RESEARCH REPORT
SWINE HEALTH

Title: Development of a rapid, swine-specific test to simultaneously detect multiple immune proteins (cytokines) affected by PRRSV infection - **NPB#08-189**

Investigator: Jane Christopher-Hennings DVM, MS

Institution: South Dakota State University

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Industry Summary: Cytokines are small secreted proteins which mediate and regulate immunity to viral infections in swine and other species. Evidence suggests that PRRSV does not induce early, effective cytokine responses in the pig leading to clinical disease and viral persistence. The immune response to PRRSV occurs late in infection and is weak in comparison to the response to other swine viruses. In designing second generation PRRSV vaccines, it would be very useful to have rapid, standardized assays available to measure whether these vaccines, or viral proteins proposed to be used in the vaccines, are inducing more rapid and protective cytokine responses. The assay should also prove useful for measuring cytokines that could determine genetic resistance to PRRSV and immune responses to other swine pathogens. The objective of this study was to develop an assay to simultaneously quantify 8 porcine cytokines in serum using Luminex xMap™ technology. A multiplex assay was optimized to detect innate inflammatory (IL-1 β, IL-8, IFN-α, TNF-α, IL-12); regulatory (IL-10), Th1 (IFN-γ) and Th2 (IL-4) cytokines. The assay was optimized, compared to individual cytokine tests and used to measure cytokines in a PRRSV vaccine study. The study showed high levels of IL-12 in pigs vaccinated and challenged with PRRSV, but this high level did not correlate with protection. This study is the first describing the development of a well validated 8-plex fluorescent microsphere immunoassay (FMIA) for the detection of swine cytokines in serum.

Keywords: cytokine, multiplex, PRRSV, FMIA, ELISA

Scientific Abstract: A Luminex (Luminex Corp., Austin, TX) multiplex swine cytokine assay was developed to measure 8 cytokines simultaneously in pig serum for use in assessment of vaccine candidates. The fluorescent microsphere immunoassay (FMIA) was tested on archived sera in a porcine reproductive and respiratory syndrome virus (PRRSV) vaccine/challenge study. This FMIA simultaneously detects innate (IL-1β, IL-8, IFN-α, TNF-α, IL-12), regulatory (IL-10), Th1 (IFN-γ) and Th2 (IL-4) cytokines. These proteins were measured to evaluate serum cytokine levels associated with vaccination strategies that provided for different levels of protective immunity against PRRSV. Pigs were vaccinated with a modified-live virus (MLV) vaccine and subsequently challenged with a

non-identical PRRSV isolate (93% identity in the glycoprotein (GP) 5 gene). Protection (as defined by no serum viremia) was observed in the MLV vaccinated pigs after PRRSV challenge but not those vaccinated with killed virus vaccine with adjuvant (KV/ADJ) (99% identity in the GP5 gene to the challenge strain) or non-vaccinates. Significantly elevated levels of IL-12 were observed in the KV/ADJ group compared to MLV vaccinated and control groups. However, this significant increase in serum IL-12 did not correlate with protection against PRRSV viremia. Additional studies using this assay to measure the local cytokine tissue responses may help in defining a protective cytokine response and would be useful for the targeted design of efficacious vaccines, not only for PRRSV, but also for other swine pathogens.

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV is found in pigs worldwide since it emerged in the late 1980s in Europe and the United States. It has since caused significant economic problems including more recently, severe outbreaks in China resulting in 2 million affected pigs with about 400 thousand fatalities. The development of effective vaccines that are broadly protective against non-identical PRRSV strains has been difficult and correlates of immune protection still remain elusive. Minimal innate cytokine production and low levels of PRRSV specific IFN-γ producing T cells are described after PRRSV infection. The development of neutralizing antibodies from vaccine administration has been reported to coincide with protection from equine arteritis virus (EAV) (e.g., another *Arterivirus).* However, for PRRSV, one study described an interesting paradoxical finding that killed vaccine vigorously induced PRRSV neutralizing antibodies upon challenge although no reduction in viremia was noted. This study also described a spontaneous production of IFN-γ secreting cells in pig peripheral blood mononuclear cells (PBMCs) produced by the adjuvanted killed vaccine (KV/ADJ), but only a moderate specific IFN-γ response resulted in pigs vaccinated with the modified-live virus (MLV) vaccine. However, no detectable serum viremia was noted in the MLV vaccinated pigs even when the PRRSV challenge strain was not completely homologous (93% identical in the glycoprotein (GP) 5 gene) to the MLV strain. Therefore, this study is important for evaluation of what measurable immunological parameters, or biomarkers, might be associated with a protective response to non-identical (non-homologous) PRRSV challenge as defined by no measurable viremia after challenge. In addition, archived samples from this study provide a logical sample set for validation of a new multiplex assay for simultaneously detecting multiple immune proteins (cytokines) in a single sample.

Cytokines are small secreted proteins which mediate and regulate both humoral and cell-mediated immunity and may readily be measured in tissues and serum. Cytokines are essential biomarkers of immunity, particularly for respiratory infections. In designing second generation PRRSV vaccines, it would be useful to have rapid, standardized assays that could measure whether these vaccines, or viral proteins proposed to be used in the vaccines, are inducing effective cytokine responses for immune protection.

Currently, few standardized assays are available for determining cytokine responses in swine. Some swine cytokine enzyme-linked immunosorbent assays (ELISAs) are commercially available, but each cytokine analysis is performed separately on separate plates, requiring more sample and cost compared with a ‘multiplexed’ assay whereby multiple cytokines are evaluated at one time within a single sample.

Therefore, the first objective of this study was to develop an 8-plex FMIA cytokine panel for detection of swine cytokines using capture and detection mAbs with the Bio-Rad, Bio-Plex 200™ system and Luminex X-Map™ platform to detect innate inflammatory (IL-1 β, IL-8, IFN-α, TNF-α, IL-12); regulatory (IL-10), Th1 (IFN-γ) and Th2 (IL-4) cytokines. Secondly, this assay was applied to

measure cytokine levels in serum of pigs with different levels of protective immunity against PRRSV challenge [8] and to determine whether a serum cytokine protective response biomarker could be identified.

Objectives: **The objectives of this study** are to: 1) develop a standardized, optimized assay for the detection and quantification of 8 swine specific cytokines simultaneously within a single sample using a BioRad/Bioplex/Luminex microsphere assay. These cytokines are important in viral clearance or persistence and include some of the innate (IL-1 β, IL-8, IFN α, TNF α); regulatory (IL-10), Th1 (IL-12, IFN γ) and Th2 (IL-4) cytokines. 2) Use the BioRad Bioplex/Luminex instrument to multiplex detection and quantification of these cytokines in sera of control (non-infected pigs) and PRRSV infected pigs.

Materials & Methods:

For the FMIA, 8 monoclonal antibodies (mAbs) were used to couple carboxylated microspheres for cytokine protein capture. The optimum working dilution of the porcine test sera for dilution of swine cytokine standards was predetermined by titration to give the highest signal to background ratio aside from nonspecific reactions. Cytokine standards (recombinant proteins from commercial sources) were diluted porcine serum. Next, 50 µl of porcine test serum diluted 1:2 were added to duplicate wells of the filter plate along with 2.5×10^3 of each mAb coupled microsphere (2.0×10^4 total microspheres for the 8-plex assay) in an additional 50 µl buffer. The plate was then incubated at room temperature for an optimum time of 2 h on a plate shaker. Next, the plate was aspirated via vacuum manifold and washed three times. Then, 50 µl of each anti-cytokine, secondary, biotinylated, mAb was diluted in PBS-NB and added to the filter plate and incubated in the dark at room temperature for an optimum time of 90 min then aspirated and washed three times with PBST. Next, 50 µl of a solution containing 10 µg/ml SAPE in PBS-NB was added to each well and incubated for 30 min at room temperature with shaking. The supernatant was aspirated and the plate washed three times with PBST. Finally, the microspheres were resuspended in 125 µl of PBST per well and transferred to a clear 96 well polystyrene optical plate. Coupled microspheres were analyzed through the flow cell of a dual laser Bio-Rad, Bio-Plex 200® instrument and analyzed with the Bio-Plex Manager software version 5.0. Each singleplex assay was compared to the 8-plex assay to determine whether there was any cross-reactivity

To test the assay, archived sera from three groups of adult female mixed-breed swine were utilized for cytokine analysis. Pigs had been vaccinated with commercially available vaccines used in the field, either a MLV PRRSV vaccine, Pyrsvac-183 (Syva Labs, Leon Spain) (n=10) which was 93% similar in the GP5 gene to the wild-type challenge virus or a killed virus vaccine with adjuvant (KV/ADJ) Progressis (Merial Labs, Lyon, France) (n=10) which was 99% similar in the GP5 gene to the wild type challenge virus. GP5 sequencing is the standard, typically used to determine the relatedness of PRRSV strains. Non-vaccinated pigs (n=5) were used for the control group. Vaccine was applied twice at day 0 and 21 days post vaccination (DPV) and pigs in all groups were subsequently challenged at 28 DPV with 10^5 $TCID_{50}$ of PRRSV (Lelystad) intranasally. Sera were collected at 0, 21, 28, 32, 35 and 42 DPV. The animals in this study had been previously assessed as exhibiting different levels of protective immunity against PRRSV, ranging from a) sterilizing immunity (viremia negative, viral load in tissue negative or low (MLV vaccinated) or, b) viremia positive, viral load in tissue positive (KV/ADJ and non vaccinated controls).

Results:

Objective 1: For each cytokine, a singleplex assay was first established. Tests evaluating several different commercially available mAb for each cytokine compared the respective capture and detection mAb. A correlation coefficient was determined between the singleplex vs. multiplex standard curve values for each cytokine measurement; the coefficient determinations (R^2) were between 0.95 to 1.0 for all 8 cytokines [IL-1β (0.998); IL-4 (1.0); IL-8 (0.950); IL-10 (0.996); IL-12 (0.990); IFN-α (0.986); IFN-γ (1.0); TNF-α (0.951)]. The mean percent cross reactivity using a single capture mAb labeled microsphere with and without the associated cytokine protein where the concentration of the cytokine proteins were all at the high end of the dynamic range was 11% ± 6. The mean percent cross reactivity for the multiplex assay with and without the associated biotinylated mAb was 8% ± 6, using cytokine levels at the high end of the dynamic range.

Objective 2: Serum cytokine levels from control (n = 5), MLV (n = 10) and KV/ADJ (n = 10) vaccinated pigs were assayed using the 8-plex FMIA before (28 DPV) and after (32 DPV) PRRSV challenge. A significant difference in the mean IL-12 serum cytokine level was noted for pigs given the KV/ADJ vaccine compared to either the MLV vaccinated or control pigs at both 28 and 32 DPV. A statistically significant difference in IFN-γ levels was observed between the control pigs at 28 DPV and pigs given the MLV vaccine at 32 DPV. Cytokine levels for IL-1β, IL-4, IL-8, IL-10, IFN-α, and TNF-α were measured in the same sera from all pigs in all groups in the same multiplex assays. There was a high level of variability between the serum cytokine levels and the levels detected for the other 6 cytokines were not statistically different between pig groups or days.

Discussion:

The FMIA protocol developed and validated for this study provided a means to evaluate single, low volume serum samples for multiple cytokines simultaneously. This decreased costs and time in measuring these cytokines compared to performing individual ELISAs for each cytokine. It should be noted that many combinations of commercially available mAbs for capture and detection antibodies were evaluated, but those used for the cytokine multiplex assay either had more optimum coupling efficiencies, less background fluorescence, higher sensitivity or a more linear standard curve. In addition, all capture and detection antibodies were mAbs, rather than polyclonal reagents, thereby giving greater specificity and sensitivities. The sensitivity of the 8-plex FMIA was in the pg/ml range in spite of limited cross reactivity which was compensated for by normalizing the data (e.g., subtracting background levels). The mAbs used for this assay were also swine-specific, except an ovine-derived IL-8 capture mAb.

This study is the first describing the development of a well validated 8-plex FMIA for the detection of swine cytokines in serum. The assay should also be very useful in the measurement of cytokines in tissues and other fluids for the targeted design of efficacious vaccines for PRRSV, as well as other swine pathogens.

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Lawson, S., J. Lunney, F. Zuckermann, F. Osorio, E. Nelson, C. Welbon, T. Clement, Y. Fang, S. Wong, K. Kulas, J. Christopher-Hennings. 2010. Development of an 8-plex Luminex assay to detect swine cytokines for vaccine development: Assessment of immunity after porcine reproductive and respiratory syndrome virus (PRRSV) vaccination. Vaccine (in press).