed when expressing VHH2s in *E. coli* (Conrath et al., 2001). Furthermore, we observed molecules with about a 2-kDa higher molecular mass. This latter product is not observed when such VHH2s are produced without the myc tag (results not shown). It could represent O-glycosylation that is dependent on the presence of the myc tag.

All three VHH2s bound to FMDV as efficiently as their monovalent counterparts as assessed by ELISA and in vitro FMDV neutralization. The VHH2s bound FMDV with high affinity (K_D below 1 nM). Since we could only determine these affinities using a highly sensitive ELISA that employs biotinylated pIgG and peroxidase-conjugated streptavidin the affinities of the monovalent VHHs could not be determined. The in vitro FMDV neutralizing capacity of two neutralizing VHH2s was increased 4- to 30-fold by addition of pIg. A similar increase in the in vitro neutralizing capacity by addition of anti-antibody to a monovalent FMDV binding recombinant antibody fragment (Mason et al., 1996) or to conventional mAbs that bind other picorna viruses such as poliovirus and human rhinovirus (Parren and Burton, 2001) has been observed previously. This increased in vitro neutralizing capacity is presumably caused by a more effective steric hindrance due to an increased molecular mass of the VHH2s after complexation with pIg. This conclusion is further supported by our previous observation that chemical coupling of 40-kDa polyethylene glycol (PEG) molecules to anti-

FMDV VHHs increased their FMDV neutralizing capacity considerably (Harmsen et al., 2007).

Intramuscular injection of the VHH2 M23ggsVI-4_{Q6E} to pigs at a dose of 3 mg/kg reduced the mean daily viremia and viral shedding but did not prevent the development of FMDV clinical signs or transmission. We previously observed that guinea pigs that received a similar dose of PEGylated VHHs (4 mg/kg) were also poorly protected from FMDV challenge infection (Harmsen et al., 2007). Considering that these PEGylated VHHs are more potent at neutralising in vitro this appears consistent with the poor protection conferred by the VHH2s. However, unlike PEGylated VHHs, the VHH2s could indirectly recruit antibody effector functions such as opsonophagocytosis due to binding to pIg. Such indirect recruitment of opsonophagocytosis using bispecific recombinant antibodies has previously been demonstrated in mice (Holliger et al., 1997). Since protection against FMDV in vivo using conventional antibodies is in part accomplished by phagocytosis of virus/antibody complexes by the reticuloendothelial system (Dunn et al., 1998; Harmsen et al., 2007; McCullough et al., 1988) such indirect recruitment of opsonophagocytosis could be important for effective immunoprophylaxis. However, we do not have experimental evidence supporting indirect recruitment of opsonophagocytosis by VHH2s. Thus, their poor in vivo protection could be due to their failure to do so.

Although the VHH2 M23ggsVI-4_{Q6E} did not prevent FMDV transmission, it did reduce mean daily viremia and viral shedding. This suggests that immunotherapy of pigs with VHHs is feasible upon further improvement. Disadvantages of M23ggsVI-4_{Q6E} are (1) its relatively low production level in yeast, which limits its cost-effective dosage, (2) its poor in vitro neutralization and (3) its propensity to aggregate at high concentrations. We have now generated improved VHHs that are able to reduce FMDV transmission in pigs (M.M. Harmsen, manuscript in preparation).

Acknowledgements

This work was supported financially by the European Union (EPIZONE NoE Food-CT-2006-016236) and The Netherlands Ministry of Agriculture, Nature and Food Quality. The authors thank Franz Daus and the animal caretakers for expert technical assistance.

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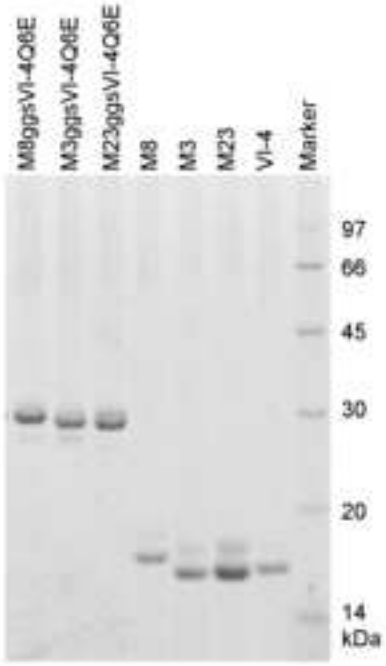
422

Figure legends

Fig. 1. SDS-PAGE analysis of yeast-produced VHHs and VHH2s. The molecular mass of the markers used is indicated.

Fig. 2. Mean Daily Virus levels in blood (panel A) and OPF (panel B) of pigs that received M23ggsVI-4_{Q6E} (open circles) or PBS (closed triangles) 24 h prior to challenge infection with FMDV O_1 Manisa. The results of both the swine challenge experiment and the transmission experiment, including all piglets that never showed clinical signs of FMD, are incorporated. Arrows indicate the cut-off level of the assay, which is assumed to be one plaque per virus isolation, corresponding to $10^{1.7}$ PFU/ml in blood and 10^{1} PFU/ml in OPF. Error bars indicate standard deviation.



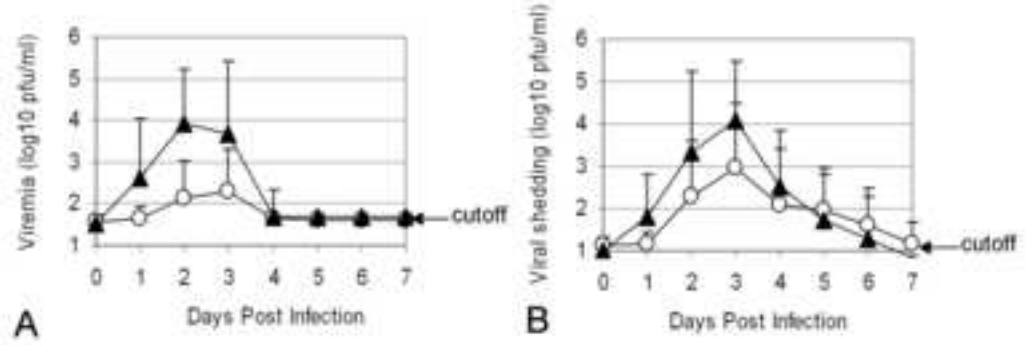


Table1.

Plasmid constructs for VHH2 production.

2-micron plasmid	MIRY plasmid	N-terminal VHH	Linker	C-terminal VHH	Epitope tags	Encoded VHH2
pRL259	pRL275	M3	GGS	VI-4_{Q6E}	myc, his6	M3ggsVI-4_{Q6E}
pRL260	pRL276	M8	GGS	VI-4_{Q6E}	myc, his6	M8ggsVI-4_{Q6E}
pRL261	pRL277	M23	GGS	VI-4_{Q6E}	myc, his6	M23ggsVI-4_{Q6E}

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Table 2.

Yeast production level, FMDV neutralization and antigen binding of VHH2s.

VHH(2)	Production in yeast[a] (g/l)	FMDV neutralization titer (g/l)		Titer in ELISA (mg/l)			Affinity for FMDV, K_D (nM)	Affinity for pIgG, K_D (nM)
		in PBS	in 50% normal swine serum[d]	FMDV	pIgG	FMDV / pIgG		
M3ggsVI-4_{Q6E}	0.02	>5 [c]	>5	0.018	0.017	0.006	0.4	1.2 ± 0.2
M8ggsVI-4_{Q6E}	0.9	0.004	0.001	0.010	0.011	0.003	0.3	1.2 ± 0.3
M23ggsVI-4_{Q6E}	0.04	0.03	0.001	0.018	0.012	0.006	0.5	0.9 ± 0.1
M3	ND[b]	>5	ND	0.010	>10	>10	ND	ND
M8	ND	0.004	ND	0.012	>10	>10	ND	ND
M23	ND	0.06	ND	0.005	>10	>10	ND	ND
VI-4	ND	>5	ND	>10	0.011	>10	ND	0.9 ± 0.1

[a] Amount of VHH2 with his6 tag secreted into the growth medium after high cell density fermentation of MIRY transformants.

[b] ND, not determined.

[c] No ELISA signal or neutralization detected at the highest concentration analysed.

[d] VHH2s were diluted to 5 g/l in 50% normal swine serum.

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Table 3.

Protection of pigs against FMD by passive intramuscular immunization with VHH2s.

VHH2	Dose	No. protected / No. of living pigs at DPI				
	(mg/kg)	1	2	3	4	14
None (PBS)	-	4/4	2/4	0/3 [b]	-	-
M3ggsVI-4_{Q6E}	3	4/4	1/4	1/4	0/1	-
M8ggsVI-4_{Q6E}	3	4/4	1/4	0/4	-	-
M23ggsVI-4_{Q6E}	3	4/4	3/4	2/4	2/2	2/2
VHH2 mixture	3[a]	4/4	4/4	1/4	1/1	1/1

[a] Each VHH2 was dosed at 1 mg/kg, resulting in a total VHH2 dose of 3 mg/kg.

[b] One pig with generalized FMD died, probably as a result of the infection, before it could be euthanized.

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Table 4.

Viremia, viral shedding, NS-ELISA and generalized FMD of pigs that received M23ggsVI-4_{Q6E} or PBS.

Animal number	Virus challenge[a]	VHH2	Viremia (DPI)	Viral shedding (DPI)	Generalized FMD (DPI)	NS-ELISA positive (DPI)	Euthanasia pig (DPI)	Transmission[d] (days)
Challenge experiment								
9026	Inoculated	M23ggsVI-4_{Q6E}	2-3	2-3	3	-	3	-
9027	Inoculated	M23ggsVI-4_{Q6E}	1-3	2-3	2-3	-	3	-
9028	Inoculated	M23ggsVI-4_{Q6E}	-	-	-	-	14	-
9029	Inoculated	M23ggsVI-4_{Q6E}	-	-	-	1-14[b]	14	-
9030	Inoculated	PBS	1	1	2	-	2[c]	-
9031	Inoculated	PBS	1-3	1-3	2-3	-	3	-
9032	Inoculated	PBS	2-3	3	3	-	3	-
9033	Inoculated	PBS	2-3	2-3	3	-	3	-
Transmission experiment								
9823	Contact	M23ggsVI-4_{Q6E}	-	-	-	-	17	-
9824	Inoculated	M23ggsVI-4_{Q6E}	-	-	-	-	17	-
9825	Contact	M23ggsVI-4_{Q6E}	-	3-10	-	-	17	1
9826	Inoculated	M23ggsVI-4_{Q6E}	3-5	2-6, 9	4-17	9-17	17	-
9833	Contact	M23ggsVI-4_{Q6E}	2, 4-5, 7	4-7	6-7	-	7	1
9834	Inoculated	M23ggsVI-4_{Q6E}	4-5	3-7	4-7	-	7	-
9835	Contact	M23ggsVI-4_{Q6E}	3-5	2-7	4-7	-	7	0
9836	Inoculated	M23ggsVI-4_{Q6E}	2-3	2-3, 5	2-7	-	7	-
9837	Contact	M23ggsVI-4_{Q6E}	13	4, 7-9,	14-15	-	15	2
9838	Inoculated	M23ggsVI-4_{Q6E}	-	2-6, 8-9	6-15	9-13	15	-
9827	Contact	PBS	4-6	4-6	6-7	-	7	2
9828	Inoculated	PBS	2-3	2-6	3-7	-	7	-
9829	Contact	PBS	-	-	-	-	17	-
9830	Inoculated	PBS	-	-	-	-	17	-
9831	Contact	PBS	-	3-4, 6	-	-	17	2
9832	Inoculated	PBS	1-3	1-3	2-4	-	4[c]	-

[a] Each contact pig was housed together with the inoculated pig in the row immediately below.

[b] This piglet was weakly positive in NS-ELISA already at the time of infection and the ELISA signal did not increase during the experiment.

[c] These pigs with generalized FMD died, probably as a result of the infection, before they could be euthanized.

[d] Transmission was calculated as day contact animal first shed virus minus day inoculated animal first shed virus.