Establishment of ELISpot technique for detection of cellular immunity against PRRSV

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Abstract The porcine reproductive and respiratory syndrome, has a high mortality and morbidity. The vaccination of PRRS is still an important means to control the epidemic disease. The purpose of this paper is to set up an effective method to evaluate cellular immunity against PRRSV. Because the GP5 protein of PRRSV is the largest structure protein and the M protein is the most conserved protein in PRRSV, we selected GP5 protein and M protein to be screened for finding the effective epitope in stimulating T cell response against PRRSV infection. The epitope of GP5 protein and M protein was predicted by using Net-MHC4 server polypeptide MHC I molecules with Artificial Neural Network (ANN). The ELISpot technology was established to detect IFN-γ secreting T lymphocyte, which provides an effective method for evaluation of the cellular immunity in PRRS. The results showed that the polypeptide pool, prepared by mixing multiple polypeptides from GP5 and M protein of PRRSV, was better than any single polypeptide in stimulating T lymphocyte to produce IFN-γ, and the total IFN-γ level in the supernatant of cell culture detected by ELISA was consistent with the result of ELISpot. The ELISpot technique to detect the T cell-mediated immunity of PRRS will pave the way to understand the pathogenesis of PRRS and evaluate the cellular immunity against PRRSV after PRRS vaccination.

1. Introduction
Porcine reproductive and respiratory syndrome (PRRS) continues to be a threat for the pig industry, causing a huge amount of direct loss in the USA [1,2] It leads to abortion of pregnant sows and respiratory disorders in pigs of all ages with high morbidity and mortality [3,4]. PRRSV is a linear, single, enveloped positive-stranded RNA virus, and it is similar to the equine arteritis virus [5]. PRRS viruses (PRRSV) can be divided into two major genotypes, Type 1 and Type 2 [6,7]. In 2006, a highly pathogenic PRRSV infection outbroke in China with high fever, mortality and
morbidity [8,9], which caused extremely serious economic losses to the pig industry in China.

PRRSV membrane proteins assemble into heterotetramers (GP2a-EGP3-GP4, minor proteins) and heterodimers (GP5-M, major proteins) [10]. The effective polypeptide epitopes of GP5 (glycoprotein 5) and M proteins initiate immune response to GP5 and M proteins. The same PRRS subunit vaccine also triggers memory cell-mediated immune responses following the challenge of classical PRRSV [11]. The GP5 expression of HP-PRRSV by orally recombinant Kluyveromyces lactis-expressing induced PRRSV-specific cell-mediated immune responses in mice [12]. The Interferon gamma (IFN-γ) plays a important role in immune response [13]. Therefore, this study aims to establish an enzyme-linked immuno-spot assay (ELISpot) technique to detect PRRSV-specific IFN-γ levels, for evaluating cellular immunity against PRRSV.

2. Materials and methods

2.1 Cell isolation and culture

The peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood samples by Ficoll-Hypaque lymphocyte separation medium, washed twice by RPMI 1640 (Gibco, USA) and resuspended in RPMI 1640 with 10% FCS (Gibco, USA) at 37°C in 5% CO2 for 18-24 h.

2.2 Designing 9-mer peptide from PRRSV

We selected the Class I MHC supertype representative alleles (SLA-10401, SLA-20401 and SLA-30401) for the prediction of specific T-cell epitopes of 9 mer length as available in the NetMHCpan 4.0 online prediction server program (http://www.cbs.dtu.dk/services/NetMHCpan/). Seven peptides were chosen for synthesis, for they have the highest predicted binding affinity (Table 1,2).

Table 1. The peptides of the GP5 protein of PRRSV with the highest predicted binding affinity with SLA-10401, SLA-20401 and SLA-30401

| Pos | Peptide     | ID    | nM   | Rank | nM   | Rank | nM   | Rank | %Ranks | Binder level |
|-----|-------------|-------|------|------|------|------|------|------|--------|--------------|
| 93  | ATVSTAGYY   | Seq   | 113.3| 0.09 | 559.8| 0.01 | 41414.4| 26.00| 0.027  | SB           |
| 11  | SRLLEFLWCI  | Seq   | 39138.1| 32.00| 36845.9| 27.00| 1100.7| 0.07 | 0.209  | SB           |
| 34  | NSSHIQLIY  | Seq   | 6171.1| 2.50 | 1642.9| 0.08 | 14649.9| 1.60 | 0.222  | SB           |
| 144 | LLDTKGRGLY | Seq   | 129.0| 0.10 | 23321.3| 6.50 | 45967.4| 60.00| 0.295  | SB           |

Table 2. The peptides of the M protein of PRRSV with the highest predicted binding affinity with SLA-10401, SLA-20401 and SLA-30401

| Pos | Peptide     | ID    | nM   | Rank | nM   | Rank | nM   | Rank | %Ranks | Binder level |
|-----|-------------|-------|------|------|------|------|------|------|--------|--------------|
| 50  | LNCAFTFGY   | Seq   | 2802.0| 1.2  | 1060.4| 0.04 | 5312.2| 0.4  | 0.106  | SB           |
| 23  | ITYTPVMIY  | Seq   | 1949.4| 0.9  | 1513.1| 0.07 | 18263.3| 2.5  | 0.190  | SB           |
| 17  | VLLAFSITY  | Seq   | 1071.6| 0.6  | 3512.8| 0.25 | 22805.6| 4.0  | 0.507  | WB           |

Note: The strong binders (SB) have an IC50 less than 50 nM. The weak binders have an IC50 more than 50 nM and less than 500 nM.
2.3 IFN-γ ELISpot assay

A porcine IFN-γ ELISpot Kit (MABTECH USA) were used for ELISpot assay. Briefly, ELISpot wells were washed 4 times with sterile PBS (200μl/well). Condition the plate with medium (200μl/well) containing 10% of the same serum as used for the cell suspensions. Incubate for at least 30 minutes at room temperature. PBMCs were seeded into 96-well plates (2×10^5 cells/well). PBMCs were pretreated with either a single peptide of GP5(A), GP5(B), GP5(C), GP5(D), M(A), M(B), M(C) or GP5 peptide pool, M peptide pool, with the end concentration of each peptide for 10 μg/ml. 24h later, removed the cells by emptying the plate and wash 5 times with PBS, leaved the plate to dry and counted spots in an ELISpot reader (AID, Germany). The results were expressed as spot-forming units. The ELISpot analysis was done triplicated for each peptide. The mean number of spots was adjusted to 1×10^6 PBMC.

2.4 IFN-γ measurement by ELISA assay

The IFN-γ in the culture supernatants of PBMC that treated by different group peptide were quantified using the porcine IFN-γ ELISA (R&D Systems, USA). The optical density A450nm of each well was determined by using Multi-function enzyme marker (Bio Tek, USA).

2.5 Statistical analyses

One-way ANOVA analysis were used for comparing the mean difference among groups by Prism 7.0 (GraphPad Software Inc., USA). Differences were considered significant at p < 0.05.

3. Results

3.1 ELISpot

The CTL epitopes can induce secretion of IFN-γ by specific PBMCs, which can be detected by ELISpot, as described [14]. We isolated PBMCs from different 9-mer control peptide and the pool of peptides by ELISpot. As shown in Figure 1 and Figure 2, the mixed peptide pool stimulated the T cell response better than the single polypeptide in GP5 and M proteins.

![Figure 1](image1.png)

*Figure 1.* The frequency of cells secreting IFN-γ in response to different peptide from GP5 protein detected in the PBMC.

![Figure 2](image2.png)

*Figure 2.* The frequency of cells secreting IFN-γ in response to different peptide from M protein detected in the PBMC.

3.2 ELISA

The IFN-γ level in cellular supernatant was detected by ELISA assay. The results are shown in Figure 3 and 4, We found that the mixed peptide pool stimulated the T cell response better than any of the...
single polypeptide of GP5 and M proteins. These results were consistent with the ELISpot results.

![Figure 3](image1.png) **Figure 3.** IFN-γ secreted in the supernatant of PBMCs induced by different peptides from GP5 protein.

![Figure 4](image2.png) **Figure 4.** IFN-γ secreted in the supernatant of PBMCs induced by different peptides from M protein.

### 4. Discussion

ELISpot is a cytokine detection technology developed in recent years. It combines cell culture technology with ELISA to analyze the frequency of single effector cells secreting cytokine (IFN-γ) after activation by specific antigens. One spot reflects one single cell to secret IFN-γ. ELISpot assay has advantages over the standard chromium-release method because it is more sensitive, and can measure the number of antigen-specific cells [15]. And it is simpler, more time saving, less labor-intensive, and the results are available within 36-48h [16,17]. In recent years, ELISpot technology has been widely used in the detection of vaccines and drugs, autoimmune diseases, allergic diseases, infectious diseases and development of vaccine. Furthermore, it can also be applied to screen for high-risk populations of tuberculosis [18,19]. At present, IFN-γ ELISpot assay is one of the main techniques in immunological research [20]. The ELISpot technique for detecting the level of IFN-γ was established to detect the cellular immune function against PRRSV, which provided a new means for the study of the pathogenesis of PRRS and the evaluation of immune effect after vaccination.

Among the structural proteins of PRRSV, the GP5 and M proteins play an important role in the formation of virions. GP5 can interact with the sialic adhesion, which is a prerequisite to enter the target cells. GP5 and M protein is also usually used for phylogenetic analyses [21]. The PRRSV GP5 and M proteins can form a heterodimeric complex which is important in infectivity of the virus[22]. The result showed that the epitopes of GP5 and M protein in the mixed peptide pool was better than the single peptide in stimulated the T cell response (Figure 1-4). We assumed that the peptides in the mixed peptide pool got cumulative effect in stimulating T lymphocyte. Additionally, the frequency of IFN-γ secreting cell by ELISpot assay was consistent with the level of IFN-γ in the supernatant of cell culture by ELISA technique.

### 5. Conclusion

The polypeptide pool, prepared by mixing multiple polypeptides from GP5 or M protein of PRRSV, was better than any single polypeptide in stimulating T lymphocyte to produce IFN-γ, and the total IFN-γ level in the supernatant of cell culture detected by ELISA was consistent with the result of
ELISpot. The ELISpot technique to detect the frequency of the specific IFN-\(\gamma\) secreting cell in the T cell-mediated immunity of PRRS will provide a way to better understand the pathogenesis of PRRS and evaluate the cellular immunity against PRRSV after PRRS vaccination.

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