RESEARCH REPORT
SWINE HEALTH

Title: Identification of conserved T-cell epitopes contained in the non-structural genes of PRRSV which contribute to broad protective immunity – **NPB #10-115**

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Industry Summary:

Effective vaccine protection against infection by any of the multiple PRRSV strains that circulate in the field is of major importance for industry. This proposal was designed at tackling such aspect of swine immunology.

As a result of this grant we have identified certain small portions (also referred in this report as "peptides" or "epitopes") of two nonstructural (NSP) proteins of PRRSV (def: NSPs are those viral proteins that not part of the actual PRRS virus particle but that are present in the cells and tissues infected by this virus). The identified epitopes are highly conserved and we show that they are "seen" (recognized) by the immune system of the pig when infected by PRRSV. It should be noted that these NSPs are not typically considered to be first-choice candidate proteins to formulate a vaccine. The feasibility of adding these NSP proteins or their epitopes to a multi-antigen/multi-epitope vaccine X PRRSV is novel and a notion directly derived from this Pork Check-off grant. The results of this one-year project are of immediate applicability and interest to PRRSV vaccinologists. Likewise, the results of this proposal contribute fundamental building blocks for the development of a future, more effective broadly protective vaccine X PRRSV. In addition, the results obtained from this proposal combine nicely with information being pursued in other NPB and USDA projects, and will provide excellent complementary information enhancing the objectives proposed for CAP2 project on PRRSV strain diversity.

Keywords: PRRSV, Broadly-protecting vaccines,T-cell epitopes, multi-epitope vaccines

Scientific Abstract:

Porcine reproductive and respiratory syndrome virus (PRRSV) is a significant swine pathogen which exhibits considerable sequence diversity. In an attempt to identify highly conserved T-cell epitopes contained in proteins of this virus, we examined heptadecamer peptides spanning the sequence of the PRRSV nonstructural proteins (NSPs) 9 and 10, both of which are highly conserved, for their ability to elicit a recall proliferative and interferon-gamma response in peripheral blood mononuclear cells obtained from pigs immunized against the type-II PRRSV strain FL-12. These studies led to the identification of four peptides, two from each NSP9 and NSP10 that appear to contain T-cell epitopes. Comparison of the amino acid sequence of these four peptide sequences to the analogous sequences from a diverse sample of type-II PRRSV strains indicated that these sequences are highly conserved and thus contain highly conserved T-cell epitopes. The identified epitopes may be important in the formulation of immunogens to provide broad cross-protection against diverse PRRSV strains.

Introduction:

Extensive genetic heterogeneity and antigenic variation occurs amongst PRRSV strains in the field which represents a major obstacle for the development of vaccines to control PRRSV circulation in the field. Therefore, it has become essential to understand what constitutes the basis for protective immunity in PRRSV and how this protection can be broadened when designing new PRRSV vaccines.
In the case of several other highly genetically diverse and variable RNA viruses such as hepatitis C virus (HCV) and HIV the use of highly conserved T-cell epitopes as immunogens have been found capable of conferring broad protective immunity. Such conserved regions often are found in nonstructural proteins (NSPs) of these RNA viruses that are synthesized early during the early phase of the virus replication cycle. These NSPs typically constitute structurally constrained, conserved proteins involved in replication of the virus. If animals are preferentially immunized against conserved epitopes of NSPs, it is possible that such immunization could result in a highly "pan-strain specific" protective immunity. This is the principle applied in the development of several multi-epitope vaccines recently reported for "hard-to-immunize" RNA viruses such as HCV or HIV and also against chronic hepatitis B virus infection.

Accordingly, we selected a subset of PRRSV NSPs based on their functional role in viral replication and virulence. The ORF1 (b) region of the PRRSV genome contains the NSPs 9, 10, 11 and 12 genes. The NSP9 is the viral RNA-dependent RNA polymerase (RdRp) required for genome replication and transcription and the NSP10 encodes for helicase function (Fang and Snijder, 2010). The main criteria used to select NSP9 and NSP10 for this study was their highly conserved nature which is consistent throughout the North American PRRSV strains, thus constituting good candidates for the identification of T-cell epitope that could provide target antigens capable of eliciting cross-protective immunity against PRRSV. To achieve this goal, overlapping heptadecapeptides spanning the entire length of the PRRSV NSP9 and NSP10 were screened respectively using T-cell proliferation and IFN-γ ELISpot assays. Herein we report the identification of T-cell epitopes mapping to these PRRSV NSPs. Two distinct regions each in NSP9 and NSP10 were identified as putative T-cell epitopes. As expected, these epitopes were found to be highly conserved among sixty-five North American PRRSV type II sequences.

Objectives:
Identify candidate T-cell epitopes of PRRSV by peptide scanning of the non-structural gene region in the genome of FL12 infectious clone-derived PRRSV

Materials & Methods:

General experimental design:

Figure 1 illustrates the experimental design used in our study to identify T-cell epitopes in PRRSV nonstructural proteins NSP9 and NSP10. The screening tests for NSP9 and NSP10 were performed independent of each other at different time points and with different sets of pigs. To screen the NSP9 peptides for their ability to stimulate a recall T-cell response, a total of sixteen 4-5 weeks old, Landrace x Large White were obtained from a PRRSV-free farm. Eight of those animals were immunized against PRRSV and the other eight were used as non-immune specificity controls. Pigs were immunized against PRRSV FL-12 by infection with a single intramuscular injection of $10^{6.5}$ $TCID_{50}$ of PRRSV FL-12 followed by two additional boosters of the same dose of PRRSV FL-12 given several weeks apart (see below). The first booster of the NSP9 group was administered at 21 days post-infection (d.p.i.) and the second at 96d.p.i. The first booster was administered emulsified in Freund's complete adjuvant and the second was administered in incomplete Freund adjuvant. For NSP10 screening, we used a total of six mixed-breed animals (same farm of origin, age, and genetic background as previous experiment, 4 PRRSV-immunized principals and 2 uninfected controls). In this case the first booster was administered at 57d.p.i. and the second at 114 d.p.i. For both NSP9 and NSP10 experiments, starting at day 7 d.p.i. and for the entire length of immunization in each case, blood was collected weekly for the following assays: 1) confirmation of viral infection parameters (viremia, PRRSV antibodies, T-cell proliferation and IFN-γ secreting cells by ELISpot) and 2) testing the set of peptide pools as well as to confirm individual peptides. For all experiments, the infected and the non-infected control groups were housed separately in isolated bio-safety level 2 (BL-2) rooms to avoid cross-contamination and supplied with standard diet ad libitum. All the rooms were well-ventilated with independent proper waste disposal systems to avoid cross-contamination at all times.

Results:

Screening of PRRSV NSP9and NSP10 for T-cell epitopes using T-cell proliferation assay:

Twenty peptide pools from NSP9 were used for the first round of peptide screening (figure 2). Based on the criteria described in the materials and methods for analysis of functional assays, four out of twenty NSP9 peptide pools encompassing amino acid positions 103-143, 135-175, 199-239 and 519-559 were identified with stimulation indices at least twice background (Table 2). As a result of the T-cell proliferation assay of the individual peptides present in those four NSP9 peptide pools (figure 3), it was found that four peptides beginning with amino acid positions 119, 151, 207 and 519 elicited positive proliferation results (Table 3).

Fourteen peptide pools from NSP10 were used for the first round of peptide screening (figure 2). Two out of fourteen NSP10 peptide pools, encompassing amino acid positions 129-169 and 193-233, were identified which elicited positive stimulation indices (Table 2). As a result of the T-cell proliferation assay of the individual peptides present in those two NSP10 peptide pools (figure 3), it was found that two peptides encompassing amino acid positions 209-225 and 217-233 respectively exhibited positive stimulation indices (Table 3).
None of the PBMCs isolated from the control pigs responded to the specific peptides obtained after two rounds of T-cell peptide screening.

Id*entification of PRRSV immunogenic individual peptides from NSP9 and NSP10*

Table 4 provides a summary of the results obtained from cryopreserved PBMCs stimulated with NSP9 and 10 peptides inducing IFN-γ. Out of 4 NSP9 peptides tested (those that gave a positive response in the lymphoproliferation assay), 2 peptides at amino acid position 119-135 and 151-167 (KEEIALSAQIIQACDIR and VRGNPERVKGVLQNTRF) showed specific immunospots as compared with their non-immunized counterparts (Table 4). The remaining two out of four infected pigs responded to each of these NSP9 peptides (Table 4). During this analysis, the remaining two peptides did not stimulate IFN-γ response in PBMC populations. In case of NSP10, both the individual peptides stimulated proliferation of IFN-g secreting cells at different time points (Table 4). The peptide at amino acid position 209-225 (VRILAGGWCPGKNSFLD) elicited an increase in IFN-γ ELISpot positive cells greater than three standard deviation over the control and thus is considered a candidate epitope. The peptide at amino acid position 217-233 (CPGKNSFLDEAAYCNHL) was able to elicit specific immunospots from PBMC collected following the second booster dose (121 days post-initial infection and 7 days after second booster).

Conservation of PRRSV NSP9 and NSP10 peptides identified throughout North American PRRSV Type II strains.

Table 5 illustrates the conservation of these identified NSP9 and NSP10 peptides in a sample of diverse PRRSV field isolates. The exact NSP9 peptide sequence KEEIALSAQIIQACDIR of FL-12 strain at amino acid position 119 was conserved in 22 % of the strains throughout the North American isolates (Table 5).

Discussion:

Both neutralizing antibodies and CMI contribute towards the establishment of PRRSV protective immunity. While the mechanisms and viral determinants of protective immunity against PRRSV are incompletely characterized, protection likely results from recognition of viral epitopes by both antibodies and T-cells. Identification of T-cell epitopes mediating heterologous protection, and the nature of variation in those epitopes will directly enhance prediction of vaccine efficacy and guide the rational design or selection of broadly protective vaccines through estimation of the optimal composition of epitopes for the formulation of vaccines efficacious against the majority of PRRSV strains. This would represent a significant advance in the control of PRRSV. Our present report deals with functional T-cell epitope mapping focused on selected nonstructural PRRSV proteins. These NSPs constitute fundamental, highly conserved proteins used for the early stages of viral replication.
The strictly functional approach that we followed in this study has been based on two bonafide CMI assays: lymphocyte proliferation and frequency of IFN-γ secreting cells. It has been reported that lymphocyte proliferation from PRRSV-infected pigs can be detected starting at 4 weeks post infection. Those proliferating cells can also secrete IFN-γ and hence, T-cell response measured by IFN-γ secreting cell frequency has been shown to be central in clearing viral infections.
The emphasis on PRRSV T-cell epitope research and identification has centered, until now, exclusively on important structural components of this virus. Several papers have been published suggesting the existence of T-cell epitopes in GP5, GP4, N and M protein. In our case we selected two PRRSV NSPs (NSP9,the RdRp, and NSP10, a

helicase) based on their functional role in viral replication and virulence. NSP9 and NSP10 seem to be highly conserved, conserved among North American PRRSV strains, thus constituting good tentative candidates for T-cell epitope mapping to provide cross-protection immunity against PRRSV infection.

The conservation of the identified epitopes was investigated by aligning sixty-five amino acid sequences of the NSP9 and NSP10 of North American genotype-II PRRSV strains. Considering the above points, our data suggests that the T-cell epitopes herein identified range in amino acid sequence identity among 22% in NSP9 (amino acid position 119), 84% in NSP9 (amino acid position 151), 95% in NSP10 (amino acid position 209) and 97% (amino acid position 217) (Table 5). From these results, we can confirm that even though these epitopes are not absolutely conserved, they remain conserved in most of the North American isolates except for a few mutations in a very low percentage of the strains.

In summary, we have identified candidate T-cell epitopes present in the NSP9 and NSP10 proteins of PRRSV. The degree of conservation of these epitopes suggests they may be highly useful in the rational design of broadly efficacious vaccines against PRRSV.

Outputs from this grant:

Manuscripts submitted:

Parida,R, Choi, IS, Peterson,DA, Pattnaik,AK, Laegreid,W, ,Zuckermann, F and Osorio FA 2012 Location of T-cell epitopes in nonstructural proteins 9 and 10 of type-II porcine reproductive and respiratory syndrome virus Submitted to Virus Research, manuscript under review.

Presentations:

Parida R., Choi I.S., Peterson D., Laegreid W., Pattnaik AK and Osorio, FA. Identification of T-cell Epitopes Present in Nonstructural Proteins 9, 10 and 11 of Porcine Reproductive and Respiratory Syndrome Virus Presented at XII International Symposium on Nidovirus Research, Traverse Michigan, USA 2011
Date: 06/08/2011

Grants submitted based on these results :
“T-Cell Epitopes and PRRSV Heterologous Protection” (No. 2011-02938)
by W. Laegreid (PI), F Osorio and F Zuckermann.
Submitted to USDA, Agriculture and Food Research Initiative Program (Animal Health and Production and Animal Products) Animal Health and Disease
Proposal Number: Amount requested:$ 490,899, Date: 4 November 18, 2011.
Rank: High Priority(within upper 24 our of 101), **Not funded, resubmission encouraged**

Key to figures:

Figure.1. Strategy to locate T-cell epitope in PRRSV NSPs. This is a schematic diagram depicting the methods and strategy being used during T-cell epitope mapping of PRRSV nonstructural proteins 9 (NSP9) and 10 (NSP10).

Figure.2. Overview of the first round of screening of NSP9 and NSP10 synthetic peptides using their respective peptide pools. T-cell proliferation results from the first round of peptide pool screening of NSP9 (a) and NSP10 (b). The average value of three results obtained from the PBMCs on 14 d.p.i, 21 d.p.i and 28 d.p.i are represented in this bar graph. A total of 4 x 10^6 cells/ml from individual pigs were incubated with 10 µg/ml final concentration of peptide pools (four heptadecamer peptides in one peptide pool) from each NSPs. PBMCs were treated with Thymidine after 18 hrs and then incubated at 37°C for 16 hours. The proliferated cells were measured as cells count per minute per million PBMCs (cpm/million PBMCs) using scintillation counter. Maximum response (dark bar) is the number of proliferating cells detected in PBMC from the highest responder pig, and average response (light bar) is the sum of all the T-cell proliferating cells (minus background) detected in the PBMC samples divided by the number of pigs tested. The results are analyzed on the basis of mean ± std. deviation of non-infected PBMCs. The asterisks are the peptide pools which proved positive for the T-cell proliferation assay.

Figure. 3. Second round of screening with NSP9 and NSP10 individual synthetic peptides identified from the first round of screening. T-cell proliferation results from the second round of peptide pool screening of NSP9 (a) and NSP10 (b). A total of 4 x 10^6 cells/ml from individual pigs were incubated with 10 µg/ml concentration of heptadecamer peptides from each NSPs. PBMCs were treated with Thymidine after 18 hrs and then incubated at 37°C for 16 hours. The proliferated cells were measured as cells count per minute per million PBMCs (cpm/million PBMCs) using scintillation counter. Maximum response (dark bar) is the number of proliferated cells detected in PBMC from the highest responder pig, and average response (light bar) is the sum of all the proliferating cells (minus background) detected in the PBMC samples divided by the number of pigs tested. The results are analyzed on the basis of mean ± std. deviation of non-infected PBMCs. The asterisks are the peptide pools which proved positive for the T-cell proliferation assay.

Figure-1

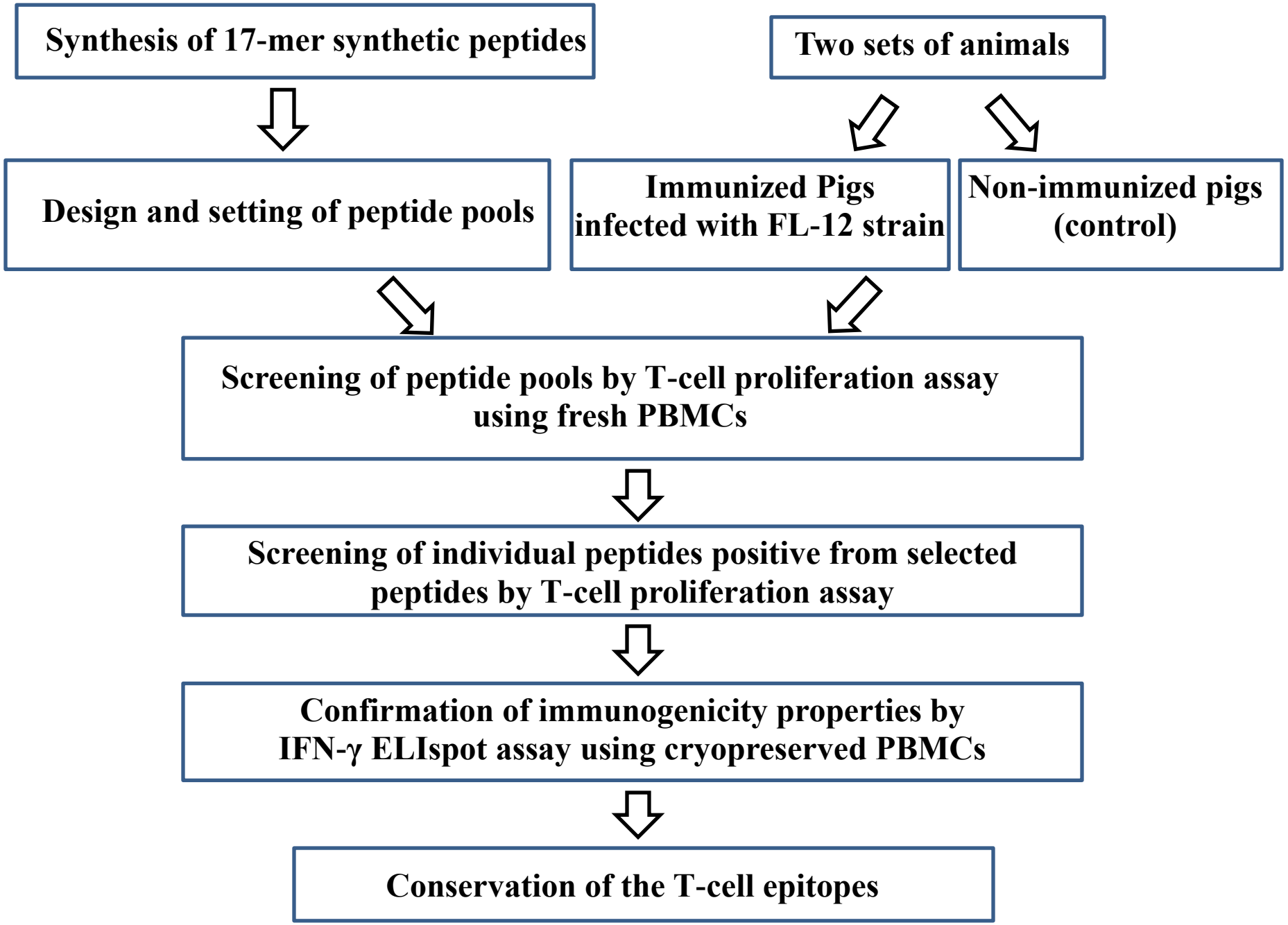
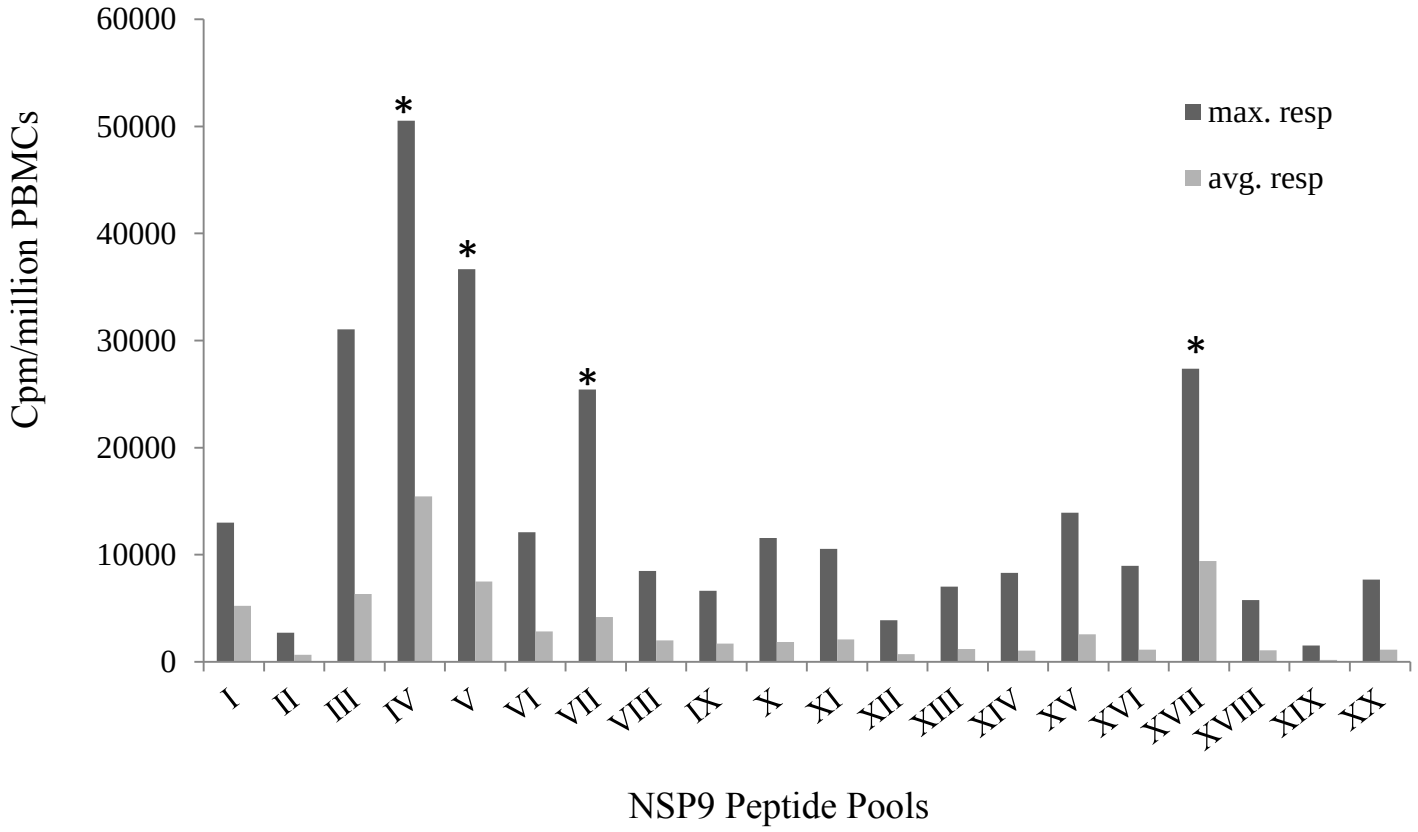

Synthesis of 17-mer synthetic peptides
Two sets of animals
Design and setting of peptide pools
Immunized Pigs infected with FL-12 strain
Non-immunized pigs (control)
Screening of peptide pools by T-cell proliferation assay using fresh PBMCs
Screening of individual peptides positive from selected peptides by T-cell proliferation assay
Confirmation of immunogenicity properties by IFN-γ ELIspot assay using cryopreserved PBMCs
Conservation of the T-cell epitopes

Figure-2

(a)


60000
50000
40000
30000
20000
10000
0
Cpm/million PBMCs
max. resp
avg. resp
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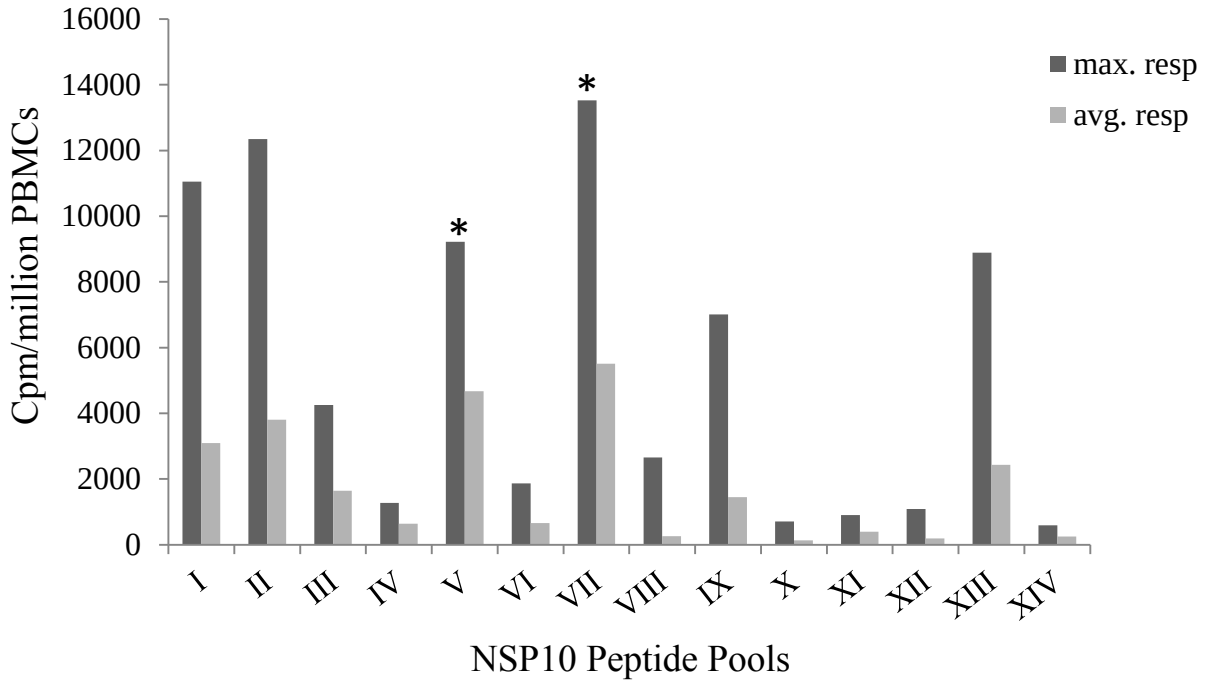
I
II
III
IV
V
VI
VII
VIII
IX
X
XI
XII
XIII
XIV
XV
XVI
XVII
XVIII
XIX
XX
NSP9 Peptide Pools

(b)


16000
14000
12000
10000
8000
6000
4000
2000
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Cpm/million PBMCs
max. resp
avg. resp
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I
II
III
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V
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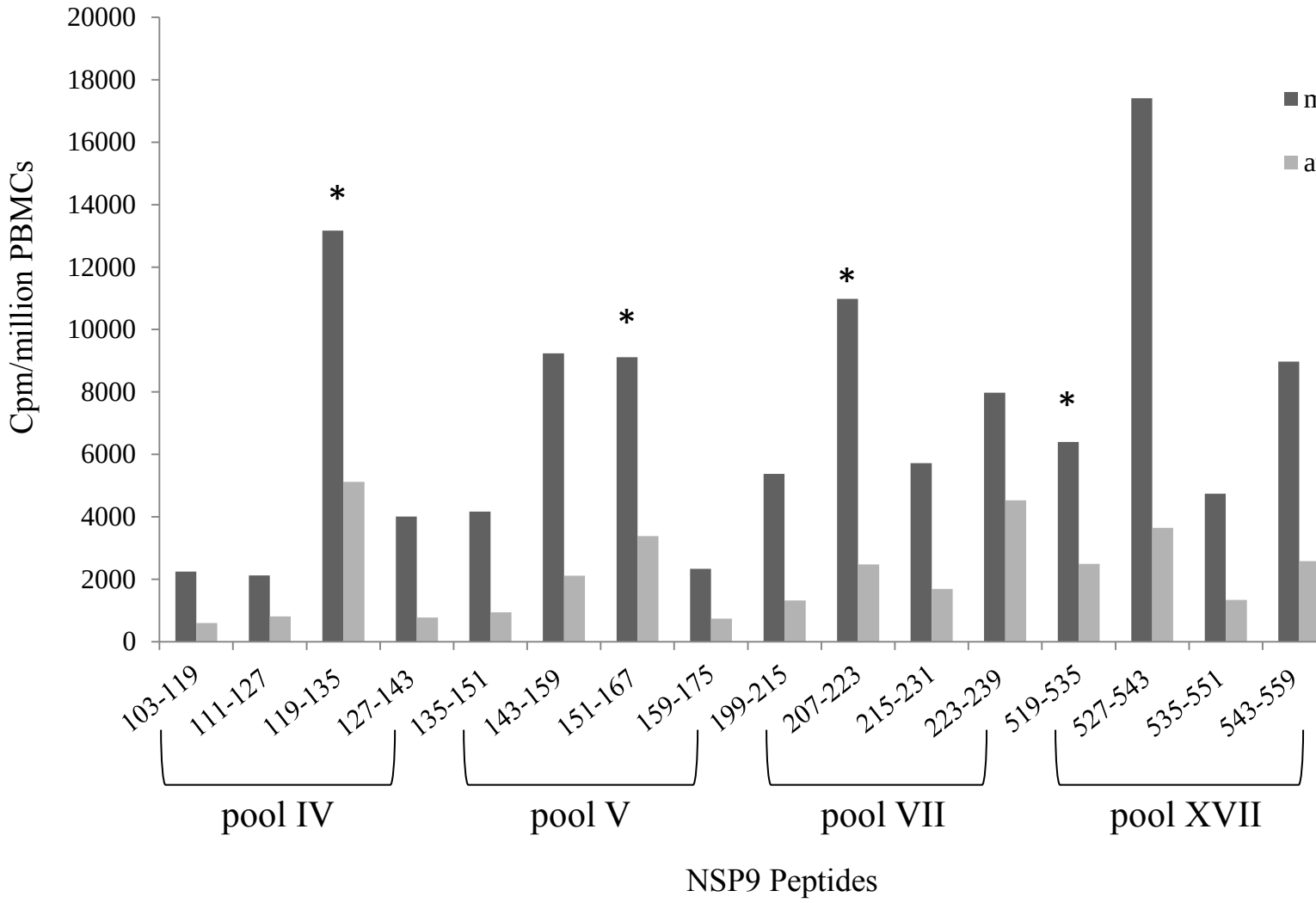
VII
VIII
IX
X
XI
XII
XIII
XIV
NSP10 Peptide Pools

Figure-3

(a)


20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
Cpm/million PBMCs
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*
103-119
111-127
119-135
127-143
135-151
143-159
151-167
159-175
199-215
207-223
215-231
223-239
519-535
527-543
535-551
543-559
pool IV
pool V
pool VII
pool XVII
NSP9 Peptides

(b)

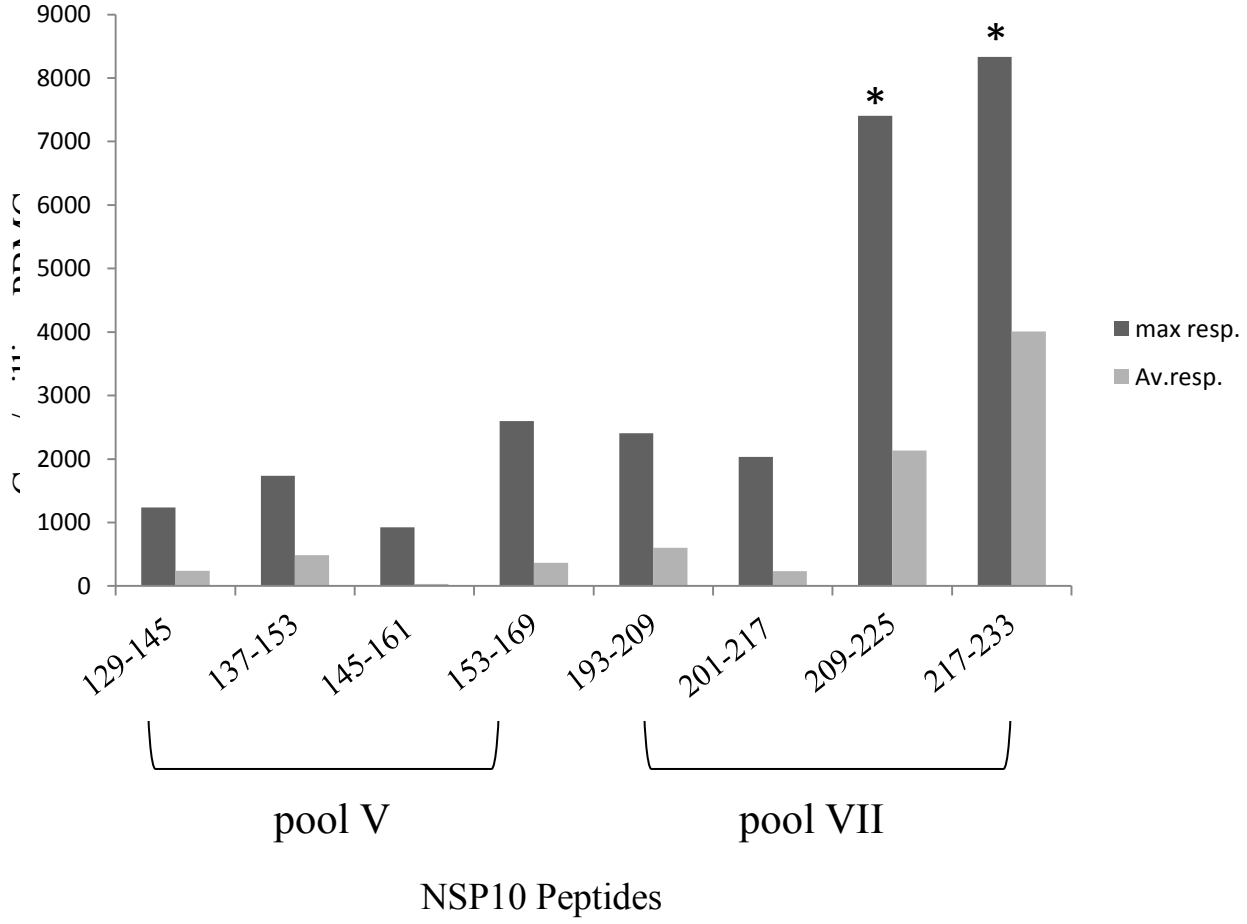
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
*
*
max resp.
Av.resp.
129-145
137-153
145-161
153-169
193-209
201-217
209-225
217-233
pool V
pool VII
NSP10 Peptides

Table1. Name of the peptide pools and individual peptides used for T-cell proliferation and ELIspot assays.

NSP9 Peptide pool no.	I	II	III	IV	V	VI	VII	VIII	IX	X
Peptide	7-23	39-55	71-87	103-119	135-151	167-183	199-215	231-247	263-279	295-311
	15-31	47-63	79-95	111-127	143-159	175-191	207-223	239-255	271-287	303-319
	23-39	55-71	87-103	119-135	151-167	183-199	215-231	247-263	279-295	311-327
	31-47	63-79	95-111	127-143	159-175	191-207	223-239	255-271	287-303	319-335
Peptide pool no.	XI	XII	XIII	XIV	XV	XVI	XVII	XVIII	XIX	XX
Peptide	327-343	359-375	391-407	423-439	455-471	487-503	519-535	551-567	583-599	615-631
	335-351	367-383	399-415	431-447	463-479	495-511	527-543	559-575	591-607	623-639
	343-359	375-391	407-423	439-455	471-487	503-519	535-551	567-583	599-615	631-646
	351-367	383-399	415-431	447-463	479-495	511-527	543-559	575-591	607-623	

NSP10 Peptide pool no.	I	II	III	IV	V	VI	VII	VIII	IX	X
Peptide	1-17	33-49	65-81	97-113	129-145	161-177	193-209	225-241	257-273	289-305
	9-25	41-57	73-89	105-121	137-153	169-185	201-217	233-249	265-281	297-313
	17-33	49-65	81-97	113-129	145-161	177-193	209-225	241-257	273-289	305-321
	25-41	57-73	89-105	121-137	153-169	185-201	217-233	249-265	281-297	313-329
Peptide pool no.	XI	XII	XIII	XIV						
Peptide	321-337	353-369	385-401	417-433						
	329-345	361-377	393-409	425-441						
	337-353	369-385	401-417							
	345-361	377-393	409-425							

Table 2. Identification of peptide pools using lymphoproliferative [H^3] incorporation assay.

protein name	peptide pool no.	overlapping aa sequence in a peptide pool	no. of resp. pigs for each peptide pool
NSP9	IV	NTGIDGTLWDFEAEAT**KEEIALSAQIIQACDIR**RGDAPEIG	3\8
	V	RRGDAPEIGLPYKLYP**VRGNPERVKGVLQNTRF**GDIPYKT P	1\8
	VII	RSVLATTM**PSGFELYVPTIPASVLDYLD**SRPDCPKQLTEH G	1\8
	XVII	**ESPTMPNYHWWVEHLNL**MLGFQTDPKKTAITDSPSFLGC RI	1\8
NSP10	V	CKEINMVAVASNVLRSRFIIGPPGAGKTYWLLQQVQDGDV I	4\4
	VII	AGTTLQFPAPSRTGPW**VRILAGGWCPGKNSFLDEAAYC NHL**	3\4

Notes: Pigs in the principal group were inoculated with PRRSV FL-12 and blood samples were collected starting at 7 d.p.i. The results shown here is the average of the PBMCs collected on 14 d.p.i., 21 d.p.i. and 28 d.p.i. This table shows the nine amino acid overlapping sequence length of peptide pools identified from PRRSV NSP9 and 10 at their respective amino acid positions after the first round of T-cell epitope mapping. The number of responding pigs is the immunized animals whose PBMC exhibited a peptide-specific proliferation with a stimulation index ≥ 2 along with a zero background response. The response to the peptides had to be ≥2 than the proliferated cells of non-immunized control pigs to be considered positive. None of the PBMCs samples isolated from the control pigs responded to the peptides pools herein shown.

Table 3. Identification of heptadecamer PRRSV peptides likely to contain of NSP9 and NSP10 epitopes.

protein name	peptide name	peptide sequence	no. of resp. pigs for each individual peptide
NSP9	119-135	KEEIALSAQIIQACDIR	4\8
	151-167	VRGNPERVKGVLQNTRF	2\8
	207-223	PSGFELYVPTIPASVLD	1\8
	519-535	ESPTMPNYHWWVEHLNL	2\8
NSP10	209-225	VRILAGGWCPGKNSFLD	2\4
	217-233	CPGKNSFLDEAAYCNHL	2\4

Notes: Pigs in the principal group were inoculated with PRRSV FL-12 and blood samples were collected 7 days post first booster dose (boosters were applied at 21 d.p.i for NSP9 study and at 57 d.p.i for NSP10 study). This table shows the heptadecamer sequence length of the individual peptides identified from PRRSV NSP9 and 10 at their respective amino acid positions after the second round of T-cell epitope mapping. The number of responding pigs is the immunized animals whose PBMC exhibited a peptide-specific proliferation with a stimulation index ≥ 2 along with a zero background response. The response to the peptides had to be ≥2 than the proliferated cells of non-immunized pigs to be considered positive. None of the PBMCs samples isolated from the non-immunized control pigs responded to any of these individual peptides.

Table 4. T-cell epitopes in PRRSV NSP9 and NSP10 identified by the IFN-γ induction criteria.

Protein name	aa position	peptide sequence	[a]max. resp	[b]tot. resp	[c]avg. resp	[d]no. resp. pigs	[e]avg. resp pigs
NSP9	**119-135**	**KEEIALSAQIIQACDIR**	**8**	**10.6**	**1.7**	**2\8**	**12.8**
NSP9	**151-167**	**VRGNPERVKGVLQNTRF**	**8. 6**	**5.3**	**0.8**	**2\8**	**8.5**
NSP9	207-223	PSGFELYVPTIPASVLD	3.7	9	1.5	1\8	0.7
NSP9	519-535	ESPTMPNYHWWVEHLNL	2.7	3	0.5	2\8	4.7
NSP10	**209-225**	**VRILAGGWCPGKNSFLD**	**7.3**	**4.3**	**1.0**	**1\4**	**14**
NSP10	**217-233**	**CPGKNSFLDEAAYCNHL**	**27.3**	**33.9**	**8.4**	**2\4**	**21.2**

Notes: Pigs were initially infected with PRRSV FL-12 and subsequently were administered two additional boosters (at 21 dpi and 96 d pi for the NSP9 experiment, and at 57 and 114 dpi for the NSP10 experiment. The ELIspot results shown here is the compilation of data obtained from the interferon response from the PBMCs cryopreserved at different time points post first and second booster doses. The ELIspot experiments for each protein were performed independently.

[a]Max.resp: number of IFN-producing cells detected in PBMC from the highest responder pig among all 2 PBMC samples tested.
[b]Tot.resp: sum of all of the IFN-producing cells (minus background) detected in the 2 individually tested PBMC samples.
[c]Avg. resp: sum of all the IFN-producing cells (minus background) detected in the 2 PBMC samples tested divided by 2, the number of pigs tested.
[d]No. Resp. pigs: number of pigs exhibiting a frequency of peptide-specific IFN-γ secreting cells with a stimulation index ≥ 2 than the cells only. If background response was zero the response to the peptide had to be ≥2 to be considered positive.
[e]Avg Resp pigs: average of the peptide-specific IFN-specific IFN-response (minus background) of all the pigs exhibiting a response to the individual peptide.
[f]Mean±Std.D: mean ± standard deviation of the values shown for each of the four T-cell peptide candidate for each of the selection criteria were concluded to contain an immunodominant peptide (Vashisht et al., 2008).

Peptide sequences in bold represent the positive peptides which showed maximum IFN-γ secretion. Controls used in this assay were: concanavalin A (positive control), FL-12 infected cell homogenates (positive control) and PBMCs only, without peptide (negative control). None of these peptides reacted with the PBMCs of the non-immunized control pigs (mean ± std.dev. = 0±3).

Table 5. Conserved T-cell epitopes in NSP9 and NSP10.

Sequence	Count	*Proportion
NSP9 (119-135)		
KEEVALSAQIIQACDIR	28	0.43
KEEIALSAQIIQACDIR	14	0.22
KEEIALSAQIIQACGIR	8	0.12
KEEIALSAQIIQACDMR	5	0.08
KEEVALSAQIIQACDMR	2	0.03
KEEVALSAQIIQACGIR	2	0.03
KEEIELSAQIIQACGIR	2	0.03
KEEIALSTQIIQACDIR	1	0.02
KEEIALSAQIIQACSIR	1	0.02
KEEIALSEQIIQACDIR	1	0.02
KEEVALSTQIIQACDIR	1	0.02
NSP9 (151-167)		
VRGNPERVKGVLQNTRF	55	0.85
IRGNPERVKGVLRNTRF	1	0.02
VRDNPERVKGVLKNTRF	1	0.02
VRGDPERVKGVLKNTRF	1	0.02
VRGNPERARGVLMNTRF	1	0.02
VRGNPERVNGVLQNTRF	2	0.03
VRGNPERVKGVLRNTRF	3	0.05
VRGNPERVKGVLKNTRF	1	0.02
NSP10 (209-225)		
VRILAGGWCPGKNSFLD	62	0.95
VRILAGGWCPGRNSFLD	2	0.03
VRILAGRWCPGKNSFLD	1	0.02
NSP10 (217-233)		
CPGKNSFLDEAAYCNHL	63	0.97
CPGRNSFLDEAAYCNHL	2	0.03

Notes: Sequences in bold define the T-cell epitopes obtained in our study after stringent categorization of the heptadecamer peptides present in NSP9 and NSP10. Other heptadecamer sequences were considered from various other PRRSV isolates among the 65 sequences aligned. The count number denotes the number of identical sequences out of 65 sequences aligned using JALVIEW. *Proportion of the sequence represents the conservation of those T-cell epitopes by applying the following formula:

no. of count of similar sequences/total no. of sequences used for multiple alignment.