RESEARCH REPORT
SWINE HEALTH

Title: Expanding the immune toolkit for assessing pig health and improving swine disease and vaccine studies - **NPB 05-015**

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[with major collaboration with Dr. Serge Muyldermans, Free Univ. Brussels, Belgium]

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Abstract:

Swine disease and vaccine research has been advanced by the development of sophisticated tools to measure physiologic parameters associated with immunity, pathology, and disease prevention. Our goal is to expand the immune toolkit for pigs, by developing and characterizing reagents that can be used to identify and quantify a major class of immune proteins, the antibodies or immunoglobulins (Igs). Swine produce antibodies, or Igs, in response to infection or vaccination. Scientists measure pathogen exposure and vaccine efficacy by quantitating Ig levels in serum. But not all Igs are equal. We know for porcine reproductive and respiratory syndrome virus (PRRSV) infections that there is a well-characterized antibody response as measured by the IDEXX ELISA. However, the more relevant test is whether infected or vaccinated pigs produce neutralizing Igs against the virus. These Igs may be a specific class of IgG. Neutralizing Igs are known to take longer to develop but are important in recovery from PRRSV infection.

The goal of this grant is to develop a broader panel of anti-swine Ig reagents to verify exactly which Ig classes, in particular IgG subclasses, are critical. Reseachers require such reagents to determine Ig function; diagnostic laboratories use them to measure Ig levels. Currently most investigators rely on polyclonal antisera that are tedious to prepare, lack immortality, are usually not class specific, and vary between batches. For NPB project #05-015 we started the process of characterizing and developing new monoclonal antibody (mAb) reagents that uniquely recognize the various Ig isotypes and IgG subclasses.

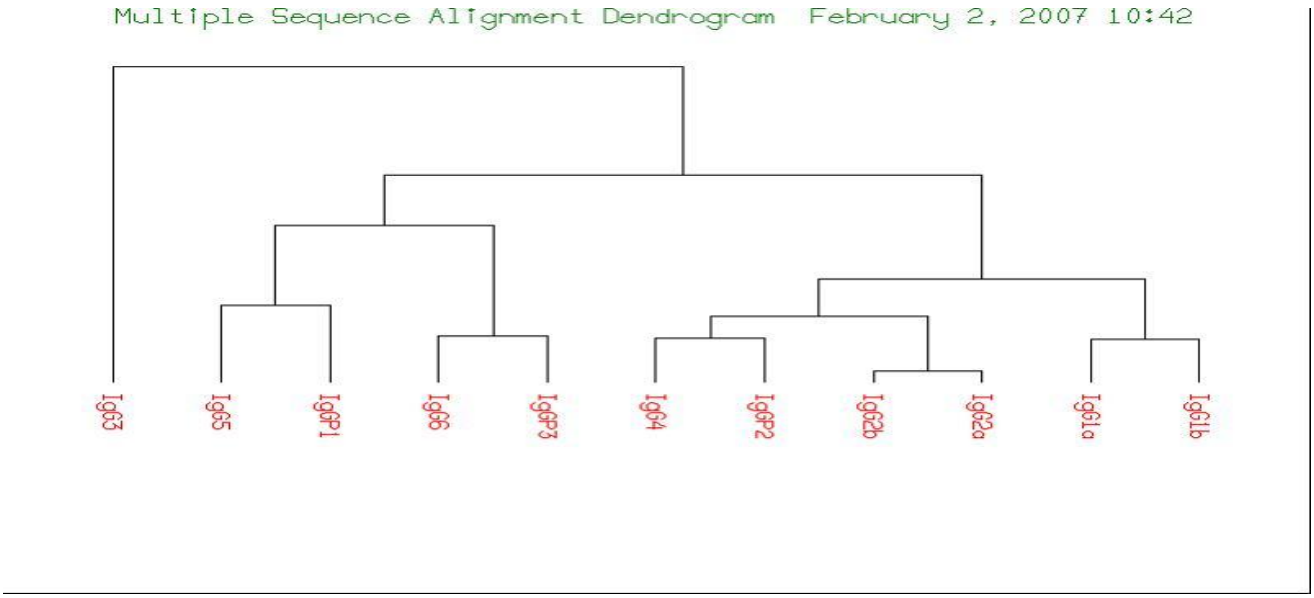
To accomplish our goals we expressed the previously known five swine IgG genes as cDNAs and actually discovered numerous other IgG genes, which are still in process of analyses. We worked with a collaborator, Dr. Serge Muyldermans, in Belgium to express the 5 previously known swine IgG subclass proteins in vitro using his novel camelid-swine Ig expression system that efficiently produces single chain porcine-camelid chimeric IgGs. With this camelid system we have the means to express only constant regions of the specific swine IgG heavy chain proteins to produce and characterize mAb.

For our second objective, we characterized the reactivity of the currently available mAb anti-swine Ig. We assured that Canadian anti-swine IgA and IgM hybridomas and mAb are available at the USDA APHIS NVSL lab in Ames, IA and that anti-IgG hybridomas were imported from the UK . Our overall goal is to have a full panel of well-characterized mAb that react specifically with each swine Ig isotype and IgG subclass so scientists will be able to compare accurately the functions of each swine Ig isotype and subclass. This will hopefully expand our understanding of disease control mechanisms and pathologies, as well as serve as improved tools for characterizing swine vaccine responses.

Introduction:
Keeping pigs healthy and productive is the major target for producers. Preventing disease using biosecurity and planned vaccinations are hallmarks of well-managed swine facilities. In the case of disease outbreaks, diagnostic laboratories need better tools to quickly determine underlying infections and causative pathogens. Veterinarians diagnose infections, and prescribe therapies and vaccines to prevent disease outbreaks. All of these functions require reagents that can quantitate pig responses, particularly to identify and quantitate swine Igs in serum or mucosal secretions. The success of virus eradication programs is a result of cooperation between producers, researchers, veterinarians and regulatory authorities. They require the use of serologic assays, and tests of specific serum or mucosal antibodies for program efficacy. This grant was aimed at producing more effective Ig quantitation tools for serum and mucosal secretions.

The expression of a full, heterodimeric Ig requires expression of two sets of genes, the Ig heavy and light chain genes. Molecular studies have verified that swine have two light chain types, kappa (IgLκ) and lambda (IgLλ), one known heavy chain gene each for IgM, IgA, IgD and IgE, but multiple IgG heavy chain subclass genes (see Fig. 1 below; and Kacskovics et al. J. Immunol. 153: 3536, 1994; Butler and Wertz, J. Immunol.177: 5480, 2006; Butler et al, in preparation.). While porcine IgG subclasses cannot be separated by biochemical methods, their genes had previously been cloned at Dr. Butler's lab at the University of Iowa.

Figure 1. Swine IgG cDNA sequence alignment.



To develop and characterize mouse monoclonal antibodies (mAbs) to each swine Ig we will first need to either purify, or utilize recombinant DNA technology, to obtain each pig Ig isotype and IgG subclass. For this grant five swine IgG heavy chain constructs were expressed by collaborating with Dr. Serge Muyldermans and his colleagues in Belgium who have perfected the technology to efficiently produce single chain porcine-camelid chimeric IgGs (Nyugen et al., EMBO J. 19: 921, 2000; Immunology 109: 93, 2003). The advantages of this porcine-camelid chimeric expression system is that only the swine IgG heavy (H) chain gene construct is needed. No light chain gene is required since camel Igs have a unique single chain structure. The camelid-chimera structure produces functional antibody with known antigen binding specificity (for lysozyme) which can facilitate purification of chimeric Ig from culture and means that all camelid-swine chimeric Igs bind the same antigen. The resultant swine-camelid constructs can be used to prepare mAb for swine research.

Our work started by characterizing known anti-swine Igs as well as developing products to generate and characterize new, IgG subclass-specific mAb. Our goal was to develop more sensitive and specific tools to measure swine antibody production, so that they can be used to understand disease mechanisms, develop new therapeutics and vaccines, and improve disease diagnostic assays.

Stated Objectives from original proposal

1. Express Ig proteins for each swine immunoglobulin (Ig) isotype and IgG subclass gene. Prepare Ig proteins as standard references.
2. Characterize the reactivity of known anti-swine Ig monoclonal antibody (mAb) reagents with each Ig gene product.
3. Begin to develop new mAbs that are specific for each of the expressed Ig proteins.

Materials & Methods:

a. Preparation of swine Ig cDNAs

At the beginning of this grant 5 swine IgG heavy chain genes were known. These were cDNA clones for IgG1a, IgG1b, IgG2b, IgG3 and IgG5 that had been identified at Univ. IA. As research progressed, additional porcine IgG genes have been characterized. Figure 1 provides a dendogram for porcine IgG genes; the larger the distance between each gene the greater the sequence difference. Each branch is expected, at this juncture, to represent unique IgG subclasses; based on their sequence some are noted as IgGP# because they cannot be properly expressed and thus are known to be pseudogenes.

b. Preparation of swine-camelid Ig constructs

The original 5 swine IgG cDNA clones for IgG1a, IgG1b, IgG2b, IgG3 and IgG5 were transferred to Belgium for expression as camelid-pig proteins. These cDNA clones were resequenced in Belgium and then engineered into the camelid-swine Ig expression system. Specifically, primers were designed to clone each swine IgG hinge-CH2-CH3 region in fusion with a camel specific antigen binding variable region (VHH). The VHH chosen encoded the cAb-Lys3 [the single domain antigen-binding fragment of a Heavy-chain camel antibody (HCAb) with specificity for chicken lysozyme]. To perform this work USDA APHIS permits were needed for legal transfer of cell lines and materials from Belgium to BARC and Univ. IA; all of these administrative procedures have been completed.

The swine-camelid constructs were produced in pCDNA or pCI-2 DNA mammalian expression vectors (Invitrogen), sequenced to control for faithful cloning, and transfected in NSO cell-lines. The stably transfected cell-lines were grown in medium with fetal bovine serum that was first depleted of bovine IgG by passage over protein G columns. The supernatants of these cultures contained swine IgG molecules as shown by lysozyme binding ELISA. The camelid-swine Ig constructs were then purified by passage of the culture supernatant over a protein-G column, washed, eluted at low pH, neutralized and dialysed. A yield of approximately ~1 mg per liter of culture was obtained; the protein seemed to be pure HCAb based on stained SDS-gel electrophoresis results. The protein-G column was used because the planned purification scheme using binding to lysozyme columns was tried, but unfortunately, the bivalent HCAbs could not be eluted from the columns due to avidity effects.

c. Prepare anti-swine IgG mAb proteins as standard references.

Hybridomas producing well-characterized mAb to porcine IgM (M160) and IgA (1459) were transferred from the inventory of Dr. Klaus Nielsen, Animal Diseases Research Institute, Canadian Food Inspection Agency, Nepean, Ontario to the USDA APHIS National Veterinary Services Lab (NVSL), Ames, IA. Dr. Linda Schlater and her technician, Rick Dewald, Center for Veterinary Biologics, NVSL, expanded those cell lines using the MiniPerm BioReactor. In Nov. 2005 aliquots of these hybridomas and supernatants were sent to BARC, expanded further and frozen. This sets the stage for establishment of a process to provide valuable anti-swine Ig mAb to porcine IgM and IgA to investigators by the NVSL.

Hybridomas producing anti-swine IgG mAb have been legally imported from Dr. Karin Haverson in the UK and expanded at BARC. Other hybridomas producing anti-swine IgG mAb (e.g. from Dr. Prem Paul, UNL,

Am J Vet Res. 50: 471, 1989) have been requested or have been received and were processed similarly. Supernatants from available hybridomas were prepared and mAb purified on a Pharmacia FPLC with a Pharmacia (now GE Heathcare) Hitrap protein G column at BARC. In general, the relevant hybridoma supernatant (200-500ml) is directly pumped onto the column, washed with PBS and eluted with 0.1 M Glycine into 100mM Tris for pH neutralization. Eluted mAbs are further concentrated and transferred into borate buffer using centracon 50s filters for long term storage. Protein concentrations are calculated based on OD280. Prior to reuse the FPLC column is stripped with 0.1M acetic acid and then regenerated in PBS and stored in 20% ethanol. Purified anti-swine IgG mAb are now available for testing once ELISAs using the camelid-porcine IgG constructs are standardized at the Univ. IA.

Results:

Objective 1. Express Ig proteins for each swine Ig isotype and IgG subclass gene. Prepare Ig proteins as standard references.

a. Preparation of swine Ig cDNAs

It was not proposed to discover additional IgG genes during this grant. However, another IgG subclass was identified as an indirect consequence of preparing the originally described five IgG subclass cDNAs for shipment to Belgium. As research progressed, additional porcine IgG genes have been characterized at Univ. IA. Figure 1 provides a dendogram of the currently known porcine IgG genes. Each of the 6 branches is expected, at this juncture, to represent unique IgG subclasses. Some duplications within a branch are noted. These are different IgG subclasses, such as the known swine IgG1a and IgG1b; others are presumed pseudogenes, based on their nucleotide sequence and noted as IgGP#. Each IgG branch or subclass is presumed to be an expressed swine IgG protein; two of these, IgG5 and IgG6 had not been previously described.

b. Preparation of swine-camelid Ig constructs

The preparation of mg quantities of five camelid-porcine IgG constructs has been achieved in Belgium. The camelid-swine IgGs, or HCAbs containing the hinge and Fc of pig IgG and V region from camel Igs, were successfully cloned. Stable transfectants were obtained for each clone. The culture supernatants contained functional antibodies (lysozyme antigen-binding and recognized by anti pig IgG antibodies). A purification scheme was designed. Two batches of mg quantities of HCAbs products for each of the original five porcine IgG cDNAs were produced and delivered to BARC in June and November of 2006. These were shared between BARC and Univ. IA. ELISAs are now underway for checking reactivity of known anti-swine mAb on these expressed camelid-porcine IgG constructs.

Efforts are continuing to produce clones with a higher production yield and improved solubility. At least two additional swine IgG genes (and several pseudogenes) have been characterized at Univ. IA. As part of our grant renewal #06-043 we proposed to provide the remaining cDNAs encoding these new IgGs to Dr. Muyldermans for chimeric IgG production.

Delay in timeline: For this international collaborative research project separate funding agreements and appropriate paperwork had to be prepared both for materials and funds transfers within the US (BARC and Univ. IA) and between the US and Belgium. A grant agreement and material transfer agreement (MTA) was established at BARC with Dr. Serge Muyldermans, Free Univ. Brussels, Belgium, and his collaborator Dr. Rudy Dekeyser, Vice-general Director, Flanders Interuniversity Institute for Biotechnology (VIB), Zwijnaarde, Belgium. [It should be noted that the first funds transfer to Belgium was delayed due to hurricane Katrina. The check from the USDA accounting headquarters, previously located in New Orleans, was only received in Belgium at the end of Nov. 2005.] This process also included all necessary USDA APHIS permits approved for transfers of cDNAs, expression vectors and final purified camelid-pig proteins so that swine chimeric Ig constructs could be legally received at USDA BARC and Univ. IA. These agreements, and the difficulties with expression and purification, resulted in HCAbs products only being delivered to BARC in June and November of 2006.

Objective 2. Characterize the reactivity of known anti-swine Ig mAb reagents with each Ig gene product.

a. Anti-swine IgA and IgM; USDA APHIS interactions

A number of mAb specific to porcine IgM and IgA have been tested and two of these designated as the "Gold Standards" for anti-pig IgM and anti-pig IgA mAb, M160 for IgM and M1459 for IgA. These hybridomas have now been transferred to the NVSL, APHIS labs from Dr. Nielsen in Canada. Meetings in Ames, IA at the NVSL involving Drs. J. E. Butler, Cyril Gay, Cynthia Baldwin, Peter Johnson, Randall Levings and Beth Lautner concluded that the NVSL would maintain hybridomas deemed valuable in producing specific mAb to porcine Ig isotypes and subisotypes. Other anti-swine Ig mAb have been legally brought into the US and purified proteins are now available for testing anti-pig Ig reactivity at BARC and Univ. IA.

b. Importation and preparation of purified known anti-swine Ig mAb

Several imported hybridomas have been legally brought into the US, expanded, frozen and supernatants produced for mAb purification. Multiple anti-pig IgG mAb have been purified on FPLC and concentrated at BARC. Aliquots of these anti-pig IgG mAb have been sent to Univ. IA for ELISA testing on the porcine IgG subclass specific HCAbs

Objective 3. Begin to develop new mAbs that are specific for each of the expressed Ig proteins.

We are awaiting the availability of more of the camelid-porcine IgG chimeric proteins prepared in eukaryotic cells to determine the specificity of these mAbs. More progress on this aim is expected as other swine-camelid Ig construct proteins are produced and become available for immunizations, hybridoma fusions and screening. Additionally, the closure of the Univ. IA hybridoma facilities has meant that alternate facilities must be identified. The Univ. IA has designated alternative Iowa sites; efforts are underway to establish immunization plans now.

Discussion:

As a result of this grant, and a second proposal to the NPB to study the IgG subclass-associated response to PRRSV (NPB #05-174), there was a re-investigation of the porcine IgG subclass issue at the genetic level. These revealed major discoveries, multiple swine IgG C gamma genes, some appearing to be the result of recent gene duplications in domesticated swine, a large number of allelic variants of each IgG C gamma gene (Figure 1). Thus, the swine IgG system could be the most polygenic and polymorphic antibody system ever described. National and international collaborations have now been established to help resolve this complexity by looking into genomic organization of the porcine heavy chain locus and breed distributions of the major allotypes of swine IgG. Therefore, the work that we originally proposed (to clone and express all swine IgG genes and verify reactivity of known mAb on each of them) has grown more complex, to an extent beyond our imagination.

It was originally believed that it would take many years before swine IgG subclass function could be studied. This would be dependent on the availability of the camelid-porcine IgG chimeras. However, with the availability of the sequences of more IgG genes (Fig. 1) in silico analyses can be performed. Some modeling can now be done that could be predictive of the biological function of each encoded swine IgG subclass, by comparison to the structure of human IgG subclass proteins for which function is known (as discussed in Butler et al., Dev Comp Immunol. 30: 199-221, 2006).

Lay Interpretation:

Swine disease and vaccine research will be advanced by the development of sophisticated tools to measure swine antibodies or immunoglobulins (Igs). Swine produce antibodies, or Igs, in response to infection or vaccination. But not all Igs are equal. Scientists measure infectious disease exposure and vaccine efficacy by quantitating Ig levels in serum or mucosal secretions. We know for porcine reproductive and respiratory syndrome virus (PRRSV) infections that there is a well-characterized antibody response as measured by the IDEXX ELISA. However, the more relevant test is whether infected or vaccinated pigs produce neutralizing Igs against the virus. These Igs may be a specific class of Igs or a subclass of IgGs. Neutralizing Igs are known to take longer to develop but are essential for recovery from PRRSV infection.

Our NPB project's goal was to expand the immune toolkit for pigs, by developing and characterizing reagents that identify and quantify the major classes of swine Igs, IgA and IgM, and the subclasses of IgGs. This grant was aimed at developing a broader panel of anti-swine Ig reagents to determine exactly which Ig classes, in particular IgG subclasses, are critical for vaccine and disease responses. Reseachers require such reagents to determine Ig function; diagnostic laboratories use them to measure Ig levels. Currently most investigators rely on polyclonal antisera to identify the swine Ig subclasses. These are tedious to prepare, are rarely class specific, lack immortality, and vary between batches. Thus we proposed to produce mouse monoclonal antibody (mAb) reagents made by hybridoma technology. MAbs recognize only one epitope, thus the name monoclonal. It is generally stated that a mAb can "*recognize a needle **in** a haystack*" whereas a polyclonal recognizes "the needle **and** the haystack". Furthermore, mAb provide a sustainable and renewable resource, so data from laboratories around the world can be compared.

For this project we started by first using molecular techniques to express the 5 known swine IgG genes. [In the process we actually found cDNA evidence for several new swine IgG genes; these are being further characterized and will be used for our renewal grant's expression work.] We worked with a collaborator, Dr. Serge Muyldermans, in Belgium to express each of the 5 previously known swine IgG subclass cDNAs as swine-camelid IgG proteins in vitro using his novel camelid-swine Ig expression system. With this system he expressed each of the 5 specific swine IgG heavy chain genes as camelid-swine Ig constructs. Once developed and purified these constructs were shipped to BARC. Portions of these constructs were shipped to Univ. IA and are now being used to characterize the IgG binding specificity of known mAb. In our next grant we will use them to immunize mice to prepare new IgG subclass specific mAb.

Our overall goal is to have a full panel of well-characterized mAb that react specifically with each swine Ig isotype and IgG subclass so scientists will be able to compare accurately the functions of each swine Ig isotype and subclass. For our second objective, we characterized the reactivity of the currently available mAb anti-swine IgA and IgM as well as anti-IgGs. We identified mAb with excellent specificity for swine IgA and IgM and have worked with USDA APHIS to start to develop a repository for such hybridoma lines and mAb reagents. The anti-IgG mAb are being tested now on the HCAbs, as noted above. Overall, we expect that these reagents will help to expand our understanding of disease control mechanisms and pathologies, as well as serve as improved tools for characterizing swine vaccine responses.

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