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Production of Monoclonal and Polyclonal Antibodies Against PRRSV MLVD23-S tag Peptide Sequence

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Abstract

Porcine reproductive and respiratory syndrome virus (PRRSV) is an RNA virus which belongs to the family *Arteriviridae.* Since its emergence in the late 1980's and early 1990's, PRRSV has had a major economic effect on the swine industry. The most notable effects of this disease are respiratory distress in piglets and late-term pregnancy failures in sows. To track and control spread of this disease, the industry must be able determine which animals have been naturally infected with PRRSV as this contributes to major disease outbreaks and localized pandemics. While vaccines help control the spread of a disease, they also interfere with tracking of disease outbreaks. A DIVA test allows for the differentiation of animals which were naturally infected from those that were infected via immunization and is used in conjunction with a vaccine. Our primary objective was to develop rabbit polyclonal and mouse monoclonal antibodies against a highly immunogenic, heterologous gene not native to PRRSV to be used as reagents for a PRRSV specific DIVA (Differentiating Infected from Vaccinated Animals) test. To start the project, non-structural protein PRRSV-NSP2 was modified to include an immune marker (S-tag genetic marker) to be used as a positive selection tag that allows for the

diagnostic detection of the vaccine virus strain. This protein was cloned into a plasmid expression vector, expressed in E. coli bacteria, and purified in order to be used as an antigen to immunize mice and rabbits. The immunization of rabbits was effective for producing polyclonal antibodies to be used for further research and diagnostic purposes, while the immunization of the mice was used to generate monoclonal antibodies through the formation of hybridoma cells. A hybridoma is produced by fusing splenic B cells from the immunized mice with mouse tumor cells (NS-1 myeloma). These hybridomas produced monoclonal antibodies which were expected to be specific to PRRSV MLVΔ23-S tag epitopes. These monoclonal antibodies were characterized and their ability to recognize PRRSV MLVΔ23-S tag epitopes was assessed via enzyme linked immunosorbent assay (ELISA), immunofluorescence assay (IFA), and Western blot assay. Overall, we recovered eighteen separate hybridoma cell lines which were immunoreactive via ELISA and Western blotting. Eight of these were also immunoreactive via IFA. We also obtained approximately 75 mL of anti-PRRSV S-tag specific rabbit polyclonal antisera. These resulting monoclonal and polyclonal antibodies will be useful to develop a DIVA test for the veterinary diagnostic industry. This test can be used to monitor the effectiveness of the PRRSV vaccine and spread of the disease in order to assist in reducing the major economic impact of PRRSV on the swine industry.

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV), discovered in the United States in 1987, is a single-stranded, positive-sense RNA virus from the family *Arteriviridae* [1]. Its genome contains a minimum of 10 open reading frames (ORFs). ORF 1a and ORF 1b make up

¾ of the viral genome and produce 14 non-structural proteins after enzymatic cleavage while ORFs 2-7 code for structural proteins (Figure 1) [2]. This disease causes respiratory distress in piglets and late-term pregnancy failures. It can be transmitted through direct contact with placental and intranasal membranes and replicates in regional macrophages, which then spreads throughout the body and causes viremia and infection of other macrophages [1]. This disease is economically devastating to the swine industry, even more so than diseases that previously impacted it. PRRSV is estimated to cost the swine industry around 560 million dollars annually compared to classical swine fever (364 million) and pseudorabies virus (36 million). This loss is due to a significant reduction in the number of piglets which survive to weaning as well as an increase in secondary infections, reduction in growth efficiency, and even death in growing-finishing pigs [3]. The substantial economic effects of PRRSV necessitate a need for improved technologies to study and manage the disease's effects in order to move closer to the hopeful eradication of the disease.

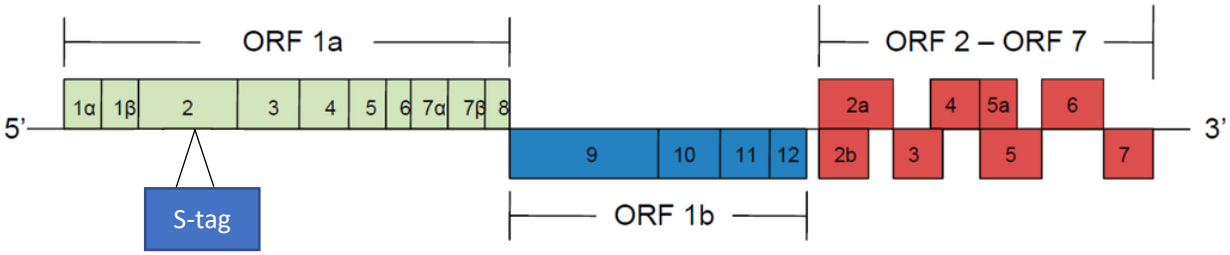

Figure 1. Organization of porcine reproduction and respiratory syndrome virus (PRRSV) genome. ORF1a and 1b code for 14 non-structural proteins, including our protein of interest: non-structural protein 2 (NSP2) in which we inserted a heterologous S-tag sequence for virus identification. [2]

An important tool used in the eradication of disease is vaccines, which can be incredibly useful to decrease or even prevent infection for many diseases. The eradication of a disease also relies on comprehensive and accurate serological surveillance of that disease within a population. However, vaccinations can hinder serological surveillance of a disease as many vaccines induce an identical antibody response to that of natural infection. This issue can be resolved through the development and usage of a DIVA vaccine [4][5]. A DIVA vaccine is accompanied by a diagnostic test which allows the veterinary industry to determine which animals were naturally infected versus those that received a vaccine consisting of a modified live virus. This is useful for continued epidemiological investigation of PRRSV among the porcine population, and also enables continuous monitoring of the safety and success of the vaccine [6].

The first step in the development of a DIVA test is to develop diagnostic detection reagents. Our primary objective was to develop rabbit polyclonal and mouse monoclonal antibodies against a highly immunogenic, heterologous gene not native to PRRSV to be used as reagents for a PRRSV specific DIVA test. To develop the monoclonal antibodies necessary for a DIVA test, we created hybridomas which produce monoclonal antibodies against PRRSV MLVΔ23-S tag protein [7]. A common method used to generate a DIVA vaccine and test is to take a protein from a virus and delete a portion of it, otherwise known as a negative selection marker [8]. Our technique differs from typical methods as we inserted an immune marker (an mRNA sequence that codes for a 15 amino acid S-tag genetic marker peptide sequence), known as a positive selection tag, into the genome of the PRRSV-NSP2 protein (Figure 1). The genetic sequence was then cloned into an expression vector which allowed it to be transformed in *E.*

coli bacteria. The bacteria produced the S- tag protein which was then purified & used as antigen for immunization of mice and rabbits. After a 68-day immunization regimen, we harvested splenic B-cells from mice and fused them with NS-1 myeloma cells to form hybridomas which produced our monoclonal antibodies.

Monoclonal antibodies are monovalent antibodies which are derived from a single B-lymphocyte and have specific affinity for the same epitope. The most effective way to obtain these antibodies is by using a technique involving hybridoma formation [9]. Hybridomas were first successfully formed and used for monoclonal antibody production in 1975 by George Kohler and Cesar Milstein [10]. The process of forming a hybridoma starts with the immunization of a species with the antigen of interest. The B-lymphocytes from the immunized animal are then harvested from its spleen and fused with an immortal myeloma cell line. The myeloma cells lack the Hypoxanthine-guanine-phosphoribosyltransferase (HGPRT) gene [9]. HGPRT is expressed when the hybridomas are placed in selective medium. This medium contains hypoxanthine-aminopterin-thymidine (HAT), which blocks unfused myeloma cells from using their salvage pathway to produce nucleotides due to their missing HGPRT gene. However, the myeloma cells which fused with B-cell inherit the HGPRT gene from the B-cells and are able to use it to produce pyrimidine nucleotides. The result of using this process is that only the hybridomas survive [11].

Initially, the culture of hybridomas contains a variety of B-lymphocytes, each producing antibodies for different antigens. This provides a polyclonal mixture of antibodies. In order to isolate the desired monoclonal antibody, the hybridomas are diluted into a multi-well culture plate. This allows a greater likelihood that each well will contain a monoclonal hybridoma. Each

well is then screened individually to determine if it contains antibodies specific to the target antigen. The positive wells are then recloned and undergo secondary screening to ensure they still have specificity for the antigen. The hybridomas can then be expanded to produce an abundance of monoclonal antibodies [9].

Monoclonal antibodies can be utilized in many ways. Most applicable to our research is their use in diagnostic tests, specifically indirect immunofluorescence assay (IFA), enzyme-linked immunosorbent assay (ELISA), and Western blot. For an IFA, mammalian cells are transfected with DNA for the target antigen. The antibodies are put on the cells along with a fluorescent dye where they bind transfected cells. This allows the visualization of these cells using a fluorescent microscope. Instead of using DNA like in the IFA, an ELISA uses proteins. A specific protein from the target pathogen is attached to the surface of a plate and then antibodies specific to that protein are applied. These antibodies bind the protein. Next, a secondary antibody which is linked to an enzyme is plated which binds the primary antibody. Finally, a reagent containing the substrate specific to the enzyme on the secondary antibody is plated which results in a measurable color change. A Western blot assay is performed by first running a linear protein on a gel using electrophoresis. Next, primary antibodies are added to the gel which bind to the protein. Enzyme-conjugated secondary antibodies are added to the membrane next which bind to the primary antibodies and can be visualized via a color change imparted by the addition of a chromogenic substrate [12]. Using monoclonal antibodies in the context of IFA, ELISA, and Western blot allows us to evaluate the immunoreactivity of the antibodies which will then be used to validate a DIVA test for PRRSV.

Materials and Methods

Expression of the PRRSV NSP2 S-tag protein

The 660-nucleotide codon optimized region of the PRRSV NSP2 genome which contained the S-tag gene was cloned into an expression vector (pET-28a, Novagen; Madison, WI) and then transformed into *E. coli* (BL21-Codon Plus DE3 RP competent) for expression of the protein. 50 µL of the transformed cells were plated onto agar plates and incubated at 37°C overnight. The next day, colonies were placed in 2X yeast extract tryptone (YT) culture media. Once the cultures were grown to an optical density of 0.5 at 37°C, then expression was induced using a 1.0 mM concentration of isopropyl β-D-1-thiogalactopyranoside (IPTG). This induced transcription of the Lac operon and the downstream NSP2 protein. The *E. coli* were incubated for an addition 8 hours at 37°C [12].

After the additional incubation, the bacteria were strained out and centrifuged at 10,000 x g for 10 minutes at 4°C to obtain a pellet. Resuspension of the pellet was achieved using 50 mL of lysis buffer solution (B-PER, Pierce, Rockford, IL) which was then incubated at room temperature (20°C) for 20 minutes. It was then centrifuged at 10,000 x g to separate soluble and insoluble proteins. The protein was denatured with 8 M urea and purified using nickel-NTA affinity column chromatography. Elutions were collected, confirmed using SDS-PAGE, then frozen at -80°C. Western blotting using a 6X histidine specific monoclonal antibody (Novagen; Madison, WI) was used in order to confirm the protein. The resulting NSP2 S-tag protein was used to immunize mice and rabbits [12].

Development of enzyme-linked immunosorbent assay for hybridoma screening

An Immulon 1B medium binding 96 well micro-titer plate was coated with PRRSV NSP2 S-tag protein and a heterologous HIS-tag control (SVS VP2 12-15-15). PRRSV NSP2 S-tag was diluted 1:2000 in antigen coating buffer (ACB) and 100 µL was added to odd numbered wells. SVS VP2 12-15-15 was also diluted 1:1500 in ACB and 100 µL was added to even numbered wells. The plate was allowed to incubate at 37°C for 1 hour. A 5% nonfat dry milk (Shurefine; Skukie, IL) and PBS solution was made as a blocking antibody diluent buffer (BAD) and 200 µL was applied to each well. This was incubated at 4°C overnight. In the morning, the plate was washed four times with PBS + 0.05% Tween 20 (PBST).

To screen the hybridomas, 60 µL of undiluted cell culture supernatant was added to each well. This was incubated for 1 hour at room temperature and then the plate washed four times with PBST. 100 µL of a goat anti-mouse, IgG, IgA, IgM-HRP conjugate (MP-Bio; Santa Ana, CA) diluted 1:5000 in BAD was added to each well and the plate was incubated for 1 hour at room temperature. TMB chromogenic substrate (VMRD; Whitman Country, WA) was removed from the fridge and allowed to warm to room temperature. It was then tested via a 1:150 dilution of the enzyme-containing secondary and displayed a clear blue color indicating that it worked. The plate was washed four times in PBST and then 100 µL of TMB was added to each well. Finally, the reaction was stopped using 100 µL of 2N H_2SO_4 in each well and the optical densities were measured using an ELx800 microplate reader (BioTek Instruments Inc.; Winooski, VT) at 450 nm.

Development of Western blot for hybridoma screening

Color chromogen development Western blotting was used to identify the PRRSV NSP2 S-tag protein. For this method, the PRRSV NSP2 S-tag antigen was diluted 1:6 in Laemelli sample buffer which was then heat treated at 97°C for 10 minutes. An SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of the samples was completed at 120 Volts for 75 minutes using an 8% polyacrylamide gel (Invitrogen; Carlsbad, CA). Electroblot was then used at 125 Volts for 1 hour to transfer the protein gel to a nitrocellulose membrane (Fischer Scientific; Hampton, NH). The membrane was blocked overnight using BAD at 4°C. In the morning, cell culture supernatant diluted 1:25 in BAD plus 0.1% Bovine Serum Albumin (BSA) and allowed to incubate for 2 hours at room temperature. The membrane was washed in PBST three separate times, each for 5 minutes. Then, a goat anti-mouse IR secondary antibody was diluted 1:5000 in BAD and was incubated at room temperature for 1 hour. The membrane was again washed in PBST three separate times, each for 5 minutes. Finally, chloro-1-naphol was used as the chromogenic substrate to detect the protein.

Development of immunofluorescent assay for hybridoma screening

To develop an immunofluorescent assay for PRRSV MLVΔ23-S tag hybridoma screening, African green money kidney derived cells (MARC-145 cells) were seeded onto Immulon 1B medium binding 96 well micro-titer plates (Thermo Labsystems, Franklin, MA) and incubated for 48 hours. They were then infected with the PRRSV MLVΔ23S virus strain which was modified to contain the S-tag. The infected MARC-145 cells were incubated for 24 hours and then fixed with 80% acetone.

To screen hybridomas, 50 µL of undiluted cell culture supernatant was added to each well and then the plate was incubated with CO_2 for 1 hour at 37°C. Next, the plate was washed three times with PBS and 100 µL of FITC (MP Bio; Santa Ana, CA) diluted PBS-BN + 1% Sodium Azide was added to each well. The plate was incubated for 1 hour at 37°C and then washed an additional three times with PBS. A few microliters of PBS were left in the bottom of each well after washing to ensure the cells did not dry out. The plate was then placed under an inverted microscope and UV light source to examine the cells for fluorescence.

Development of mouse monoclonal antibodies

Using 250 µg of the purified NSP2 protein mixed with an equal volume of water-in-oil adjuvant (Seppic Montanide ISA 50 V2; Seppic, France), 3 ten-week-old specific pathogen-free mice were immunized intraperitoneally 4 times in three-week intervals. After the final immunization, the mice's spleen cells were harvested and fused with NS-1 myeloma cells using 50% polyethylene glycol (GIBCO, Grand Island, NY). They were then grown in the present of hypoxanthine-aminopterin-thymidine (HAT) media in order to select for only viable hybridoma clones. The cell culture supernatant was then screened using NSP2 S-tag ELISA and IFA to determine which clones produced antibodies specific to the PRRSV MLVΔ23-S tag antigen (as described in respective sections above). Those clones which were positive were then subcloned using limiting dilution in order to isolate monoclones. They were screened a final time using NSP2 S-tag ELISA, IFA, and Western blotting (as described in respective sections above). These monoclones were then isotyped using their culture fluids on a lateral-flow immunoglobulin typing kit (Serotec; Raleigh, NC).

Development of rabbit polyclonal antisera

Two 4-month-old rabbits were immunized with a 150 µg dose of PRRSV NSP2 S-tag antigen mixed in an equal volume with water-in-oil adjuvant (Seppic Montanide; Seppic, France). The immunization was given both subcutaneously and intramuscularly in 4 separate locations. Immunization was repeated three times at three-week intervals. Rabbits were then euthanized, and their blood was collected via cardiac puncture. Their sera were tested via IFA, ELISA, and Western blot to determine if they had antibodies against PRRSV NSP2 S-tag. Animal studies were approved by the South Dakota State University Institutional Animal Care and Use Committee (17-066A Mice; 17-067A Rabbits).

Results

Expression of the PRRSV NSP2 S-tag protein

After running the PRRSV S-tag protein on a polyacrylamide gel, the gel was cut into two halves. The first half was stained using Coomassie blue (Figure 2B) while the second half underwent Western blotting (Figure 2A). The Coomassie blue staining allowed visualization of the proteins while Western blotting revealed the specificity of the PRRSV S-Tag protein, which contained a histidine label, to an anti-histidine monoclonal antibody. Overexpression of the protein was completed at a concentration of 1.5 mg/mL and showed a level of purification at 90%.

Development of mouse monoclonal antibodies

Initial screening of the PRRSV MLVΔ23-S tag hybridomas following the fusion resulted in 6 primary wells. These primaries were cloned to limiting dilution and subclones underwent

secondary screening via ELISA and IFA. Based on secondary screening, subclones were selected to be expanded. The final expanded clones were screened a final time via ELISA, IFA (Figure 3), and Western blotting (Figure 4). Each of the monoclones were also isotyped. All monoclones with an IgM isotype were positive via ELISA and Western blot but were negative via IFA. However, all of the IgG_1 isotype monoclones were positive via ELISA, IFA, and Western blot. Compiled data involving isotype and reactivity to ELISA, IFA, and Western blot of 18 monoclones is provided in Table 1.

Development of rabbit polyclonal antisera

150 mL of whole blood was collected via cardiac stick from the two New Zealand Red rabbits after immunization with PRRSV NSP2 S-tag antigen. The blood was spun down then the serum (77 mL) was removed and titered using ELISA and IFA (Figure 4). The results of this titer are listed in Table 2.

PRRSV MLVΔ23-S tag Monoclone Number	Isotype	Reactivity		
		ELISA	IFA	Western Blot
16-63	IgM	+	-	+
16-75	IgM	+	-	+
84-92	IgM	+	-	+
84-94	IgM	+	-	+
84-99	IgM	+	-	+
84-103	IgM	+	-	+
84-106	IgM	+	-	+
84-110	IgM	+	-	+
84-113	IgM	+	-	+
142-13-46	IgG_1	+	+	+
142-36-13	IgG_1	+	+	+
142-36-16	IgG_1	+	+	+
142-36-120	IgG_1	+	+	+
142-36-123	IgG_1	+	+	+
142-206-26	IgG_1	+	+	+
142-206-40	IgG_1	+	+	+
142-306-45	IgG_1	+	+	+
205-346	IgM	+	-	+

Table 1. Monoclonal antibody reactivity. Eighteen hybridoma clones. Immunoreactivity for each clone was tested via ELISA, IFA, and Western blotting. Clones which showed positive immunoreactivity are indicated by a " + " while those which were negative are indicated by a " - ".

Rabbit serum-antibody titer determination (testing platform)	Antibody Titer Rabbit A	Antibody Titer Rabbit B
IFA	1/1,200	1/1,600
ELISA	1/10,000	1/12,000

Table 2. PRRSV NSP2 S-tag rabbit antisera antibody titers. Sera collected from two New Zealand Red rabbits were titered via IFA and ELISA, these are the results.

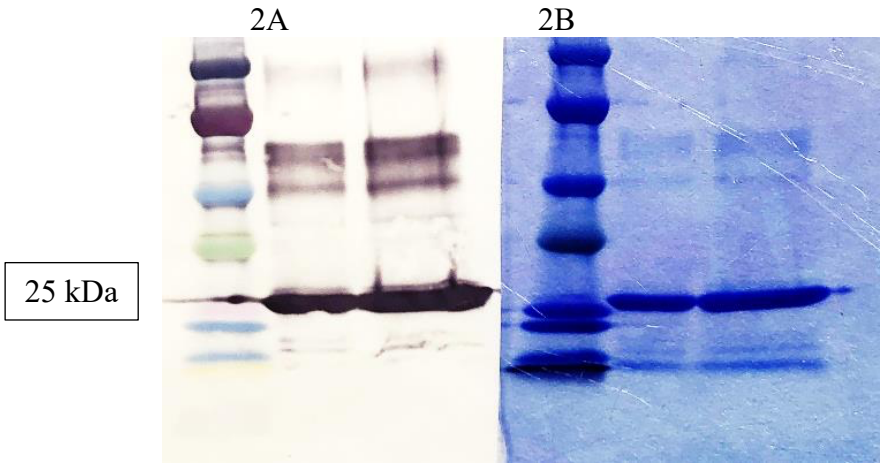

Figure 2. Western detection & Coomassie blue staining of PRRSV NSP2 S-tag protein. After protein purification, 100 µg of PRRSV NSP2 S-tag was run on a polyacrylamide gel using electrophoresis. Half of the gel underwent Western blotting (Figure 2A) while the other half was Coomassie blue stained (Figure 2B).

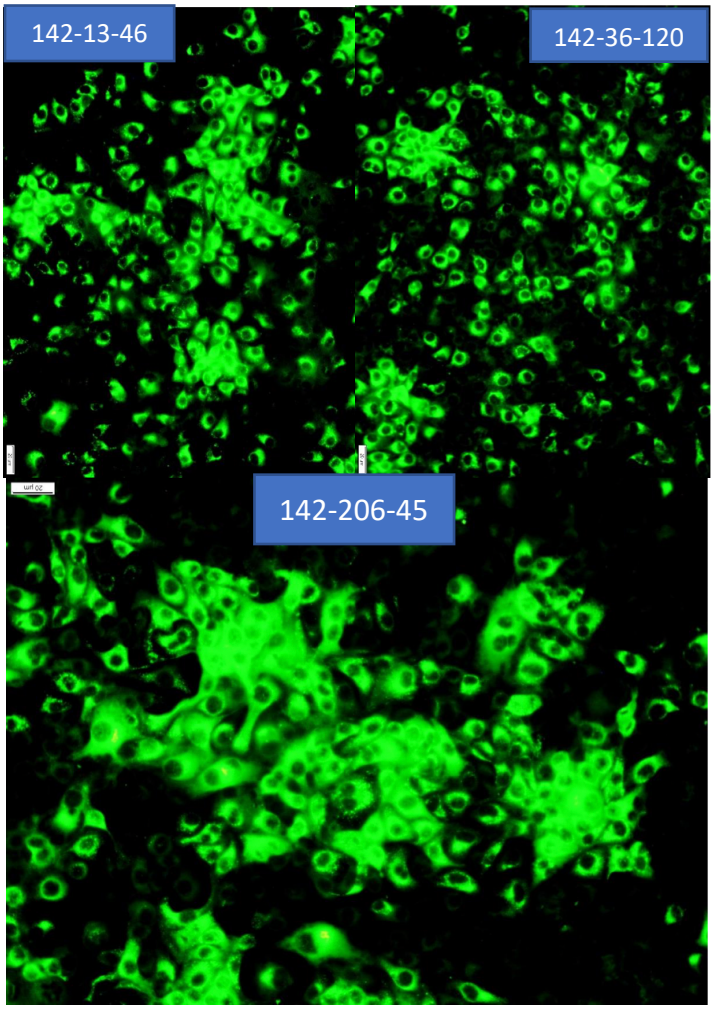

Figure 3. Immunofluorescent staining of PRRSV MLVΔ23S -infected MARC-145 cells. Cell culture supernatant was taken from monoclones to stain infected MARC-145 cells. The above images show IFA-positive results from 3 individual monoclones.

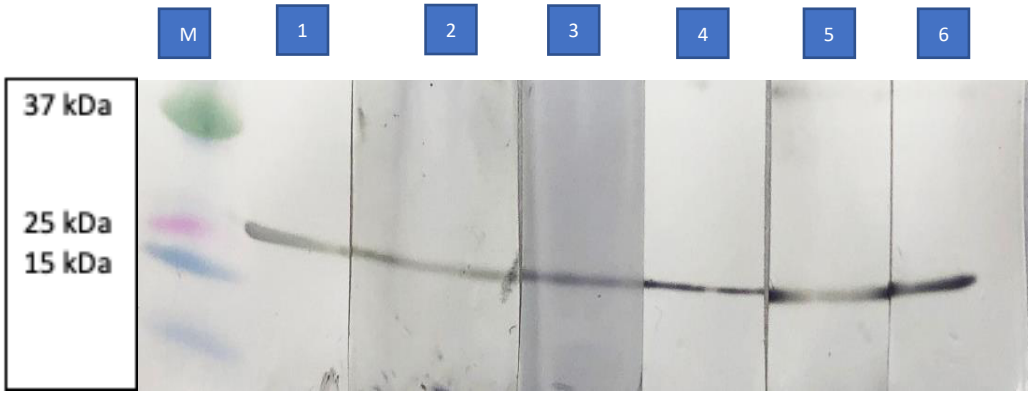

Figure 4. Western blotting of PRRSV NSP2 S-tag protein. Six mouse monoclones were subjected to Western blot detection. All six were shown to recognize the 25 kDa purified PRRSV NSP2 S-tag protein. Chromogenic (4-chloro-1-napthol) detection method was used after probing with a peroxidase-labeled, isotype-specific, anti-mouse antibody.
(M) Marker; (1) mAb 142-13-39 IgG_1; (2) mAb 142-36-120 IgG_1; (3) mAb 142-206-45 IgG_1; (4) mAb 205-346 IgM; (5) mAb 16-75 IgM; (6) mAb 84-99 IgM.

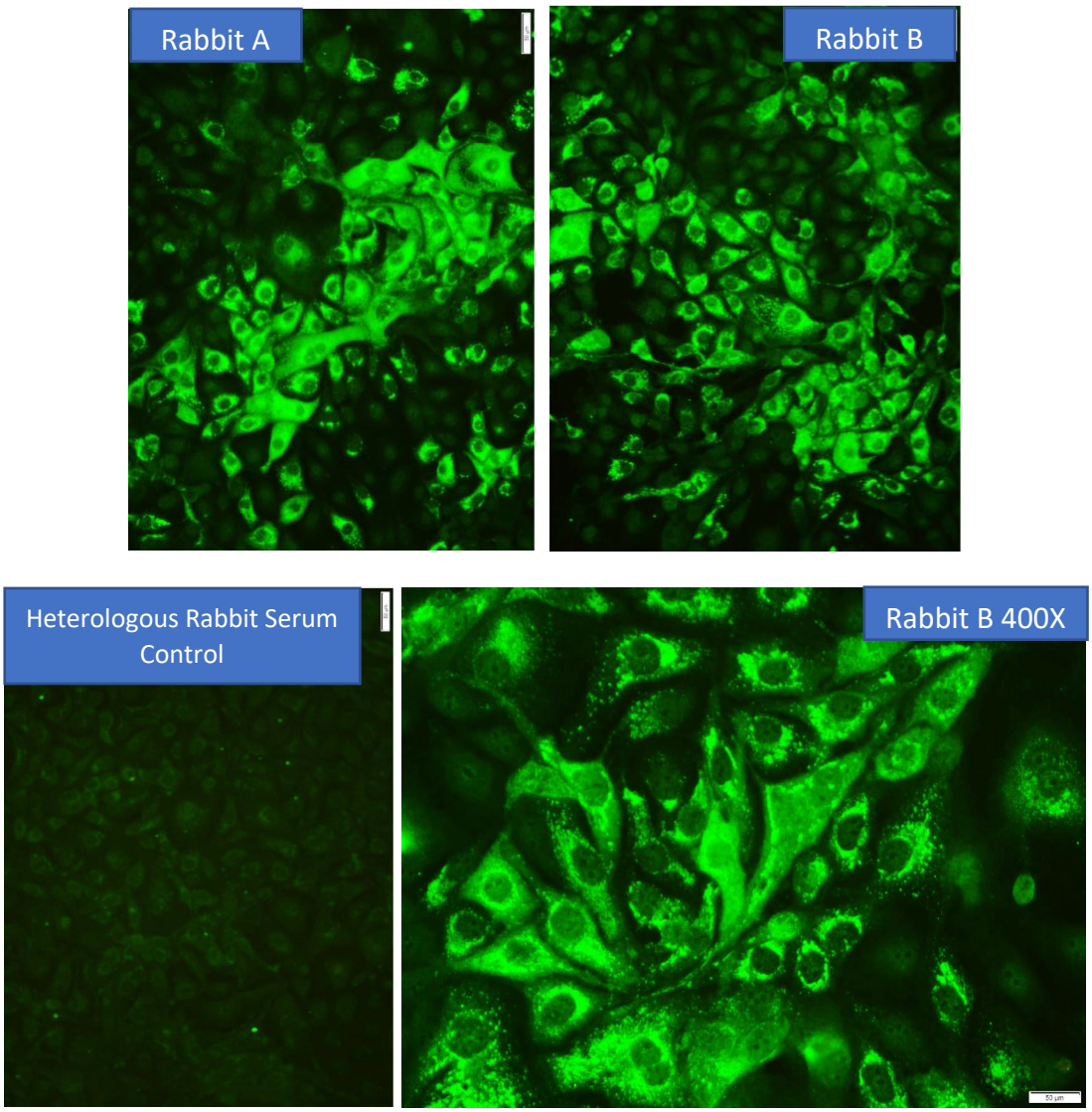

Figure 5. Immunofluorescent staining of PRRSV MLVΔ23S -infected MARC-145 cells with rabbit polyclonal antisera. Rabbit A exhibited titers at 1/1200 while Rabbit B exhibited titers at 1/1600.

Discussion

PRRSV has had a major economic effect on the swine industry since its emergence in the late 1980's and early 1990's [1]. It causes a significant reduction in the number of piglets which survive to weaning as well as an increase in secondary infections, reduction in growth efficiency, and even death in growing-finishing pigs. These losses combined are estimated to cost the swine industry around 560 million dollars annually [3]. A successful DIVA test (differentiating infected from vaccinated animals) will provide a tool necessary to move the industry one step closer to the eradication of PRRSV. Therefore, our primary objective of this study was to develop monoclonal and polyclonal antibodies which could be used in further research of a DIVA vaccine and accompanying diagnostic test.

We produced 18 mouse hybridoma monoclones against the PRRSV MLVΔ23-S tag antigen. These monoclones were isotyped and their immunoreactivity was tested via ELISA, IFA, and Western blot. All clones from the 142 series were IgG_1 and immunoreactive via ELISA, IFA, and Western blot. Clones from the 16, 82, and 205 series were IgM and immunoreactive via ELISA and Western blot, but not IFA. This tells us that clones from the 142 series are able to detect both linear and conformational epitopes while clones from the 16, 82, and 205 series can only detect linear epitopes. In addition to the 18 monoclonal hybridoma clones, 75 mL of anti-PRRSV NSP2 S-tag rabbit polyclonal antisera was collected from two rabbits: Rabbit A and Rabbit B. It was tested via ELISA, IFA, and Western blot and found to have 1/1200 titers (Rabbit A) and 1/1600 titers (Rabbit B). 500 µL of recombinant/purified PRRSV NSP2 S-tag protein was also produced.

Purified PRRSV NSP2 S-tag protein can be used for future DIVA ELISA test development and can provide a positive control for future Western blot development. Both monoclonal and polyclonal antibodies can be used in future studies to validate DIVA IFA, ELISA, and Western blot assays or to develop a DIVA Fluorescent Microsphere Immunoassay (FMIA).

Conclusion

The monoclonal and polyclonal antibodies produced in this study against PRRSV MLVΔ23-S tag antigen are currently being used as reagents in development of a DIVA test for PRRSV. A successful DIVA test for PRRSV could positively impact the swine industry and move us one step closer to the eradication of PRRSV.

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I would like to thank Dr. Steve Lawson and Dr. Eric Nelson for all their help with this project. Their expertise on the methods and background of this study were invaluable, as well their willingness to provide materials and a space to work. Thank you to Dr. Aaron Singrey for helping us to complete a successful hybridoma fusion and the accompanying screening of clones. Thank you to Molly Kroeger and Laura Munger for being excellent and patient mentors during my first few months in the lab. Molly, Laura, and Kayla Egenes were also incredible collaborators on this project and helped with all aspects of the research.

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