RESEARCHS / INVESTIGACIÓN

Preliminary study for the detection of pig specific antibodies against peptides obtained from PRRSV envelope proteins using Dot blot assay.

Estudio preliminar para la detección de anticuerpos específicos en cerdos contra péptidos obtenidos a partir de proteínas de envoltura del virus PRRS mediante un ensayo de Dot blot.

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ABSTRACT

Porcine Respiratory and Reproductive Syndrome Virus (PRRSV) is found worldwide being responsible for one the largest economic losses in the swine industry. Currently, different immunological assays are being used to detect specific anti-PRRSV antibodies, however they have some deficiencies. As an example of this is the ELISA test that usually does not work well with small molecules because it generally absorbs peptides larger than 15-20 residues in length. This study was conducted for the detection of specific antibodies in field pig sera against PRRSV using small synthetic peptides by Dot Blot assay. Those peptides were obtained from the envelope protein GP5 of PRRSV type 1 and type 2. Each sera sample was diluted using a range from 0.000625 to 0.005. The level of concordance between the results obtained by Dot-blot and commercial ELISA for pig categories oscillated from discrete to moderate against peptides. Sows showed higher antibody titers than younger pigs towards all peptides, being more noticeable against GP5 P1.

It was demonstrated that Dot-blot assay is a valuable technique for detecting specific antibodies against small synthetic peptides.

Keywords: PRRSV, pigs, Dot-blot, antibodies, synthetic peptides, envelope proteins.

RESUMEN

El Síndrome reproductivo respiratorio porcino conocido como PRRSV, posee una distribución mundial y genera una de las mayores pérdidas económicas dentro de la industria porcina. Actualmente diferentes ensayos inmunológicos se utilizan para la detección de anticuerpos específicos contra el virus de PRRS, sin embargo, estos presentas diversas deficiencias. Un ejemplo, es el test de ELISA, en el cual moléculas pequeñas no se adhieren correctamente a la placa, debido a que esta última absorbe péptidos que posean entre 15-20 residuos de aminoácidos. Este estudio fue realizado con el objetivo de detectar anticuerpos específicos en cerdos contra péptidos de pequeño tamaño, mediante un ensayo de Dot blot. Estos péptidos fueron sintetizados en base a la proteína GP5 del virus PRRS tipo 1 y 2. Los sueros fueron diluidos en un rango desde 0.000625 hasta 0.005. El nivel de concordancia entre los resultados obtenidos mediante Dot blot y ELISA comercial por cada categoría animal fue de discreto a moderado contra los péptidos. Las cerdas mostraron mayores títulos de anticuerpos hacia todos los péptidos, siendo esto más marcado para el péptido GP5 P1. Se demostró que el ensayo de Dot blot es una valiosa técnica, la cual permite identificar anticuerpos específicos en cerdos, utilizando péptidos de pequeño tamaño.

Palabras clave: PRRSV, cerdos, Dot-blot, anticuerpos, péptidos sintéticos, proteínas de envoltura.

Introduction

The reproductive and respiratory syndrome virus (PRRSV) is caused by a RNA virus which affects pigs of all ages and is responsible for one of the largest economic losses in the swine industry in North America.[1] The structural organization of PRRSV is based on a Nucleocapsid (N) protein, membrane protein (M), glycosylated envelope proteins (GP2a to GP5), E protein and the ORF5a protein.[2] GP5 is the most variable protein of the virus and is responsible for the lack of immunological cross-reaction between North American (NA) and European (EU) strains. PRRSV structural proteins play an important role for inducing an early antibody response in pigs during day 5 to 10 post infection (PI). This response is mainly directed against N protein but also toward envelope protein.[3] The first antibodies detected during PRRSV infections are IgM around day 5-7 PI and different subclass of IgGs are detected in serum around day 7-14 PI.[4] Studies have demonstrated that virus-neutralizing antibodies (NAbs) may inhibit the replication of virus in alveolar macrophages and high titers of these antibodies in serum can clear the virus from the blood.[5] The main target for NAbs is GP5 in which it has been discovered the major neutralizing epitope (NE) called Epitope B founded in both PRRSV strains. This fact leads the synthesis of small peptides based on Epítope B, in order to promote the production of NAbs by B cells.[6]

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Currently, several immunological assays are being used to detect specific anti-PRRSV antibodies. Indirect fluorescent antibody test (IFA) and Enzyme-linked immunosorbent assay (ELISA) are examples of the most used in North America. Nevertheless, all those assays show disadvantages, for example IFA presents unknown sensitivity for individual animals, it is not automated; thus, it is difficult to perform on a large number of samples. On the other hand, even though ELISA has high specificity and good sensitivity it has been reported individual unexpected positive results in seronegative herds which elicit an unacceptable level of false-positives cases in commercial ELISAs.[7] In addition, ELISA in general has low binding for small molecules since generally absorbs peptides larger than 15-20 residues.[8]

Dot-blot assay represents a good alternative for this preliminary study to detect specific antibodies in field pig sera against small synthetic peptides that represent linear epitopes from protein GP5. Dot-blot is a sensitive, specific and easy technique to perform and as employs a nitrocellulose membrane with small pore size to immobilize antigens, it is not needed of carrier larger proteins for high binding of small peptides.[9]

In this work, it was identified specific antibodies against small peptides that were synthetized based on the sequence of epitope B of protein GP5 from North American and European strain of PRRSV using a Dot blot assay. The antibody response from different pig categories were analyzed toward each peptide and those results were compared to commercial ELISA.

Materials and Methods

Synthetic peptides

Peptides from GP5 were synthesized based on published sequences for the genes encoding PRRSV structural glycoproteins (Table 1). These sequences were collected into a database (Geneious Pro 5.5, Biomatters, New Zealand), homology was determined on a nucleotide and amino acid level, and conserved sites in the PRRSV structural glycoproteins were selected. Their exposure to the immune system was predicted in silico by the 3D protein structure (HHPred, Max-Planck-Institute for Developmental Biology, Tübingen, Germany). Each peptide sequence (9 to 10-mer peptides) representing potentially immunogenic epitopes were chosen and were commercially synthesized (CanPeptide, Pointe-Claire, QC, Canada).

Table 1. Summary of synthetic peptides. NA: North American strain EU: European strain.

	Sequence	Position (aa)	Strain	Molecular Weight (KDa)	Charge at pH 7	Water Solubility	Company
GP5 P1	MRCSHKLGRF	1-10	EU	1234.52	3	Good	Peptide 2.0
GP5 P3	HKLGRFLTP	5-14	EU	1068.29	2.1	Good	Peptide 2.0
GP5 P12	ELNGTDWLKD	49-61	NA	1580.7	-2	Good	Peptide 2.0
GP5 P29	FLLDTKGRLY	144-153	NA	1225.46	1	Good	Peptide 2.0

Pig serum samples

Twenty-eight pig serum samples with known PRRS-serostatus with a commercial ELISA (IDEXX, Westbrook, ME) belonging to 4 pig farms from the province of Alberta, Canada were screened against the peptides using a Dot-blot assay (Table 2). The sera came from three different pig categories: 20 from sows (6 PRRS-positive and 4 negative), 7 grower pigs (5 PRRS-positive and 2 negative) and 9 finisher pigs (6 PRRS-positive and 3 negative). A positive-PRRSV grower pig was used as positive control and serum sample from a newborn piglet belonging to negative farm was selected as the negative control.

Swine sera screening against peptides through Dot blot assay

Protran BA79 Nitrocellulose Blotting Membrane 0.1µm (GE Healthcare, Pittsburgh, PA) assembled in Bio-Dot Microfiltration System (Bio-Rad, Hercules, CA) was coated with 100 ng/well of peptides and incubated at 4°C overnight. Membrane was blocked adding bovine albumin serum (BSA) 1% in TBS buffer for 1 hour (h) at room temperature (RT). Swine sera from all pig categories were diluted in Log2 serial dilutions from 1:200 to1:1600 and pre-absorb in BSA 5% during 1 h at RT. Consequently, wells were incubated with 100ul of the pig sera at 37°C during 1 h. Rabbit anti-pig IgG (Sigma-Aldrich, St Louis. MO 63103 USA) was diluted 1:4000 in PBS buffer and 100 ul/well of the conjugated antibody was added to the membrane and incubated at 37°C for 1 h. ECLTM was added for color development with an incubation time of 5-10 minutes. Color intensity was measured using VersaDocTM MP 4000Imaging System, (Bio-Rad, Hercules, CA), and the analysis was performed using software Quantity One®.

Data analysis

Data obtained from software Quantity One® was analyzed to calculate the cut-off value for the samples based on the negative control. It was established that samples with intensity values (intensity*mm2) 3.5 times greater or equal than negative control were considered positive. On the other hand, concordance level between Dot blot and commercial ELISA was determined by calculating Kappa index, through the program VassarStats.

Comparison between commercial ELISA and Dot Blot results by animal category against GP5 P1, GP5 P3, GP5 P12 and GP5 P29

The results showed that sows category exhibited a higher level of concordance (kappa index between 0.55 to 0.60) with commercial ELISA results, in comparison to the other two pig categories which obtained a level of concordance between discrete to moderate. Finisher pigs showed the lowest level of concordance with a Kappa index of 0.25 (Table 3).

On the other hand, there was not a significant difference between the level of concordance in sows per peptide. In grower pigs the lowest concordance was observed for peptide GP5 P3 and finisher pigs exhibited the same level of concordance for all peptides (Table 3).

Table 2. Individual serum samples organized based on pig category (sows, grower and finisher pigs) and antibody PRRSV status (positive and negative).

Animal ID	Pig Category	Commercial ELISA Results
A3	Sow	Positive
C2	Sow	Positive
A2	Sow	Positive
A1	Sow	Positive
B1	Sow	Positive
C1	Sow	Positive
A4	Sow	Negative
B2	Sow	Negative
D1	Sow	Negative
C3	Sow	Negative
B4	Grower pig	Positive
A6	Grower pig	Positive
A5	Grower pig	Positive
A7	Grower pig	Positive
B3	Grower pig	Positive
D3	Grower pig	Negative
D2	Grower pig	Negative
C5	Finisher pig	Positive
B5	Finisher pig	Positive
A8	Finisher pig	Positive
B7	Finisher pig	Positive
B6	Finisher pig	Positive
A9	Finisher pig	Positive
D5	Finisher pig	Negative
D6	Finisher pig	Negative
C4	Finisher pig	Negative
Positive Control	Grower pig	Positive
Negative Control	Newborn piglet	Negative

The difference between the results obtained in this study and the commercial ELISA can be attributed to the different cut off point used in each assay. Cases of false-negative and even the amount of antibodies present in the sample it can also be involved in those results. Some studies have been reported differences between results obtained with one or other assay even using the same antigen for both of them. This could be attributed to the fact that each assay differs in the protein tested (N protein in ELISA and GP5 in Dot-blot) thus, specific antibodies against GP5 P1, GP5 P3, GP5 P12 and GP5 P29 may have been detected by Dot-blot assay in those samples that resulted to be positive in this study but originally were negative by the commercial ELISA. Differences between the sensitivity and specificity in each assay, it can also be associated to those results.[9,10]

Results and Discussion

Comparison between positive pig's categories toward peptides

The results for GP5 P1described that sows showed higher antibody titers than grower and finisher pigs because it was the only pig category from which 50% of the samples reacted positively to the peptide from the lowest concentration of serum tested (0.000625). Also, it was observed that only against GP5 P1, the percentage of positive sows decreased when swine sera were more concentrated therefore may be the detection capacity of the Dot-blot assay reaches a point from where the system is saturated because of a high concentration of GP5 P1 or the high concentration of sera (Figure 1a).

In contrast, the results for GP5 P3 showed that not only sows reacted positively from concentrations of serum of 0.000625, but also grower and finisher pigs were positive against the peptide. These group of pigs also exhibited higher antibody titers as well as the sows. Nevertheless, the percentage of sows that were positive from that concentration is still higher than the younger pigs (Figure 1b).

Table 3. Level of concordance between commercial ELISA results and Dot-blot results per animal category against GP5 P1, GP5 P3, GP5 P12 and GP5 P29. + (Positive); - (Negative); * (positive and negative concordant samples in both tests). Kappa: < 0 (No concordance); > 0.00 - 0.20 (Insignificant); 0.21 - 0.40 (Discrete); > 0.41 - 0.60 (Moderate); 0.61 - 0.80 (Substantial); 0.81 - 1.00 (Almost perfect).

	ELISA Results	Dot-blot Results								
		Sows			Grower pigs			Finisher pigs		
		+	-	Total	+	-	Totall	+	-	Totall
GP5 P1	+	5*	1	6	5*	0	5	5*	1	6
	-	1	3*	4	1	1*	2	2	1*	3
	Total	6	4	10	6	1	7	7	2	9
	Kappa		0.58			0.59			0.25	
GP5 P3	+	6*	0	6	4*	1	5	5*	1	6
	-	2	2*	4	1	1*	2	2	1*	3
	Total	8	2	10	5	2	7	7	2	9
	Kappa		0.55			0.3			0.25	
GP5 P12	+	5*	1	6	5*	0	5	5*	1	6
	-	1	3*	4	1	1*	2	2	1*	3
	Total	6	4	10	6	1	7	7	2	9
	Kappa		0.58			0.59			0.25	
GP5 P29	+	5*	0	5	5*	0	5	5*	1	6
	-	2	3*	5	1	1*	2	2	1*	3
	Total	7	3	10	6	1	7	7	2	9
	Kappa		0.6			0.59			0.25	

In GP5 P12 it was found that 50% of positive sows exhibited higher antibody titers than grower and finisher pigs. Half of the grower pigs (50%) and most finisher pigs (57.14%) were positive to that peptide from higher concentrations. In addition, there was none grower and finisher pig that reacted positively from concentration of serum of 0.000625 to this peptide (Figure 1c).

Finally, results for GP5 P29 showed that sows exhibited higher antibody titers than the other pig categories. Forty percent of positive sows reacted positively against GP5 P29 from concentration of serum of 0.000625 and a small percentage of grower pigs were positive from the same concentration (Figure 1d).

In general, at least for all peptides it was found a positive sow from the lowest concentration of serum tested (0.000625). Sows exhibited higher antibody titers than younger pigs toward all peptides.

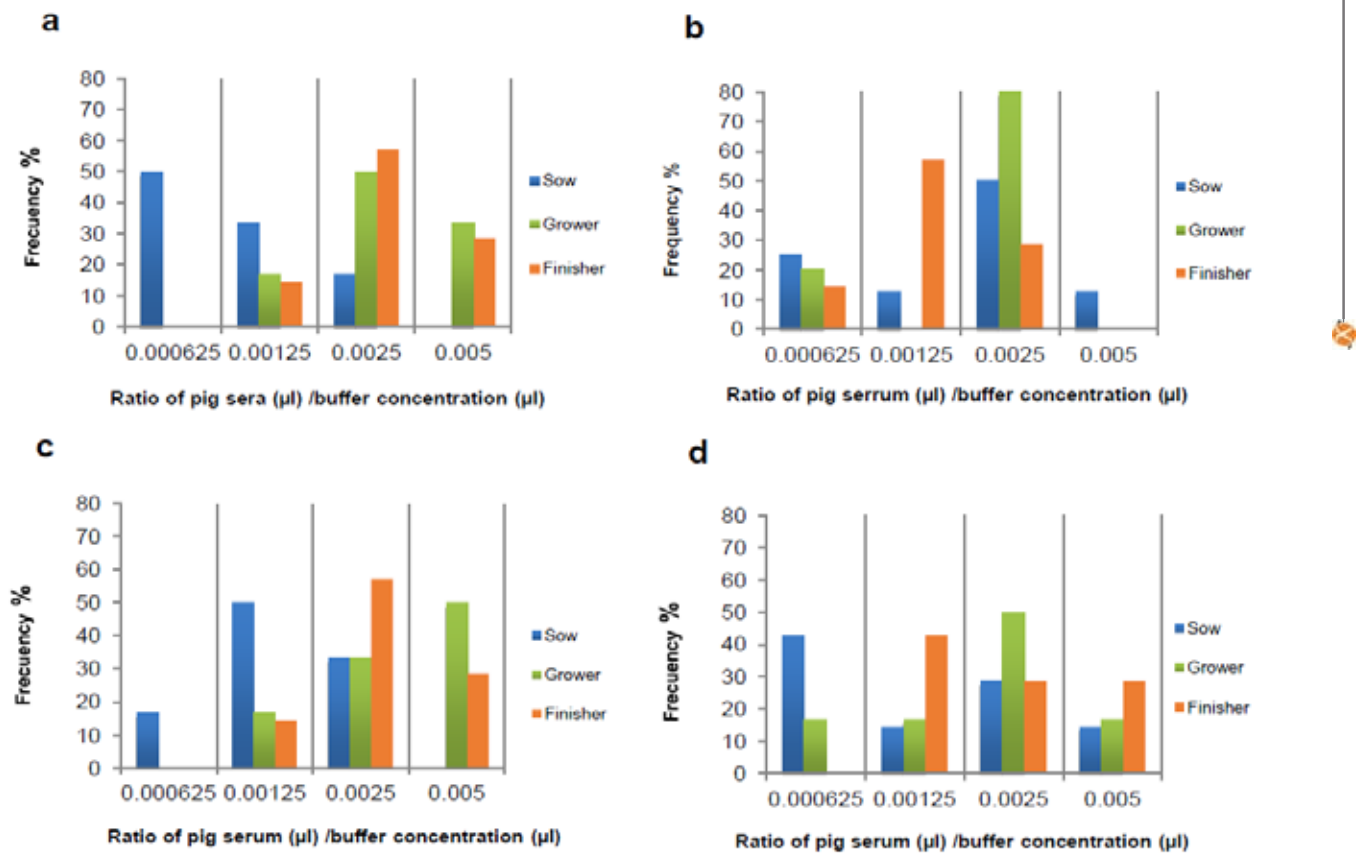

Figure 1. Percentage of positive pigs to the lowest ratio of pig sera/buffer concentration from different age groups tested against GP5 P1 (a), GP5 P3 (b), GP5 P12 (c) and GP5 P29 (d).

These results suggested that age of pigs may be involved in the amount of antibodies contained in serum. This could be explained because sows had a longer productive life than grower and finisher pigs, thus they have been exposed to different types of pathogens during their lives. Older animals have a more developed immune system capable of elicit a better response than young animals against pathogens.[11]

A study performed by Klinge et al. 2009 [12] where it was assessed whether age of pigs is involve or not in the resistance to PRRSV, suggested that older pigs can mount a faster and better immune response against the virus. Those animals showed higher antibody titers than younger ones

On the other hand, the results in this work also showed that from concentrations of serum of 0.0025, all pig categories (over 20% of the samples) reacted positively to all peptides which can suggest that this concentration could be the optimal to use in future screening using those pig categories. This can be supported by previous studies performed in ELISAs where have been tested concentrations of problem sera of 0.0025 obtaining good results.[13,14]

The level of concordance between Dot blot and commercial ELISA results for sows was higher than those obtained in the other pig's categories. Furthermore, specific antibodies against the peptides GP5 P1, GP5 P3, GP5 P12 and GP5 P29 were detected using Dot-blot assay, nevertheless those results require also be compared with other assays. Finally, as sows needed less concentration of serum to be positive toward all peptides, it may suggest that they presented higher antibody titers than younger pigs. However, due to the small number of samples used in this study it will be considered to assay a higher n in a future work.

Conclusions

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