Vaccine

Characterization of antigenic regions in the porcine reproductive and respiratory syndrome virus by the use of peptide-specific serum antibodies

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ARTICLE INFO

ABSTRACT

The porcine reproductive and respiratory syndrome virus (PRRSV) is an RNA virus that causes reproductive failure in sows and boars, and respiratory disease in pigs of all ages. Antibodies against several viral envelope proteins are produced upon infection, and the glycoproteins GP4 and GP5 are known targets for virus neutralization. Still, substantial evidence points to the presence of more, yet unidentified neutralizing antibody targets in the PRRSV envelope proteins. The current study aimed to identify and characterize linear antigenic regions (ARs) within the entire set of envelope proteins of the European prototype PRRSV strain Lelystad virus (LV).

Seventeen LV-specific antisera were tested in pepscan analysis on GP2, E, GP3, GP4, GP5 and M, resulting in the identification of twenty-one ARs that are capable of inducing antibodies upon infection in pigs. A considerable number of these ARs correspond to previously described epitopes in different European- and North-American-type PRRSV strains. Remarkably, the largest number of ARs was found in GP3, and two ARs in the GP3 ectodomain consistently induced antibodies in a majority of infected pigs. In contrast, all remaining ARs, except for a highly immunogenic epitope in GP4, were only recognized by one or a few infected animals.

Sensitivity to antibody-mediated neutralization was tested for a selected number of ARs by *in vitro* virus-neutralization tests on alveolar macrophages with peptide-purified antibodies. In addition to the known neutralizing epitope in GP4, two ARs in GP2 and one in GP3 turned out to be targets for virus-neutralizing antibodies. No virus-neutralizing antibody targets were found in E, GP5 or M. Since the neutralizing AR in GP3 induced antibodies in a majority of infected pigs, the immunogenicity of this AR was studied more extensively, and it was demonstrated that the corresponding region in GP3 of virus strains other than LV also induces virus-neutralizing antibodies. This study provides new insights into PRRSV antigenicity, and contributes to the knowledge on protective immunity and immune evasion strategies of the virus.

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1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) was discovered during the late eighties as the cause of severe reproductive failure in sows, and is considered today as one of the most important pathogens in the swine industry [1–3]. Infection of sows can lead to late term abortion, early farrowing and the birth of weak-born piglets, while infected boars show decreased sperm quality and virus excretion in the semen [4–6]. On the other hand, PRRSV is involved in the porcine respiratory disease complex, causing respiratory problems in combination with secondary viral

and bacterial infections [7,8]. The virus shows a restricted *in vivo* cell tropism for certain subsets of macrophages, with alveolar macrophages being the main target cell [9]. PRRSV is an enveloped positive single-stranded RNA virus of the family Arteriviridae and order Nidovirales [10]. The virion consists of a nucleocapsid core that is built up by nucleocapsid protein (encoded by open reading frame 7, ORF7) in association with the viral RNA. The nucleocapsid is surrounded by a lipid envelope in which six structural proteins are embedded: the glycoproteins GP2 (ORF2a), GP3 (ORF3), GP4 (ORF4) and GP5 (ORF5), and the non-glycosylated proteins M (ORF6) and E (ORF2b). GP5 and M are considered to be the most abundant proteins in the envelope, while the other envelope proteins are present in lower amounts [11–14]. Similar to many other RNA viruses, PRRSV shows a large genetic variability, which is amongst other things reflected in variation in virulence, interaction with the immune system and antigenic properties of viral proteins. Virus strains are usually classified within a European (EU) and a North-American (NA) genotype, based on ORF5 and/or ORF7 sequences,

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although a high degree of variability exists within genotypes nowadays [15–19].

Although PRRSV only emerged in the swine industry rather recently, the virus seems to have acquired a number of properties that allow escape from the host's protective immunity. In addition to an imbalanced cytokine response, impaired cytotoxic T-cell activity and absence of antibody-dependent complement-mediated cell lysis, the PRRSV-specific neutralizing antibody response shows an aberrant course [20–22]. Virus-specific antibodies are produced after one or two weeks upon PRRSV infection. However, these antibodies are not able to reduce *in vitro* virus replication in primary porcine alveolar macrophages (PAM). Low levels of virus-neutralizing antibodies appear not earlier than three to four weeks post infection, which is probably too late to influence the acute phase of viremia [23–26]. Despite this weak virus-neutralizing antibody response, it is known that the presence of sufficient amounts of virus-neutralizing antibodies at the onset of infection can offer certain protection against virus replication in the lungs, viremia and transplacental spread of the virus, indicating that PRRSV-specific antibodies can contribute to protective immunity [27,28]. The search for antigenic regions (ARs) that are potential inducers of virus-neutralizing antibodies therefore is a main topic of interest in PRRSV research. Antigenic characterization of PRRSV by the use of mouse monoclonal antibodies (mAbs) has led to the discovery of neutralizing epitopes in GP4 of EU-type PRRSV and GP5 of both EU- and NA-type PRRSV strains, and it has been suggested that also M and GP3 can act as targets for neutralizing mAbs [29–33]. Although mAbs can greatly contribute to the identification and characterization of ARs on viral proteins, they cannot be used to evaluate the potential immunogenicity of these ARs in pigs. For this latter purpose, antibody responses against viral proteins and epitopes upon PRRSV infection have been evaluated in different studies, including studies that provide large maps of ARs [25,31,34–44]. Furthermore, some correlations between epitope-specific antibody responses and the appearance of virus-neutralizing antibodies have been described [31,35,41]. Still, the involvement of the different PRRSV proteins and epitopes in the induction of virus-neutralizing antibodies in pigs is not completely resolved, and substantial evidence points to the presence of yet unidentified neutralizing antibody targets in the PRRSV envelope proteins [30,44–48]. A more detailed antigenic characterization of the entire structural PRRSV proteome can create insights into the antiviral immune response, immune evasion mechanisms, the viral replication cycle and evolutionary properties of the virus, and it can play its part in the development of new vaccines and diagnostic tools.

In a preceding study, the antibody response against ARs in GP4 upon PRRSV infection in pigs was studied, and the susceptibility of each AR to antibody-mediated neutralization was investigated by the use of peptide-purified antibodies [49]. A similar approach was used in the current study to characterize ARs within all other envelope proteins of the EU prototype PRRSV strain Lelystad virus (LV). The specificity of the serum antibody response against ARs within E, GP2, GP3, GP4, GP5 and M was determined in LV-infected pigs. Next, antibodies against every separate AR were purified and their virus-neutralizing capacity was determined. Finally, one AR in GP3 that was target for virus-neutralizing antibodies and induced antibodies in a majority of infected pigs was studied more extensively.

2. Materials and methods

2.1. Cell cultures and viruses

Primary porcine alveolar macrophages (PAM) were obtained from four-week-old pigs from a PRRSV-negative herd as described earlier [3]. The cells were cultivated in RPMI 1640, supplemented with 10% fetal calf serum, 2 mM L-glutamine, 1% non-essential amino acids, 1 mM sodium pyruvate and a mixture of antibiotics at 37 °C in a humidified atmosphere with 5% CO_2. The EU prototype PRRSV strain LV and the EU-type PRRSV field isolates 07V063 and 08V204 were used in this study. 07V063 was isolated from an aborted fetus, derived from a Belgian farm during an outbreak of PRRSV-associated reproductive disorders, while 08V204 was isolated from a Belgian farm from the serum of eight-week-old healthy piglets that showed PRRSV-specific antibodies. The viruses were propagated in PAM, derived from either gnotobiotic piglets when used for inoculation of animals or conventional piglets when used in virus-neutralization tests (LV: 5th passage, 07V063: 2nd passage, and 08V204: 3rd passage) [3,49]. ORF2–ORF6 sequences of the LV stocks and ORF3 sequences of the 07V063 and 08V204 stocks were determined as formerly described (primer sequences are available upon request) [49]. GP3, GP4, GP5 and M amino acid (aa) sequences of all LV stocks were identical to the sequences that are available in GenBank (accession number M96262), and the same was true for GP3 of 07V063 and 08V204 stocks (accession numbers GU737264 and GU737266). The GP2 sequences of both LV stocks were identical to the GenBank sequence, except for a conserved aa change in the putative signal peptide at position 28 (proline to serine).

2.2. Experimental inoculation of animals and collection of serum and broncho-alveolar lavage fluid

All pigs used in this study were offspring of hybrid sows (JSR Genepacker 90, English Landrace × Large White) and Piétrain boars. The pigs were derived from a PRRSV-negative farm and the seronegative status of the animals was confirmed by IPMA [26]. At 14 weeks of age, the animals were intranasally inoculated with 10^6 $TCID_{50}$ PRRSV. Eleven piglets (pigs A–K) were inoculated with the PRRSV strain LV, blood was drawn at 0, 7, 14, 20, 26 and 31 days post inoculation (dpi), and sera were collected. Six more piglets were inoculated with LV, of which sera were collected at either 52 dpi (pigs L–N) or 141 dpi (pigs O–Q) to maximize the chance of identifying slowly developing antibody populations. The different time points of end serum collection were randomly assigned to the animals, without pre-selecting in any way. LV-inoculated pigs A, B, E, I and K were euthanized at 45 dpi by intravenous injection of an overdose of Na-Pentobarbital 20% (Kela Laboratories) after which serum was collected and lungs were isolated. Broncho-alveolar lavage (BAL) fluids were collected from the right lung by flushing with 120 mL of PBS [50]. In addition to LV, 2 times six pigs were inoculated with either PRRSV strain 07V063 (pigs 7A–7F) or 08V204 (8A–8F), and sera were collected at 45 dpi. Sera and BAL fluids were heat-inactivated (56 °C, 30 min) and stored at −70 °C before use.

2.3. Pepscan analysis and peptide ELISA

Pepscan analysis with porcine serum and peptide ELISA with purified antibodies, porcine serum or BAL fluid was essentially performed as described earlier [49]. Sets of overlapping dodecapeptides with an offset of 4 and an overlap of 8 aa were designed, based on the complete sequences of GP2, E, GP3, GP4, GP5 and M of LV, and the GP3 aa 49–76 region of 07V063 and 08V204. Biotinylated peptides (BioTides) were chemically synthesized by JPT Peptide Technologies, using the SPOT synthesis approach on cellulose membranes. Synthesis was performed using a capping step after each amino acid coupling to enable biotinylation of target peptide only, which allows selective immobilization of correct peptides in streptavidin-coated plates. A selected number of peptides were applied to liquid chromatography–mass spectrometry analysis to substantiate proper technical synthesis performance and identity. Streptavidin-coated 96-well plates (VWR International)

were coated with 0.1 µg of BioTides in PBS with 0.5% Tween-20, washed, and blocked with 1% bovine serum albumin (R&D systems) in 0.5% Tween-20. Test samples (porcine sera, purified antibodies or BAL fluids) were diluted in blocking buffer and incubated on the peptide-coated plates. Sera were diluted 1/100 or used in two-fold serial dilutions, BAL fluids were used in two-fold serial dilutions, and purified antibodies were used in a concentration of 25 µg/mL. Serum dilutions and antibody concentrations were chosen in such a way that clear specific signals and minimal background signals were obtained, based on preliminary experiments. After incubation with the test samples, plates were washed and incubated with an optimal dilution of peroxidase-conjugated goat-anti-swine polyclonal antibodies (Jackson ImmunoResearch), after which they were washed and developed with a substrate solution of tetramethylbenzidine and H_2O_2 (R&D systems). The reaction was stopped after 10 min with 1 M H_2SO_4, and the optical density at 450 nm (OD450) was measured. All wash steps were performed with PBS with 0.5% Tween-20, and all incubation steps were carried out at room temperature for 1 h.

Serum or protein A-purified antibodies from a PRRSV-negative pig were included as negative control for sera or peptide-purified antibodies, and PBS was used as control for BAL fluids. OD450 values obtained with test samples at a certain peptide were always expressed relative to the OD450 value obtained with the negative control sample at the same peptide (OD450 sample/negative, OD450 s/n). In pepscan analysis, the mean OD450 s/n over all peptides within a protein was calculated. If the OD450 s/n at a certain peptide was more than 2 times the mean over all peptides within the same protein, the signal was considered specific. In peptide ELISA on a limited number of peptides, an OD450 $s/n > 2$ was considered a specific signal.

2.4. Purification of peptide-specific serum antibodies

Purification of peptide-specific serum antibodies was performed by peptide affinity chromatography as described before [49]. Sera with the highest amounts of antibodies of interest were chosen to maximize purification yields. 1 mg of a 12-mer peptide (>80% purity, JPT Peptide Technologies) was covalently coupled to a N-hydroxysuccinimide-activated sepharose column (HiTrap NHS-activated HP, GE Healthcare), following the manufacturer's instructions. Heat-inactivated serum was clarified by centrifugation, the pH was adapted with 200 mM Na_2HPO_4 (pH 7.0), and the serum was filtrated over a 0.2 µm filter. After equilibration of the column with 20 mM Na_2HPO_4 (pH 7.0), serum was run over it and the column was subsequently washed with 20 mM Na_2HPO_4 buffer. When no more protein could be detected by spectrophotometry at 278 nm in the wash fractions, peptide-specific antibodies were eluted from the column with 0.1 M glycine (pH 2.7) and collected in 1 M Tris (pH 8.0). Elution fractions that contained protein, as measured by spectrophotometry at 278 nm, were pooled, dialyzed against PBS and stored at −70 °C.

2.5. Single-replication virus-neutralization test on PAM

Single-replication virus-neutralization test on PAM was performed as described earlier [49]. Two-fold serial dilutions of peptide-specific serum antibodies, sera or BAL fluids were mixed with equal volumes of PAM-grown virus (either LV, 07V063 or 08V204) resulting in a final titre of 10^5 TCID$_{50}$/mL, and in case of neutralization with purified antibodies, a final antibody concentration range of 400–13 µg/mL. Protein A-purified antibodies from a serologically PRRSV-negative pig, negative serum or PBS were included as mock condition in neutralization tests with purified antibodies, sera or BAL fluids respectively. Virus-antibody mixtures were incubated for 1 h at 37 °C and transferred to a 96-well plate

(100 µL/well) with PAM (10^5 cells/well) that were cultivated during 48 h prior to use. The inoculum was removed after 1 h and replaced by medium, after which the cells were further incubated for another 10 h, fixed by drying, and stored at −20 °C. The cells were stained for PRRSV infection with mAb 13E2 [51] against the nucleocapsid protein of PRRSV and peroxidase conjugated goat-anti-mouse polyclonal antibodies (Dako), followed by development with 3-amino-9-ethylcarbazole. The number of infected cells in each well was counted in three fields at 200× magnification, and expressed relative (%) to the mean number of infected cells for all mock conditions within the same experiment. Neutralization experiments with peptide-purified antibodies were performed in four-fold. The relative % of infected cells was analyzed by 2-way analysis of variance, followed by Bonferroni's post-tests to determine statistically significant differences between treatment and mock antibody conditions for a given antibody concentration. $P < 0.01$ was chosen as level of statistical significance. Statistical analysis was performed using GraphPad Prism version 5.0a. Neutralization experiments with sera and BAL fluids were performed in duplicate, and titres were determined as the reciprocal of the highest dilution that resulted in more than 50% reduction of infected cells.

3. Results

3.1. Reactivity of LV antisera with linear peptides of all envelope proteins

Seventeen PRRSV-negative piglets were inoculated with LV at the age of six weeks, and all pigs showed viremia during two to four weeks (data not shown). To determine the reactivity of serum with linear dodecapeptides covering all envelope proteins upon infection with LV, a pepscan analysis was performed on GP2, E, GP3, GP4, GP5 and M with a 1/100 dilution of sera that were collected at 31 ($n = 11$), 52 ($n = 3$) or 141 ($n = 3$) dpi. Thirty-nine peptides in total within GP2, GP3, GP4, GP5 and M were recognized by one or more sera, while no peptides of the E protein were recognized by any of the samples. Peptide reactivity patterns of individual animals are available as supplementary data. Reactive peptides were grouped into antigenic regions (ARs) of 12–38 amino acids (aa) based on reactivity of individual sera with successive overlapping dodecapeptides (Table 1). Five ARs were defined in GP2, eight in GP3, one in GP4, four in GP5 and three in the M protein. Four of these ARs are encoded by overlapping coding regions: GP2-V (ORF2/ORF3) and GP3-VII, GP3-VIII and GP4-I (ORF3/ORF4). Thirteen ARs (GP2-IV, GP3-I, GP3-II, GP3-III, GP3-IV, GP3-V, GP3-VIII, GP4-I, GP5-I, GP5-III, GP5-IV, M-I and M-II) were recognized by at least 2 sera and were selected for further study. The remaining ARs, that only induced antibodies in one out of seventeen pigs were not included in further experiments, since it is questionable whether these can be considered relevant ARs or epitopes. AR GP2-II was only recognized by serum of one pig, collected at 141 dpi, but because of the interesting position of this region in the N-terminal end of the putative ectodomain of the GP2 protein, this region was exceptionally also selected for further analysis [52]. Within the selected ARs, a core of 12 aa was defined by the peptide that showed reactivity with the highest number of sera, or in case of an equal number of reactive sera, by the peptide that gave the strongest signals in pepscan. As an exception, the cores of ARs GP3-III and GP3-IV were defined as peptides 22 and 25 respectively to avoid overlap between ARs. The selected ARs and their core peptides are indicated in bold in Table 1. The AR that was found in GP4 has already been extensively studied for EU-type PRRSV, and was included in the current study as gold standard [29,33,40,49,53–55].

Table 1
Antigenic regions in GP2, GP3, GP4, GP5 and M of LV. Seventeen six-weeks old piglets were intranasally inoculated with LV, serum was collected at 31, 52 or 141 dpi and serum reactivity with linear dodecapeptides in the envelope proteins GP2, GP3, GP4, GP5 and M was tested in pepscan analysis. Reactive peptides were grouped into antigenic regions (ARs), based on reactivity of individual animals with successive overlapping dodecapeptides. For each AR, the number of reactive pigs out of 17 is given, together with the numbering, amino acid sequence and positioning of the peptides that define the AR. References are given for ARs that have earlier been identified by the use of mAbs (in italics) or porcine antisera, in either EU- or NA-type viruses (*non-neutralizing epitope; **neutralizing epitope). ARs that were recognized by at least 2 pigs as well as AR GP2-II were selected for further study. Core sequences (bold) within these ARs were defined, based on the number of reactive pigs with every peptide and with avoidance of overlap.

Protein	AR	#Reactive pigs (out of 17)	Peptide	Amino acid sequence (position)	Previously described (in EU- or NA-type virus)
GP2	GP2-I	1	1	MQWGHCGVKSAS (1–12)	
	GP2-II	1	9	PYCLGSPSQDGY (33–44)	Oleksiewicz et al. [38] (EU)
			10	**GSPSQDGYWSFF (37–48)**	de Lima et al. [44] (NA)
	GP2-III	1	23	KHPLGMFWHMRV (89–100)	
	GP2-IV	2	**30**	**EHSGQAAWKQVV (117–128)**	Oleksiewicz et al. [38] (EU)
					de Lima et al. [44] (NA)
	GP2-V	1	51	FRQWLISVHASI (201–212)	
GP3	**GP3-I**	13	14	ICMPCSTSQAAR (53–64)	Oleksiewicz et al. [40] (EU)
			15	CSTSQAARQRLE (57–68)	Oleksiewicz et al. [38] (EU)
			16	**QAARQRLEPGRN (61–72)**	de Lima et al. [44] (NA)
					Zhou et al. [42] (NA)
	GP3-II	12	18	PGRNMWCKIGHD (69–80)	Oleksiewicz et al. [40] (EU)
			19	**MWCKIGHDRCEE (73–84)**	Oleksiewicz et al. [38] (EU)
			20	IGHDRCEERDHD (77–88)	Van Breedam et al. [51] (EU)*
			21	RCEERDHDELLM (81–92)	de Lima et al. [44] (NA)
					Zhou et al. [42] (NA)
	GP3-III	2	**22**	**RDHDELLMSIPS (85–96)**	de Lima et al. [44] (NA)
			23	ELLMSIPSGYDN (89–100)	Zhou et al. [42] (NA)
	GP3-IV	2	24	SIPSGYDNLKLE (93–104)	de Lima et al. [44] (NA)
			25	**GYDNLKLEGYYA (97–108)**	Zhou et al. [42] (NA)
	GP3-V	5	**27**	**GYYAWLAFLSFS (105–116)**	Zhou et al. [42] (NA)
	GP3-VI	1	36	QFICAEHDGHNS (141–152)	
	GP3-VII	1	46	EWLRPLFSSWLV (181–192)	
	GP3-VIII	2	**62**	**KFPSESRPNVVK (245–256)**	Oleksiewicz et al. [39] (EU)
			63	ESRPNVVKPSVL (249–260)	
GP4	**GP4-I**	17	13	DINCFRPHGVSA (49–60)	Meulenberg et al. [29] (EU)**
			14	FRPHGVSAAQEK (53–64)	Oleksiewicz et al. [40] (EU)
			15	**GVSAAQEKISFG (57–68)**	Costers et al. [33] (EU)**
			16	AQEKISFGKSSQ (61–72)	Vanhee et al. [49] (EU)**
			17	ISFGKSSQCREA (65–76)	de Lima et al. [44] (NA)
GP5	**GP5-I**	3	9	DGNGDSSTYQYI (33–44)	Van Breedam et al. [51] (EU)*
			10	**DSSTYQYIYNLT (37–48)**	Ostrowski et al. [31] (NA)**
			11	YQYIYNLTICEL (41–52)	Plagemann et al. [41] (NA)**
					de Lima et al. [44] (NA)
	GP5-II	1	37	NFIVDDRGRVHR (145–156)	Rodriguez et al. [56] (EU)*
	GP5-III	5	41	IVVEKLGKAEVD (161–172)	Rodriguez et al. [56] (EU)*
			42	**KLGKAEVDGNLV (165–176)**	Zhou et al. [57] (NA)
			43	AEVDGNLVTIKH (169–180)	
	GP5-IV	2	**48**	**QPLTRTSAEQWEA (189–201)**	Oleksiewicz et al. [38] (EU)
					Rodriguez et al. [56] (EU)*
					de Lima et al. [44] (NA)
					Zhou et al. [57] (NA)
M	**M-I**	2	**1**	**MGGLDDFCNDPI (1–12)**	
	M-II	2	32	SASGNRAYAVRK (125–136)	Oleksiewicz et al. [38] (EU)
			33	**NRAYAVRKPGLT (129–140)**	
	M-III	1	39	LVLGGKRAVKRG (153–164)	Oleksiewicz et al. [38] (EU)
					de Lima et al. [44] (NA)

3.2. Virus neutralization by peptide-specific serum antibodies

Peptide-specific antibodies against the core peptide of each separate AR were purified from polyclonal sera by peptide affinity chromatography. All purifications yielded positive protein fractions as measured by spectrophotometry at 278 nm, except for purification against peptide GP3.27, which was further omitted from this study. Peptide-purified antibody fractions were tested at a concentration of 25 μg/mL in peptide ELISA on the respective homologous and heterologous peptides to determine their specificity. All antibodies recognized the corresponding homologous peptide, although the ELISA signal obtained with the M.1-specific antibody fraction was clearly lower compared to that of the other antibody fractions, and also the GP2.30-specific antibodies showed some lower reactivity (Fig. 1). None of the antibodies clearly reacted with any of the respective heterologous peptides, further confirming their specificity, although antibodies against peptide GP3.62 showed rather high reactivity with heterologous peptides.

Peptide-purified antibodies were used in single-replication virus-neutralization tests on PAM to determine the effect of antibodies, specific to each AR on in vitro virus replication. Serum antibodies against peptide GP4.15 were able to reduce viral replication in PAM in a dose-dependent manner in a similar way as previously described (Fig. 2, gray lines) [49]. Antibodies against peptide GP2.10, GP2.30, GP3.16 and GP3.62 were also able to reduce viral replication in PAM in a concentration-dependent manner (Fig. 2, black lines). A significant reduction in viral replication was observed with antibody concentrations starting from 100 μg/mL for antibodies against GP2.10, GP2.30 and GP3.62, while GP3.16-specific antibodies already exerted a significant effect at 50 μg/mL
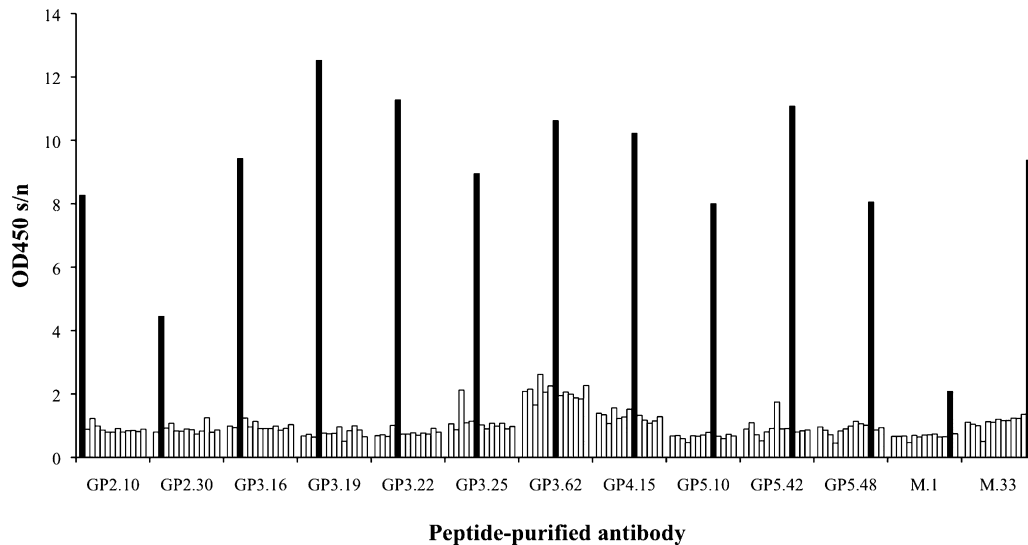
Fig. 1. Reactivity of peptide-purified antibodies with homologous and heterologous peptides in ELISA. Peptide-purified antibodies against core peptides of ARs in LV were tested in a concentration of 25 μg/mL in ELISA on the respective homologous (black bars) and heterologous (white bars) peptides. OD450 values obtained with test samples (*s*) were expressed relative to the OD450 value obtained with protein A-purified antibodies from a PRRSV-negative pig (*n*) at the same peptide (OD450 *s/n*). Groups of bars represent reactivity of single peptide-purified antibody fractions with peptides GP2.10, GP2.30, GP3.16, GP3.19, GP3.22, GP3.25, GP3.62, GP4.15, GP5.10, GP5.42, GP5.48, M.1 and M.33 (from left to right).

($P < 0.01$). Antibodies against all other peptides did not influence viral replication in PAM in any concentration tested (Fig. 2, black lines). Peptide-specific antibodies that did reduce viral replication never influenced viral infectivity as much as GP4-specific antibodies, although antibodies against peptide GP2.30 and GP3.62 were not tested in the highest concentration because of a low purification yield.

3.3. Antibodies against AR GP3-I in serum and BAL fluid upon infection with LV

Of the four newly identified ARs that are target for neutralizing antibodies, only GP3-I induced antibodies in a majority of LV-infected pigs (Table 1). The antibody response against this AR upon infection was further evaluated and compared with the antibody response against the well-characterized AR GP4-I [49]. Sera of 11 LV-infected pigs (A–K), collected at 0, 7, 14, 20, 26 and 31 dpi, were tested in a 1/100 dilution in ELISA with peptides GP3.14, GP3.15 and GP3.16 within AR GP3-I, and peptides GP4.14, GP4.15 and GP4.16 within AR GP4-I. OD450 *s/n* values of 2 or more were considered positive, and the number of positive pigs per time point is given for each peptide in Fig. 3. The earliest antibody response against AR GP3-I (full lines) was observed at 14 dpi in one pig, while 10/11 animals showed reactivity with one or more peptides of this AR at 26 dpi. Antibodies against peptide GP3.16 were detected earlier and in more animals than antibodies against peptides GP3.14 and GP3.15, sustaining the choice of peptide 16 as core of AR GP3-I. Antibodies against AR GP4-I (dashed lines) were detected in one pig at 7 dpi, in more than half of the pigs at 14 dpi and in all animals at 20 dpi, with peptide GP4.16 as main target within this AR.

BAL fluids were collected from lungs of pigs A, B, E, I and K at 45 dpi, and virus-neutralizing antibody titres were determined by single-replication virus-neutralization test on PAM. Antibody titres in BAL fluids were compared with titres in the serum at the same time point (Table 2). Virus-neutralizing antibodies were detected in BAL fluids from all but one animal, and these titres were lower than in sera. Next, antibody titres against peptides 14, 15 and 16 of both GP3 and GP4 were determined with ten-fold serial dilutions of sera and BAL fluids in ELISA. All sera and BAL fluids contained

antibodies against both ARs, and antibody titres against AR GP4-I were always equal or higher than GP3-I-specific antibody titres. Also here, antibody titres were higher in sera than in BAL fluids.

3.4. Investigation of AR GP3-I in PRRSV field isolates 07V063 and 08V204

It was shown so far that AR GP3-I of LV induces antibodies in a majority of infected pigs on the one hand, and that this AR is target for virus-neutralizing antibodies on the other hand. To investigate whether this is true for EU-type PRRSV strains other than LV, two Belgian PRRSV field isolates, 07V063 and 08V204, were selected. These viruses respectively show 89.0% and 84.5% GP3 aa homology with LV, and are 81.5% homologous to each other. The aa sequences

Table 2

Neutralizing antibody titres and ELISA titres against antigenic regions GP3-I and GP4-I in sera and BAL fluids of LV-infected pigs at 45 dpi. Sera and BAL fluids of pigs A, B, E, I and K were collected at 45 days post inoculation with LV. Virus-neutralizing antibody titres ($\log_2$) were determined by single-replication virus-neutralization test on PAM. Serum and BAL fluid reactivity with peptides 14, 15 and 16 of both GP3 and GP4 was quantified by peptide ELISA with ten-fold serial dilutions (*s*) of serum or BAL fluid. Serial dilutions of a negative (*n*) serum or BAL fluid sample were included, and titres ($\log_2$) were determined as the reciprocal of the highest dilution for which an OD450 *s/n* of 2 or more was observed.

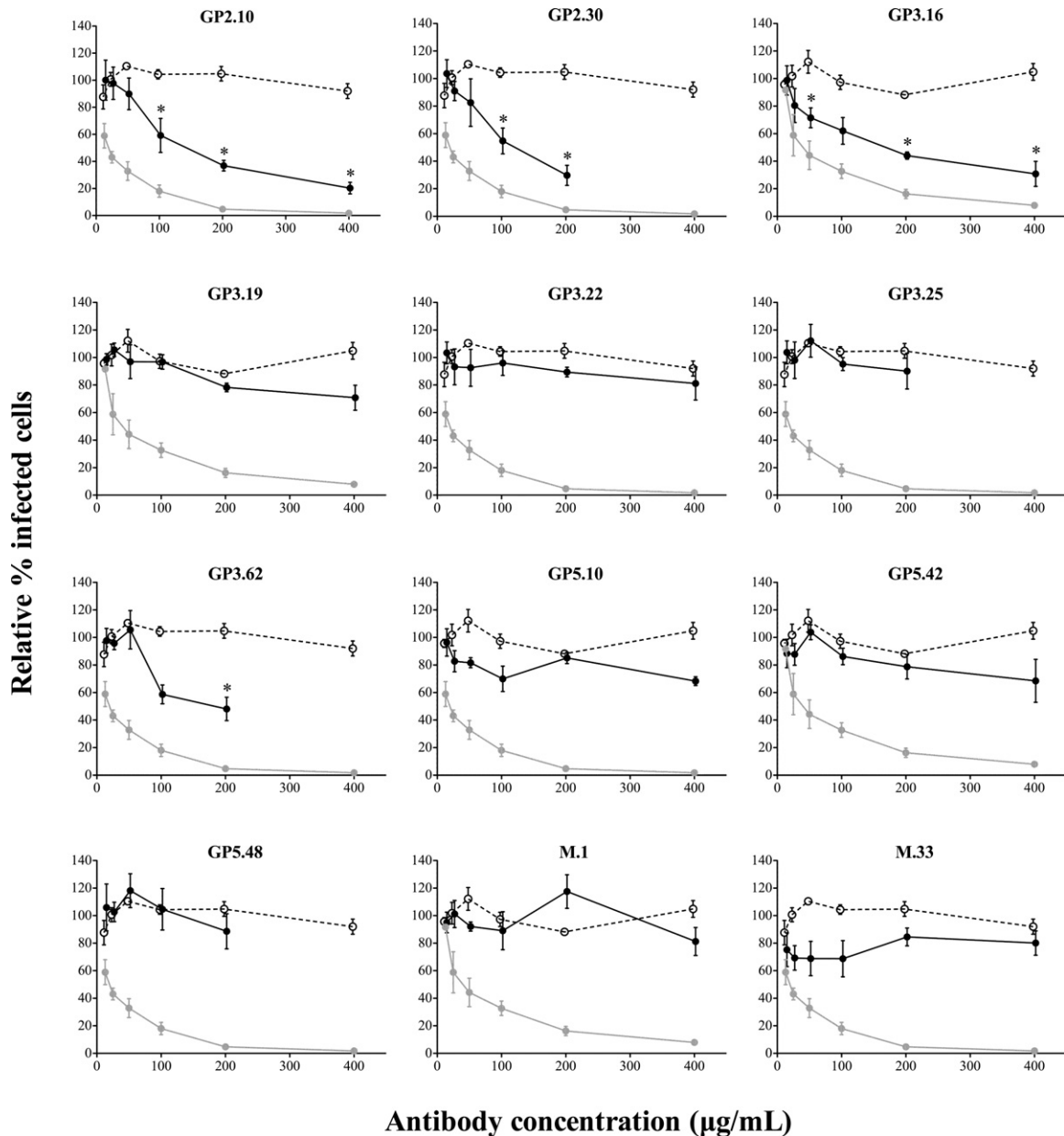| Pig	Sample	Neutralization titre ($\log_2$)	Peptide ELISA titre ($\log_2$)					
			GP3			GP4		
			14	15	16	14	15	16
A	Serum	5.0	<3.3	6.6	10.0	10.0	10.0	13.3
	BAL	1.6	<3.3	<3.3	3.3	3.3	3.3	6.6
B	Serum	7.0	<3.3	10.0	6.6	10.0	10.0	10.0
	BAL	4.6	<3.3	3.3	3.3	3.3	3.3	3.3
E	Serum	3.0	<3.3	10.0	10.0	6.6	10.0	10.0
	BAL	<1.0	3.3	3.3	3.3	3.3	3.3	3.3
I	Serum	4.6	3.3	10.0	10.0	13.3	13.3	13.3
	BAL	1.0	<3.3	3.3	3.3	3.3	3.3	6.6
K	Serum	4.0	<3.3	10.0	10.0	13.3	13.3	10.0
	BAL	1.0	<3.3	<3.3	3.3	6.6	3.3	6.6

Fig. 2. Reduction of LV replication in PAM by peptide-specific antibodies. Affinity-purified serum antibodies against peptides GP2.10, GP2.30, GP3.16, GP3.19, GP3.22, GP3.25, GP3.62, GP5.10, GP5.42, GP5.48, M.1 and M.33 of LV were used in single-replication virus-neutralization tests on PAM. Two-fold serial dilutions of antibodies were pre-incubated with LV (final titre 10^5 TCID$_{50}$/mL) and incubated on PAM. Antibody concentrations ranged between 13 µg/mL and 200 or 400 µg/mL, depending on the purification yield. Cells were fixed after 11 h and infected cells were stained, quantified and expressed relative (%) to the mean number of infected cells in the presence of mock antibodies. The mean relative % infected cells of four experiments is given together with the standard error of the mean for each antibody concentration. Black symbols represent treatment antibody conditions, open symbols represent mock antibody conditions, and gray symbols represent neutralization by GP4.15-specific antibodies. Statistically significant differences ($P < 0.01$) between treatment and mock conditions are indicated by asterisks.

of AR GP3-I are given for LV, 07V063 and 08V204 in Fig. 4 (GenBank M96262.2, GU737264.1 and GU737266.1).

3.4.1. Reactivity of 07V063 and 08V204 antisera with peptides in AR GP3-I

Six antisera against either 07V063 (pigs 7A–7F) or 08V204 (pigs 8A–8F), collected at 45 dpi, were used in a 1/100 dilution in ELISA on peptides 13–17 of GP3 of the respective homologous virus (Table 3). Sera of LV-infected pigs A, B, E, I and K, collected at 45 dpi, were also included. All five LV antisera recognized both GP3 peptides 15 and 16 of the homologous virus, while peptide 14 was not recognized. Sera of 5/6 07V063-inoculated pigs showed reactivity with peptide 15 of 07V063 GP3, and one of these sera also showed

reactivity with peptide 16. The remaining 07V063 antiserum did not react with any of the peptides within AR GP3-I of the homologous virus. All 08V204 antisera reacted with peptide 15 of the 08V204 AR GP3-I. Three of these sera showed additional reactivity with peptide 14, one with peptide 16, and the two remaining sera with both peptides 14 and 16. None of the LV, 07V063 or 08V204 antisera showed reactivity with peptide 13 or 17, flanking the AR (not shown). From this, it was clear that all three virus strains induced antibodies against peptides in AR GP3-I. However, in contrast to LV in which peptide 16 was considered the core of AR GP3-I, peptide 15 was considered the core of AR GP3-I of both 07V063 and 08V204, since this peptide was recognized by the highest number of animals.
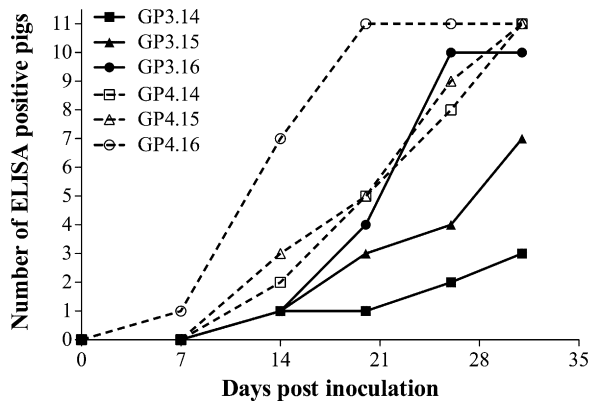
Fig. 3. Serum reactivity of LV-infected pigs with antigenic regions GP3-I and GP4-I at different time points post inoculation. Sera of 11 LV-infected pigs, collected at different time points post inoculation, were tested in peptide ELISA on peptides 14, 15 and 16 of either GP3 or GP4. OD450 values obtained with test samples were considered to be specific when they exceeded 2 times the OD450 value obtained with mock serum at the same peptide. The number of reactive pigs at each time point is given for the different peptides of the AR (full lines: GP3-I, dashed lines: GP4-I).
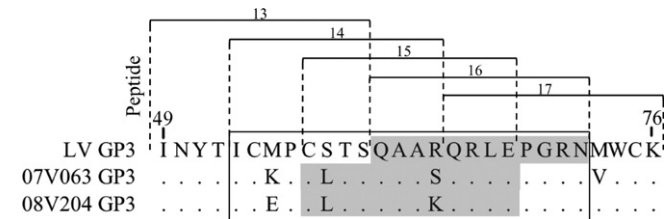
Fig. 4. Amino acid sequence alignment of antigenic region GP3-I of LV, 07V063 and 08V204. GP3 amino acids 49–76 of LV, 07V063 and 08V204 are given, and the positions of peptides 13–17 are indicated. Dots represent identical residues, the box indicates AR GP3-I, and the gray sequences indicate the core peptides (LV: peptide 16, 07V063 and 08V204: peptide 15) of the AR for each virus.

Table 3
Reactivity of LV, 07V063 and 08V204 antisera with antigenic region GP3-I of homologous virus. Antisera against LV (pigs A, B, E, I and K), 07V063 (pigs 7A–7F) and 08V204 (pigs 8A–8F) were tested in peptide ELISA on GP3 peptides 14, 15 and 16 of homologous viruses. Sera were diluted 1/100 and OD450 values were determined at each peptide for test samples (s) and a negative control serum (n). Signals were considered positive when the OD450 s/n ratio was 2 or more.

Inoculum	Pig	GP3 peptides of homologous virus, recognized by serum in peptide ELISA (OD450 s/n > 2)		
		14	15	16
LV	A	−	+	+
	B	−	+	+
	E	−	+	+
	I	−	+	+
	K	−	+	+
07V063	7A	−	+	−
	7B	−	+	−
	7C	−	−	−
	7D	−	+	+
	7E	−	+	−
	7F	−	+	−
08V204	8A	+	+	−
	8B	+	+	−
	8C	+	+	+
	8D	+	+	−
	8E	−	+	+
	8F	+	+	+

3.4.2. Virus neutralization by peptide-specific serum antibodies against AR GP3-I of 07V063 and 08V204

Antibodies against peptide GP3.15 of 07V063 (CLTSQAASQRLE) or 08V204 (CLTSQAAKQRLE) were purified from corresponding polyclonal sera, and the specificity of purified antibody fractions was confirmed by peptide ELISA. Subsequently, the antibodies were used in single-replication virus-neutralization tests on PAM with homologous virus. Antibodies against peptide 15 in GP4 of 07V063 and 08V204 were previously described to neutralize homologous virus, and were included here as a positive control for virus neutralization (Fig. 5, gray lines) [49]. Similar to neutralization of LV by antibodies against GP3.16 (Fig. 3), antibodies against GP3.15 of 07V063 and 08V204 reduced replication of the respective homologous virus strain in a dose-dependent manner (Fig. 5, black lines). A clear and significant reduction in viral replication was observed with antibody concentrations starting from 50 μg/mL for both virus strains.

4. Discussion

The specificity of the antibody response upon PRRSV infection has been studied frequently in the past, at the level of either proteins, antigenic regions (ARs) or linear epitopes. Some studies covered most or all envelope proteins, while others focused on a limited selection of viral proteins. In the current study, the antibody response against linear ARs within the entire set of envelope proteins was investigated in pigs upon infection with the EU prototype PRRSV strain LV. Twenty-one ARs in total were recognized by serum of one or more pigs, either at 31, 52 or at 141 dpi. A majority of these ARs were recognized by sera collected at 31 dpi, indicating that the antibody repertoire against linear ARs at that time point was largely representative for the repertoire at later time points post infection. Remarkably, a considerable number of ARs was recognized by no more than one pig, and it is questionable whether these sequences can be considered relevant ARs or epitopes. From the remaining ARs, only GP4-I was recognized by sera of all animals. This AR was described earlier as a hypervariable region that strongly induces antibodies in pigs, at least for different EU-type PRRSV strains [40,49]. Furthermore, ARs GP3-I and GP3-II were recognized by a majority of the animals, while the remaining ARs in GP3 and all ARs in GP2, GP5 and M were recognized by not more than five out of seventeen animals. These results match to a large extent with the findings of Oleksiewicz et al. [38] that showed a high reactivity of EU-type PRRSV antisera with 'epitope sites' corresponding to ARs GP4-I, GP3-I and GP3-II [38]. NA-type PRRSV strains also induce antibodies against these three ARs, but in a study of de Lima et al. [44] AR GP4-I did not seem to be immunodominant, and ARs other than GP3-I, GP3-II and GP4-I showed equal or higher immunogenicity [42,44]. In addition to ARs GP3-I, GP3-II and GP4-I, a high number of the remaining ARs within LV also correspond to earlier described epitopes in other virus isolates of both the EU and NA genotypes. It is known that regions corresponding to ARs GP2-II, GP2-IV, GP5-III, GP5-IV and M-III of both EU- and NA-type PRRSV are able to induce antibodies in pigs, while ARs GP3-VIII and M-II were described earlier to induce antibodies for EU-type PRRSV, and GP3-III, GP3-IV, GP3-V and GP5-I for NA-type viruses [31,38,39,41,42,44,56,57]. Despite the high genetic variability of PRRSV, the antigenicity of the viral proteins seems relatively well conserved, even at highly variable sites. The antigenicity of certain regions in the envelope proteins is probably more dependent on conserved structural characteristics of the protein than on the exact aa sequence of the particular AR. This was suggested earlier for GP4-I, but now seems to suit for other ARs as well [49]. Remarkably, a large number of ARs identified in this study are situated in GP3. The GP3 ectodomain contains a considerable number of highly conserved cysteine residues and N-glycosylation sites, indicating that this protein shows a strictly
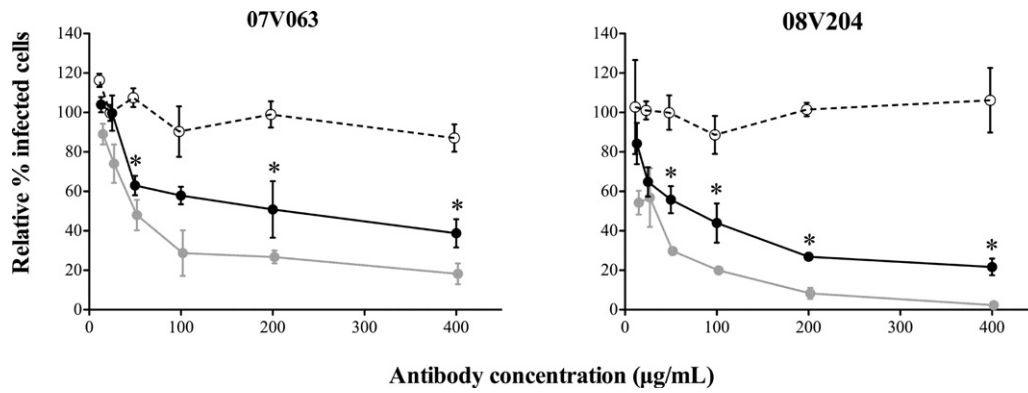
Fig. 5. Reduction of 07V063 or 08V204 replication in PAM by antibodies against peptide GP3.15 of the corresponding virus. Affinity-purified serum antibodies against peptide GP3.15 of either 07V063 or 08V204 were used in single-replication virus-neutralization tests on PAM with the respective homologous virus. Two-fold serial dilutions of antibodies were mixed with virus (final titre 10^5 TCID$_{50}$/mL) and incubated on PAM. Antibody concentrations ranged between 13 μg/mL and 400 μg/mL. Cells were fixed after 11 h and infected cells were stained, quantified and expressed relative (%) to the mean number of infected cells in the presence of mock antibodies. The mean relative % infected cells of four experiments is given together with the standard error of the mean for each antibody concentration. Black symbols represent treatment antibody conditions, open symbols represent mock antibody conditions, and gray symbols represent neutralization by antibodies against peptide GP4.15 of the corresponding virus strain. Statistically significant differences ($P < 0.01$) between treatment and mock conditions are indicated by asterisks.

ordered conformation that is conserved amongst PRRSV strains. Within the highly organized GP3 ectodomain, a large hydrophilic region is present that is not occupied by glycans. Interestingly, this particular region turned out to determine a long antigenic stretch that can be subdivided into five distinct linear ARs (GP3-I to GP3-V), comprising the more immunogenic ARs GP3-I and GP3-II.

For all ARs that were recognized by sera of two animals or more, as well as for AR GP2-II, it was determined whether antibodies against the core peptide were able to influence *in vitro* virus replication in PAM. Antibodies specific for peptides GP2.10 (AR GP2-II), GP2.30 (GP2-IV), GP3.16 (GP3-I), GP3.62 (GP3-VIII) and GP4.15 (GP4-I) reduced *in vitro* virus replication in PAM in a dose-dependent manner, while none of the other antibodies influenced virus replication in any concentration tested. Although the antibody response against GP2 was poorly studied in the past, the ARs GP2-II and GP2-IV have already been described for both EU- and NA-type PRRSV [38,44]. However, GP2 was never associated with virus neutralization, and the identification of two neutralizing epitopes in this protein shows the value of studying the antigenicity of the 'minor' PRRSV envelope proteins. Antibodies against ARs GP2-II and GP2-IV were only found in sera of 1 and 2 animals respectively, and for the former only at 141 dpi, indicating that the neutralizing antibody response against these ARs upon infection is probably of minor importance. Further investigation is needed to determine whether these ARs can be made more immunogenic in view of induction of GP2-specific neutralizing antibodies. AR GP3-I showed similar sensitivity to antibody-mediated neutralization as the ARs in GP2. However, it is clear from this and other studies that a majority of PRRSV-infected pigs develop antibodies against this AR, albeit rather late post infection [38,42,44]. GP3 has been associated with virus neutralization in the past, and it has been speculated that an 'epitope site' covering ARs GP3-I and GP3-II is involved in functional interactions with the host cell, suggesting that antibodies against this site might have some neutralizing capacity [30,38]. However, this is the first study that offers direct evidence that antibodies against AR GP3-I reduce viral infectivity *in vitro*, and thus have the potential to contribute to protection *in vivo*. AR GP3-I is situated immediately upstream of GP3-II, and both ARs show similar immunogenicity. It is however clear from reactivity of individual pigs with peptides of either GP3-I or GP3-II that these are distinct ARs, which was suggested earlier in other studies [38,42,44]. Antibodies against peptide GP3.19 within AR GP3-II did not influence viral infectivity *in vitro*, in accordance to a study of Van Breedam

et al. [51] in which mAbs against this peptide did not reduce viral replication in PAM [51].

In addition to GP3-I, AR GP3-VIII in the C-terminal end of the protein also showed some susceptibility to antibody-mediated virus neutralization. This AR is however situated in the putative endodomain of GP3 and should thus not be accessible for antibodies [52]. One possible explanation for this apparent contradiction is that the antibody fraction obtained by purification against peptide GP3.62 was not sufficiently pure. This antibody fraction showed remarkably high reactivity with other peptides, and it cannot be ruled out that remnant antibodies against other ARs, for example strongly neutralizing GP4-specific antibodies, were responsible for the observed neutralizing effect. Unfortunately, a lack of appropriate sera and weak purification yields did not allow further investigation of this issue. Another possibility however is that, contradicting with topology prediction models, this part of GP3 is indeed exposed at the virion surface, and in that case the AR would be accessible for neutralizing antibodies. In any case, a neutralizing antibody response against AR GP3-VIII would not be expected to be of major significance, since it seems that this AR in LV is not at all highly immunogenic. Interestingly, Oleksiewicz et al. [39] have shown that the corresponding region in a Danish EU-type isolate shows high immunogenicity in pigs on the one hand, but that viruses with deletions in this region easily survive in the population on the other hand [39]. While the predicted GP3 ectodomain seems antigenically well conserved, the antigenicity of the C-terminal endodomain is clearly more strain-dependent. This is sustained by the fact that all ARs in the ectodomain of GP3 identified here are also present in NA-type PRRSV, while this is not the case for AR GP3-VIII [42,44].

Compared to the neutralizing capacity of GP4-specific antibodies, antibodies against ARs GP2-II, GP2-IV, GP3-I and GP3-VIII were clearly less potent to reduce viral infectivity *in vitro*. The difference in neutralizing capacity of antibodies against different ARs at a similar concentration can be explained in two ways. On the one hand, different ARs can show different susceptibilities to antibody-mediated neutralization, inherent to the position and/or function of this AR in the virion. This would then mean that even high concentrations of antibodies with maximal affinity for the AR would never be able to exert a strong neutralizing effect. On the other hand, the peptide-purified antibodies used in this study may have a suboptimal affinity and consequently show limited neutralizing capacity. It is well possible that B-cell populations specific for cer-

tain ARs have not undergone optimal affinity maturation yet upon single infection at the time points of serum collection. Furthermore, a core of 12 aa was defined for an entire AR, while it cannot be ruled out that certain linear ARs are part of broader discontinuous or conformation-dependent epitopes. Whether there exists any potential to obtain stronger neutralizing antibodies against GP2 or GP3 is subject for further research, but it is clear from the current study that ARs GP2-II, GP2-IV, GP3-I and GP3-VIII are at least partially susceptible to antibody-mediated virus neutralization.

In contrast to GP2, GP3 and GP4, none of the ARs in GP5 and M were susceptible to antibody-mediated virus neutralization, although it should be mentioned that purified antibodies against AR M-I showed only low affinity for the corresponding peptide in ELISA. It was not unexpected that ARs GP5-III and GP5-IV were not susceptible to neutralization, since these ARs are situated in the predicted endodomain of the protein, and since it is known that mAbs against the corresponding regions in both EU- and NA-type viruses are not neutralizing [52,56,57]. The lack in neutralization that was observed with antibodies against AR GP5-I is however more controversial. The corresponding region in NA-type GP5 was described earlier as the "primary neutralizing epitope" of PRRSV, and GP5 is generally considered the main target for virus-neutralizing antibodies [41]. Several GP5-specific mAbs exist that are able to reduce viral infectivity of NA-type PRRSV strains, and one of these is directed against AR GP5-I [31,58,59]. Furthermore, correlations between serum antibody responses against AR GP5-I of NA-type viruses and seroneutralization titres have been described [31,41]. While it is clear that antibodies against GP5-I can be formed upon infection with NA-type PRRSV, de Lima et al. [44] however showed that the immunogenicity of this AR is not exceptionally high [44]. For EU-type PRRSV, a neutralizing epitope has also been identified in GP5, but it is crucial to emphasize that this epitope is not the same as the one that is described for NA-type PRRSV, and that it does not correspond at all to AR GP5-I [31,32]. The neutralizing epitope in EU-type GP5 is located upstream of AR GP5-I, and is in fact only susceptible to antibody-mediated neutralization in the context of an *in vitro* selected virus phenotype that has never been observed *in vivo* [32]. No other neutralizing epitopes have been described in GP5 of EU-type PRRSV to date. Furthermore, it was recently described that mAbs directed against AR GP5-I of LV are not able to influence virus infectivity in PAM, even at high concentrations [51]. This is in line with the results from the present study, and sustains the idea that although antibodies against AR GP5-I in NA-type PRRSV may be able to reduce viral infectivity, this is not the case for LV and possibly neither for other EU-type viruses. This discrepancy can currently not be explained, but it is very well possible that the structural properties of the GP5 protein vary amongst virus strains, leading to different antigenic properties of this protein.

Since ARs GP3-I and GP4-I were both immunogenic and target for neutralizing antibodies, the serum antibody response against these ARs was further studied. While antibodies against GP4-I appeared relatively fast upon infection in a majority of the animals, it clearly lasted longer for most animals to develop antibodies against AR GP3-I. Moreover, antibody titres in end sera and BAL fluids against GP3-I were invariably lower than against GP4-I. Although most pigs are able to develop antibodies against both ARs, GP4-I clearly shows the highest immunogenicity in the context of infection. There is evidence from recent studies that AR GP4-I is susceptible to antibody-mediated selective pressure *in vivo*, and its high variability confirms that aa changes in this region are generally well tolerated by the virus [33,53,55,60,61]. One might speculate that the high immunogenicity of this region is at the expense of the immunogenicity of other, more conserved neutralizing epitopes, which would offer the virus an evolutionary advantage. Although it can feel contradictory that a decoy epitope is associated with protective immunity, a neutralizing epitope for which the emergence

of antibody-escape mutant viruses within the quasispecies population is easily allowed, can perfectly act as decoy for neutralizing epitopes in which aa changes are less frequently tolerated. For NA-type PRRSV, it was suggested that GP5 contains a non-neutralizing decoy epitope that compromises the immunogenicity of the neutralizing epitope corresponding to AR GP5-I of LV [31]. The current study however shows no evidence for either the presence of such a decoy epitope in GP5 of LV or the susceptibility of AR GP5-I to antibody-mediated neutralization. EU- and NA-type PRRSV probably evolved separately during a considerable amount of time, and it is not unlikely that both genotypes developed different immune evasion strategies [16].

Earlier studies, in particular the studies of de Lima et al. [44] and Oleksiewicz et al. [38,40], have provided large maps of antigenic regions that are capable of inducing antibodies in infected pigs. However, these authors did not investigate the neutralizing capacity of epitope-specific antibodies. The approach that was used in the current study to characterize ARs, consisting of pep-scan analysis with porcine antisera, followed by neutralization tests with peptide-purified serum antibodies, was successfully used in the past to characterize ARs in GP4, and allows the study of ARs against which no mAbs are available [49]. Furthermore, the use of primary cells, homologous virus that has not been adapted to cell culture and antibodies from the natural host in neutralization tests maximizes the *in vivo* relevance of the results. Although caution is needed when relating *in vitro* virus neutralization with *in vivo* protection, it seems reasonable to expect that neutralizing antibodies as detected here can contribute to protective immunity in pigs. The detection of antibodies specific for ARs GP3-I and GP4-I in BAL fluids of infected animals confirms that neutralizing antibodies are present at the major site of viral replication, where they may contribute to viral clearance.

Considering the development of new generation PRRSV vaccines that aim to induce virus-neutralizing antibodies, AR GP3-I in particular shows some interesting properties. Antibodies against this AR in LV, but also against the corresponding region in the EU-type field isolates 07V063 and 08V204, are able to reduce viral infectivity, and a large majority of pigs seem able to develop GP3-I-specific antibodies upon infection. Further studies should address to what extent the sensitivity of this AR to antibody-mediated neutralization is conserved amongst a broader range of PRRSV strains.

Since different viruses induced antibodies against different peptides within AR GP3-I, it is currently not clear how this AR can be more generally defined. Furthermore, it is not known to which extent structural properties of the entire protein determine the antigenic properties and immunogenicity of AR GP3-I. This information will be essential for subunit vaccine development, and to be able to determine antigenic cross-reactivity between virus strains at the level of this epitope.

AR GP3-I is genetically relatively well conserved, especially compared to AR GP4-I [38,42,61,62]. However, it is not clear to what extent this AR is well conserved because it fulfills an important function for the virus, or because no strong selective pressure was present on this region during PRRSV evolution. In case variability in this sequence is functionally restricted, targeting the antibody response towards this AR would be an appropriate strategy, because the viability of virus mutants that escape antibody-mediated neutralization at the level of this AR will be largely compromised, and escape-mutant viruses will rarely survive. If the aa sequence of this region is however less essential for the virus viability, targeting the immune response towards this AR would enhance the selective pressure, and create a driving force in the evolution of this particular region, similar to what happens in AR GP4-I [33,53]. Hence, it should be thoroughly investigated to what extent the virus can tolerate aa changes in certain ARs before considering vaccine development. This can be done by cultivating

the virus *in vitro* in the presence of epitope-specific neutralizing antibodies while monitoring the appearance of antibody-escape mutant viruses, as earlier described for the neutralizing epitope in GP4 [33]. More generally, a vaccine against a rapidly evolving RNA virus that exists as a quasispecies population should always aim to stimulate as many different protective immune mechanisms as possible to minimize the risk of emergence of virus variants that escape the host's immune system.

Many experimental vaccines based on one or more PRRSV envelope proteins have been developed and tested with variable success [63]. Because GP5 is often considered the major target for PRRSV protective immunity, the main focus was put on this protein in subunit vaccine development for a long time. It becomes however more and more clear that other envelope proteins also contain important determinants of protective immunity, and it is encouraging to notice that recent studies also consider the minor envelope proteins for subunit vaccine development [38,45,64,65]. Some of these studies indicated the involvement of GP2 and GP3 in clinical and virological protection, although it was not assessed to what extent this protective effect could be attributed to stimulation of the GP2- and GP3-specific neutralizing antibody response [66–70].

The current study aimed to characterize linear ARs in the entire set of envelope proteins of LV, and new targets for both non-neutralizing and neutralizing antibodies were identified. This work provides new insights into PRRSV antigenicity, and contributes to knowledge on protective immunity and immune evasion strategies of the virus.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vaccine.2011.04.071.

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