SEROLOGICAL RESPONSES OF INFECTED AND VACCINATED PIGS TO RECOMBINANT INFLUENZA A PROTEINS

A THESIS

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ABSTRACT

Influenza A virus is constantly evolving in swine herds and it is important to evaluate the performance of serological diagnostic tools in the screening of pigs. Hemagglutination inhibition (HI) and ELISA are frequently used in the subtype specific detection of swine influenza virus (SIV) infections, but there is poor correlation reported between these assays. Conserved proteins of the virus, such as nucleoprotein (NP) and matrix 1 (M1), are potential antigen candidates to detect antibody response across different SIV subtypes. Although good candidates for use in diagnostic assays, the antibody profile against these proteins in infected and vaccinated animals is not fully understood. In an attempt to improve the agreement between ELISA and HI assays, and to better understand the antibody profile against M1 and NP proteins in infected and vaccinated pigs, 433 sera were screened using an indirect H3 ELISA, 384 samples were screened using a commercial NP ELISA, 433 were screened using recombinantly expressed M1 in indirect ELISA, and 304 were screened using HI. Sera were obtained from pigs vaccinated and challenged with a diverse SIV subtypes in an attempt to mimic the diversity of strains circulating in the field. Agreement between ELISA assays and HI was low, and induction of anti NP and M1 antibodies in vaccinated pigs was delayed in comparison to antibodies directed against hemagglutinin. Contrary to anti NP antibodies, anti M1 antibody levels did not increase after challenge. Although high specificity was obtained, antigenic mismatches between strains used in the vaccination and challenge of pigs seemed to interfere with the sensitivity of H3 ELISA. Anti-NP and -M1 antibodies were detected regardless of the infecting influenza subtype or the strain used for vaccination, but poor performance of M1 ELISA excluded the possibility of using this antigen in a diagnostic tool to screen swine herds for anti-influenza A virus antibodies.

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CHAPTER I: Introduction

Influenza A is a single stranded RNA virus with segmented genome belonging to the family *Orthomyxoviridae*. Influenza A has a broad host range as it is able to infect and replicate in humans, pigs, horses, ferrets, marine mammals, felids, dogs and a variety of avian species. The primary reservoirs for this virus are thought to be wild aquatic birds which are usually asymptomatic carriers responsible for the viral spread across wide geographic locations [1].

Swine influenza H1N1, H1N2 and H3N2 serotypes are endemically circulating in the pig population and are constantly undergoing genetic and antigenic variation [2-5]. Pigs are considered mixing vessels for influenza A viruses since they are susceptible to infection by avian, human, and swine lineage viruses facilitating reassortment of segments and generation of new variants [5-7]. In addition to reassortment of segments, host immunological pressure contributes to selective evolution and antigenic variability of influenza A viruses [8-11].

Swine herds employ several serological assay formats such as enzyme linked immunosorbent assay (ELISA), virus neutralization (VN), and hemagglutination inhibition (HI) to test for antibodies against influenza [12]. Among the serological tools available for screening of influenza A antibodies, HI has high sensitivity and specificity and is generally considered a “gold standard” or reference test; however, test performance can be affected by the virus antigen used, erythrocyte origin, and technician subjectivity. While both ELISA and HI detect exposure to the virus, HI detects neutralizing antibodies making it a preferred test. This assay requires periodic updating of reference strains to maintain its analytical sensitivity [12]. The rigors of the HI test make it difficult to adopt HI at the farm level. This is an inconvenience to field veterinarians who need a rapid, repeatable test for timely intervention during outbreaks or to plan for vaccinations. ELISA is a good alternate for HI as it not prone to the same problems. However, prior art suggests that there is low or no correlation between HI and commercially available ELISAs [13, 14]. Since HI is the reference assay, it is important to compare the results of any new generation tests to evaluate their performance in the field. As the evolution of SIV in swine herds is continuous, and the strains used as antigens in serologic assays are not updated frequently, the levels of antibody

reactivity detected using ELISA might not accurately reflect the serological status of the animals. The comparison between the two assays (HI and ELISA) is expected to provide critical information for updating of ELISA assays and evaluation of their accuracy in the field.

The subtype of swine H3N2 virus currently circulating in swine herds is a triple reassortant between human, avian and swine influenza viruses. This virus first emerged as a double reassortant in swine-herds around 1998. Subsequently, this subtype acquired HA from a human virus that has since evolved in the swine populations into four antigenically different clusters: Cluster I, II, III, and IV [15]. Cluster IV H3N2 viruses are currently the most prevalent in the North American swine population. Understanding the extent of cross-cluster seroreactivity among animals vaccinated or infected with influenza would aid in the interpretation of serodiagnostic test results between SIV subtypes circulating in swine-herds, and evaluate vaccine efficacy. In addition, the establishment of good and practical diagnostic tools for serological screening of influenza in swine herds is extremely important.

Inasmuch as there is rapid change in antigenic sites of hemagglutinin, we hypothesized that hemagglutination inhibition and recombinant H3 (rH3) ELISA would both be able equally to detect serotype specific seroconversion in pigs. We evaluated the extent of cross seroreactivity between animals infected or vaccinated with cluster IV H3N2 virus and those with exposure to a diverse H1N1 and H3N2 SIV. We screened sera using an ELISA developed with recombinantly expressed H3 from a cluster IV H3N2 virus and HI. Seroconversion measured by ELISA was compared with hemagglutination inhibition results. In general, HI and ELISA assay results have not been in agreement. This may be due to the detection of anti- hemagglutinin (HA) antibodies by HI assays whereas commercially available ELISA use whole viruses as antigens, which likely detect not only anti-HA antibodies but also anti–neuraminidase antibodies formed to other influenza protein. We reasoned that the use of rH3 alone in ELISA would provide a better comparison with HI and accounted for this in our study design by using a baculovirus expressed and purified recombinant H3 as the detection reagent.

Since pigs are implicated as a mixing vessel for the emergence of novel reassortant strains[16] , it is important to monitor and unambiguously classify active infections in swine herds. Combinations of diagnostic tools which can identify exposure to the virus, levels of neutralizing antibodies, and can differentiate infected from vaccinated animals would be useful in SIV surveillance and control [12]. In addition to useful and up to date diagnostic assays, vaccinations are also used to control influenza in swine. The vaccines currently used in swine herds are composed of whole inactivated viruses and as there is no replication of the virus in the host, it is expected that antibody response against internal proteins would be low. Internal proteins such as nucleoprotein (NP) and matrix 1 (M1) are relatively conserved across influenza A viruses and detection of antibodies against such proteins are suggestive of infection [17] . Commercial ELISAs are available for detection of antibodies against the NP protein, but there is not a diagnostic tool known to detect anti-M1 antibodies.

The understanding of the pig antibody profile against the M1 protein is important for several reasons. First, M1 has the potential to be used in diagnostic assays to distinguish between infected and vaccinated animals. Second, M1 has been used in the development of universal vaccines. Third, there is little information on the kinetics of antibody response to M1 in a natural infection or when inactivated vaccines are used in pigs[18]. And finally, since pigs are implicated as sources of newly emerging reassortant strains with pandemic potential to humans, it is important to to evaluate the performance of internal proteins of the virus in assays with potential to discriminate between infected and vaccinated animals. To characterize the antibody profile of pigs against the M1 protein and to evaluate the use of this protein to differentiate between infected and vaccinated animals, we screened sera from pigs infected or vaccinated with a variety of influenza viruses using an ELISA based on recombinantly expressed M1 protein. The results obtained in the assay were compared against a commercial NP ELISA, which differentiates infected and uninfected animals. We reasoned that the NP ELISA would also differentiate vaccinated from infected animals, when the history of vaccination in the herd is known. Thus we We postulated that NP ELISA, the M1_ELISA can detect exposure to influenza A virus irrespective of the infecting lineage or subtype**.** As the commercial NP ELISA has been routinely

used by diagnostic labs in the identification of animals exposed to infection, this assay will be used as a reference to compare M1 ELISA.

We tested these hypotheses under two specific aims:

Aim 1: We developed and validated H3 and M1 specific ELISAs using either recombinant H3 hemagglutinin from A/swine/Minnesota/SG-00235/2007(H3N2) (cluster IV virus) or a recombinant M1 protein from A/Wisconsin/629-D01414/2009(H1N1), respectively.

Aim 2: We evaluated antibody cross reactivity of sera from pigs infected or vaccinated with a variety of swine influenza H1N1 and H3N2 viruses using H3 and M1 ELISA, and a commercial NP ELISA. Hemagglutination inhibition (HI) results available for infected and vaccinated animals were analyzed for agreement with those obtained in the H3, M1 and NP ELISAs.

CHAPTER II: Literature review

1) Influenza A viruses

Influenza A is an enveloped RNA virus belonging to the family *Orthomyxoviridae.* The genome spans 13.5-kilobases and consists of eight single stranded RNA segments. Each segment ranges in length from 890-2341 nucleotides and the segments encode a total of 11 proteins [1]. Influenza A viruses are classified into 144 subtypes based on the combination of the currently recognized 16 hemagglutinin (HA) and 9 neuraminidase (NA) variants.

Influenza A is a zoonotic virus with a wide host range including humans, at least 105 bird species, swine, equine, canine, and felines. In the United States alone, more than 200,000 hospitalizations and 36,000 deaths annually are due to complications from seasonal influenza. Globally, it is estimated that influenza causes 300,000 to 500,000 deaths annually [19]. The impact of avian influenza (AI) on the poultry industry has increased nearly 100-fold from 23 million birds affected in a 40-year-period between 1959 and 1998 to over 200 million from 1999 to 2004 [20]. Further, since 1997, multiple cases of human infections with H5N1, H7N7, and H9N2 avian influenza viruses (AIV) have been reported and raised concerns over the pandemic potential of AIV in humans [21]. Since pigs are considered the intermediate hosts for the adaptation of avian viruses to humans increases the possibility of avian viruses causing a human pandemic.

2) Influenza A virus evolution in pigs

Influenza like illness was first identified in swine herds in the United States during 1918 when “Spanish flu” caused millions of deaths in Europe. Swine influenza H1N1 was first isolated from pigs in 1930 by Shope [15] being further classified as classical H1N1 (cH1N1). The virus was relatively stable in swine-herds until the 1998 when a H3N2 double reassortment between humans and swine influenza viruses was identified as causing severe respiratory disease in swine herds of North Carolina[26]. Subsequently, outbreaks involving H3N2 subtype were reported in Iowa, Minnesota and Texas. An avian-swine-human reassortant virus emerged as outbreaks among swine herds were ongoing. The new triple reassortant virus contained NP, M,

and NS genes from swine influenza, HA, NA, and PB1 genes from human influenza, and PA and PB2 genes from avian influenza viruses. The new virus quickly became the dominant genotype circulating in swine herds in United States completely replacing the previous double reassortant H3N2 virus [27] . The "TRIG" or combination of NP, M and NS genes from swine influenza, PB1 from human influenza and PA and PB2 from avian influenza became established in the swine population being frequently identified in progeny viruses as a result of reassortment events[26].

Triple reassortant H3N2 viruses continued to evolve and based on the similarity of their hemagglutinin with those of human influenza viruses they were subdivided into clusters I, II, III, and IV. Each cluster is considered to have originated from the introduction of a new human influenza virus into the pig population. Hemagglutinins from cluster I, II, III and IV are genetically different, but there are reports of antigenic cross reactivity in hemagglutination inhibition and virus neutralization assays between H3N2 from cluster I and III [27]. When Cluster IV H3N2 viruses are identified in swine herds, they are generally the only lineage circulating in that population [28].The reassortment between the classical H1N1 (cH1N1) and the triple reassortant H3N2 resulted in the formation of reassortant H1N1 (rH1N1) containing the TRIG from H3N2 and HA and NA from the cH1N1. The reassortment between the H3N2 and cH1N1 also gave rise to the H1N2 viruses with cH1N1 HA and NA and TRIG from the H3N2 viruses[15] . From 1998 through 2005 the rH1N1 and H3N2 were the dominant subtypes and co-circulated within swine herds of North America. The rH1N1 containing swine influenza hemagglutinin evolved to form alpha, beta and gamma clusters and are represented by the H1N1 and H1N2 viruses [26, 29]. Alpha H1 viruses remained relatively conserved for 68 years until the introduction of the triple reassortment H3N2 in swine herds in 1998. Viruses of this cluster can still be isolated from swine herds but the prevalence is very low compared to other H1 clusters. Gamma and beta H1 viruses were first isolated in swine herds in 2000 and 2001, respectively. Viruses from the gamma H1 cluster have higher prevalence in swine herds than viruses from the beta cluster.

Since 2005 new H1N1 and H1N2 reassortants with human-like (hu) hemagglutinin and neuraminidase have emerged. Such viruses had a TRIG cassette slightly different than the that

identified from the triple reassortant H3N2 and were classified as belonging to the delta cluster-H1[15]. The hemagglutinin of H1N1 and the H1N2 viruses forming the delta cluster are antigenically different forming the subdivisions Delta 1and Delta 2. Neutralizing antibodies induced by Delta 1 viruses may have low neutralization activity during infection with viruses from Delta 2 and vice versa. Viruses from the Delta cluster were first isolated in 2003 and are actively circulating in swine herds to date [28, 29].

In April 2009, a new H1N1 virus emerged in humans in Mexico and Southern California. The virus was found to cause influenza like respiratory illness in people and subsequently in pigs [30].Genome analysis of the virus identified that the new H1N1 was a triple reassortant between currently circulating swine H3N2 and H1N2 viruses from North America and Eurasian avian like swine viruses. After the initial outbreak in 2009, the virus spread throughout North America and the rest of the world. In 2010, according to reports from the University of Minnesota Veterinary Diagnostic Laboratory, the 2009 pH1N1 virus in pigs was detected in 22% from porcine samples submitted for influenza A virus diagnostic tests between January and November of 2010**(Figure 1).**

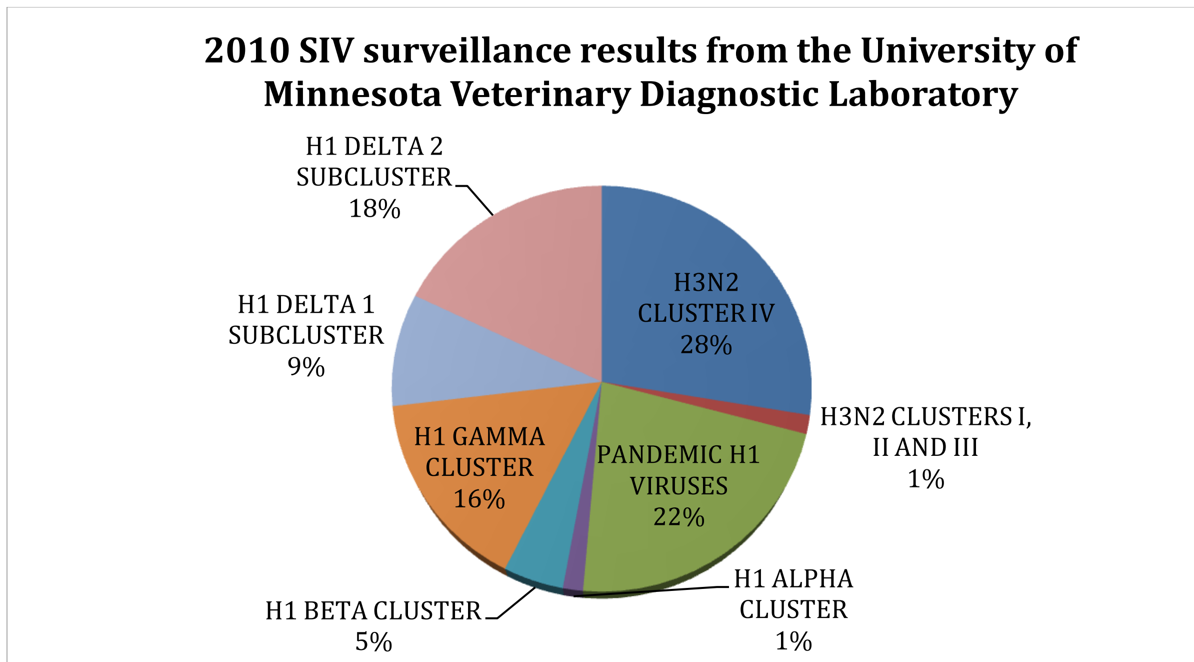

Fig 1: Influenza virus detection in swine respiratory cases received between January and November of 2010.

Figure adopted from:http://www.flusurexp.com/AboutSIV/SIV-diagnostic-trends.html

3) Immune response of the host against influenza A virus

Influenza A virus stimulates both cellular and humoral immune responses in infected hosts. Humoral response generally leads to clearance of the virus from the respiratory tract and the cellular response is involved in elimination of infected cells and partial protection against heterosubtypic influenza strains.

4) Humoral response against influenza

The humoral response against influenza A virus is usually subtype specific and directed primarily to the hemagglutinin and neuraminidase proteins. Antibodies raised against one subtype usually show low neutralization activity and cross reactivity with other subtypes.

Hemagglutinin and neuraminidase are immunogenic viral surface proteins that elicit high levels of neutralizing antibodies. Neutralizing antibodies are important in virolysis, antibody dependent cell

cytotoxicity (ADCC), complement dependent cytotoxicity (CDC), opsonization, and antibody-mediated phagocytosis. The M2 ion channel, although a surface peptide, does not elicit high levels of antibodies during infection or vaccination [31].

Production of secretory IgA immunoglobulin is particularly important in intracellular neutralization of the virus and clearance of the virus from the respiratory tract [32]. The presence of IgA in infected hosts is correlated with reduction of clinical signs, reduced shedding, and lower recovery time [33]. Presence of IgM immunoglobulin in infected hosts is correlated with decrease of viral particles in the blood and protection of infected individuals. In challenged naïve animals, IgM is produced near the beginning of the infection cycle, as early as 7 days post-infection, and decreases over time. The decrease in IgM is accompanied by increasing levels of IgG, which can be detected in the blood up to 28 days post infection. [34]. In the swine host, IgG is widely distributed and can be found in the respiratory mucosa potentially aiding in the clearance of the virus from the respiratory tract. In animals previously exposed to influenza, memory response can induce the production of IgA and IgG antibodies as early as 3 days post infection [32], with detection in the serum typically occurring 5 days post-infection.

Pigs are usually immunized with polyvalent inactivated vaccines containing different variants of H1N1 and H3N2 SIV [35]. This type of vaccine usually induces high level of neutralizing antibodies against surface viral proteins. Antibody response against internal proteins such as M1 and NP can be detected during vaccination and infection whereas antibodies against NS1 and NS2 are induced exclusively during active infection of the host [34]

4.1) Humoral response against viral proteins

4.1.1) Hemagglutinin

Hemagglutinin is highly immunogenic inducing detectable IgM and low IgG serum immunoglobulin titers before 7 days post infection. After 7 days post infection IgM titers against the hemagglutinin drop and IgG becomes the predominant immunoglobulin serologically detected in infected animals [34].

Most of the antibodies against the hemagglutinin are directed against the globular head or HA1 part of the protein. These antibodies inhibit the binding of the virus to the cellular receptors potentially blocking viral entry and subsequent replication. The HA1 part of the hemagglutinin is highly variable and antigenic changes in this region are generally correlated with the antigenic drift or immunological escape of the virus. The stalk region of the hemagglutinin is relatively conserved across diverse influenza subtypes, and is considered a good candidate for a "universal" vaccine against influenza. Antibodies directed against the stalk region of the hemagglutinin are limited during natural infection of the virus. Therefore it is unlikely that these antibodies significantly contribute to neutralization of the virus in vivo [36].

4.1.2) Neuraminidase

Akin to hemagglutinin, the neuraminidase is a highly immunogenic and variable surface protein. Anti-neuraminidase antibodies inhibit the release of viral particles during budding from infected cells and are correlated with decrease in disease severity, duration, and viral replication. Antibodies against neuraminidase are subtype specific but seem to cross react within neuraminidases of antigenic drift variants within a serotype [37]. In contrast to hemagglutinin, there is no detectable IgM response against neuraminidase early in the infection cycle. The levels of IgG start to rise at 7 days post infection and can be detected in the serum up to 28 days post infection [34].

4.1.3) Matrix 1 and nuclear protein

Internal proteins of the virus are strongly associated with stimulation of cellular immune response, especially T cell responses. The neutralization role of antibodies against proteins internal to the virus is questionable [34], but some studies describe that antibodies against such proteins play a role in reduction of viral titers and decreased mortality after challenge in mice [38].

The nucleoprotein is able to induce detectable levels of serum IgM and IgG by 7 days post infection while M1 induces a delayed IgG response compared with HA starting at 14 days post infection [34]. As replication of the virus in the host results in production of NP and M1 proteins,

one might think that the antibody response against NP and M1 proteins will be higher in infected than in vaccinated pigs. With the lack of a clear understanding about the antibody profile against these proteins it is difficult to predict if serological assays based on internal proteins of the virus can be used to discriminate between infected and vaccinated pigs. Thacker et. al described a non uniform seroconversion against M1 protein when analyzing serum samples from pigs infected and/or vaccinated with influenza using indirect M1 ELISA[18]. In her studies, there was not a clear understanding of the impact of vaccination or challenge in induction of anti M1 antibodies, which leaves room for further investigation. Zimmerman and his group described the high sensitivity of the commercial NP ELISA from IDEXX laboratories in the identification of positive pigs before 7 days post infection. Although the assay is extremely sensitive in detecting anti NP antibodies in infected pigs, there is no description of its performance when analyzing serum samples from vaccinated animals[17]. Having said that, before serological assays based on internal proteins of the virus are develop to screen swine herds, it is necessary to have a better understanding of the antibody profile of infected and vaccinated pigs against these proteins. The development of our study comes to aid in the understanding of how to interpret and use the serological assays in swine herds.

5) Cell mediated immune response against influenza

Cell mediated immune response against influenza is usually stimulated during infection of the host. Internal viral proteins such as M1, NP, PB1 and PB2 have been shown to induce T cell response during infection[39, 40]. Contrary to the surface proteins of the virus, internal proteins are relatively conserved within influenza A viruses, and cellular immunity against one subtype can provide protection against a heterosubtypic challenge. [41]. Among the internal proteins of the virus, nucleoprotein is known to induce the strongest T cell response against influenza [17, 32]. Cellular and humoral responses against influenza are interconnected and triggering of cellular immunity against internal proteins of the virus results in stimulation of antibody response against the same. Replication of the virus in the host's epithelial cells causes exposure of viral peptides in MHC-I molecules, which are recognized by natural killer cells (NK) and cytotoxic $CD8^+$

lymphocytes (CTL). Natural killer and CTLs eliminate infected cells by induction of apoptosis by the proteolytic action of granzymes in intracellular caspases, or cell lysis by introduction of porforins in the cell membrane. The phagocytosis of apoptotic bodies from infected cells by antigen presenting cells (APC) such as macrophages and dendritic cells results in exposure of viral proteins in MHC-II molecules and stimulation of $CD4^+$ lymphocytes precursors or Th0 cells. Secretion of interleukin 12 (IL-12) by the APC induces Th0 differentiation into Th1 lymphocytes, important in the production of IL-2 and interferon (IFN) interleukins. Production of IFN is especially important during viral infections because IFN stimulates secretion of IgG2 by B cells, and induces an “antiviral” state in healthy cells neighboring infected ones. Production of IL-10 by APC induces Th0 differentiation into Th2 lymphocytes producing IgA and IgG immunoglobulins [40]. Although internal proteins of the virus play a stronger role in eliciting cellular immunity, the understanding of the immune responses to these proteins can help elucidate the viral replication cycle and its connection to the antigen presentation repertoire by the host leading to clearer interpretations of diagnostic serology results.

6) Serology tests

As previously stated, the serological tests frequently used to identify influenza status in swine herds are virus neutralization, ELISA, and hemagglutination inhibition. Commercial ELISAs generally use nucleoprotein, or whole H1N1 or H3N2 virus as antibody detection reagents [42, 43]. Both ELISAs are commercialized by IDEXX laboratories.

6.1) Hemagglutination Inhibition assay

Hemagglutination inhibition is the gold standard assay most commonly used to detect antibodies against influenza A viruses in humans and animals. The guidelines for the animal assays are set forth by the World Organization for Animal Health (OIE) and the Centers for Disease Control and Prevention (CDC) provides protocols for human assays. The antigens used in the assay can vary between laboratories according to the local prevalence of influenza subtypes in the region where the laboratory is located. During the development of the assay reference strains or strains that

are antigenically related within a subtype are used in the screening of test sera. The reference strains for human influenza A viruses are regularly updated based on changes in the epidemiology of the virus in a region [12] [43]; however, such updates are not routinely available for animal influenza A viruses.

Red blood cells (RBC) have a high quantity of sialic acid covering their external surface. As the influenza virus has the ability to bind to sialic acid receptors, binding of the virus to its receptor causes the agglutination of RBC. Before being exposed to the virus, the RBCs are in suspension, Binding of the virus to its receptor on the surface of RBCs results in the formation of a "mesh" that stays in suspension instead of precipitating at the bottom of the plate. This is the principle of the **hemagglutination assay,** and the hemagglutination assay can be used to determine the hemagglutinating ability of viruses such as influenza A viruses, avian paramyxoviruses, and parvoviruses**.** This ability to agglutinate erythrocytes is also the basis for detecting antibodies in serum. When antibodies are present in the sera, the virus becomes bound to the antibodies and hemagglutination is thus inhibited. The erythrocytes settle to the bottom of round wells in the assay plates indicating a positive hemagglutination inhibition antibody serum titer..HI detects antibodies against the viral hemagglutinin mostly IgM and IgG immunoglobulin [43].

High HI titers are usually correlated with protection of the animals against the strains used in the assay, but animals with low HI titers can also show protection during challenge[44]. This probably happens due to cellular immunity or presence of cross-reactive antibodies not detected in the assay.

Although HI is considered as the gold standard assay to screen for influenza this assay is accompanied by several pitfalls. The main limitation of the assay is the low reproducibility of results between different laboratories. The low reproducibility of results might be affected by variability due to personnel developing the assay, quantification of viral titer, and type of RBC used in the assay. In addition, some influenza strains show lower capacity of agglutinating RBC which results in misinterpretation of anti influenza antibody titers in pigs.

6.2) Enzyme linked immunosorbent assay

Two types of commercial ELISAs are frequently used in the serological screening of swine herds: indirect H1N1 and H3N2 ELISA, and competitive nucleoprotein ELISA.

6.2.1) Indirect ELISA

The Indirect ELISA is a way to detect if antibodies specific for an antigen are present in serum samples submitted to the assay.

The assay is based on the coating of polystyrene plates with the antigen of interest followed by addition of the test sera. The antibodies present in sera are called primary antibodies and when specific for the antigen coating the plates there is binding with high affinity to the antigen. After washing the plates and removing antibodies which are non specific for the antigen or bind to it with low affinity, there is addition of a secondary antibody which is specific for the primary antibody used in the assay. The secondary antibody is usually conjugated with enzymes such as Horseradish Peroxidase (HRP) and addition of substrate specific for the enzyme results in the development of a colored product. The substrate used to develop color in ELISA reactions are usually hydrogen donors which will contribute with oxidation of the enzyme and formation of a colored product which is quantified using spectrophotometer. When exposed to its substrate, the HRP enzyme undergoes oxidation by H_2O_2 resulting in an intermediate referred to as HRP I. . HRP I is then reduced receiving one electron from an hydrogen donor resulting in another intermediate HRP II and a donor radical.. HRP II is further reduced by transfer of one electron by a hydrogen donor to regenerate the original enzyme an form a second donor radical. The combination of the two donor radicals yields a detectable colored product which can be quantified using the spectrophotometer. The machine emits light with the wavelength specific for the colored product formed and the amount of colored product is converted in optical density values (OD values).Higher OD values correspond to higher levels of antibodies detected in the assay. Horseradish Peroxidase is compatible with 2,2-azino-di (3-ethyl-benzathiazoline) sulphonic acid (ABTS substrate), and also tetramethylbenzidine (TMB). The reaction with the first substrate

yields a blue-green colored product, and reaction with the second substrate results in a dark blue color. The reaction with both substrates can be stopped with addition of an acid such as 1N HCL or sulfuric acid. The reaction with ABTS can be measured using 405nm and the reaction with TMB can be read at 450nm when stopped with acid solutions, or at 650nm when not [45].

In indirect ELISAs higher O.D. values represent reactive or positive samples, and low O.D. values represents nonreactive or negative samples. The H1N1 and H3N2 ELISAs available for screening of pig sera are commercialized by IDEXX Laboratories, ME, USA, and have whole viruses coating the plates. The cutoff values to classify positive and negative values are established by the company [46] .

6.2.2) Competitive ELISA

In competitive ELISA antibodies conjugated with detection enzymes (competitive antibodies) compete with primary antibody, or antibodies present in the test serum, for binding to the antigen. When the test samples have a high enough antibody concentration specific for the antigen coating the plates, the binding of competitive antibodies is inhibited, and these antibodies are eliminated from the assay during the washing of the plate. The competitive antibodies are commonly conjugated with enzymes such as HRP, and the color development can be reached by adding TMB or ABTS to the assay. The nucleoprotein ELISA is standardized for use with pig sera [17] and is commercialized by IDEXX Laboratories, ME, USA. The plates are coated with avian influenza nuclear protein, which isis relatively conserved in influenza A viruses, so that anti NP antibodies in the pig sera can be detected in the assay.

7) Development and validation of H3 and M1 specific ELISAs using either recombinant H3 hemagglutinin from A/swine/Minnesota/SG-00235/2007(H3N2) (cluster IV virus) or a recombinant M1 protein from A/Wisconsin/629-D01414/2009(H1N1), respectively.

7.1) Material and methods

7.1a) Amplification of hemagglutinin gene: The primers used in the amplification were designed based on the HA sequence of (SIV) A/swine/Minnesota/SG-00235/2007(H3N2), in GenBank (Accession CY035419; Gi: 209866393). The design was based on the open reading frame of the HA gene excluding the untranslated regions, signal peptide, transmembrane domains and cytoplasmatic tail of the protein. Viral RNA was extracted using QIAamp Viral RNA Mini Kit (Qiagen) and used to perform a cDNA reaction with one step Super script III First Strand Synthesis System (Invitrogen). The RT step was performed with gene specific reverse primer following the manufacturer's directions. The amplified cDNA was used in 100 µl PCR amplification using KOD High Fidelity HotStart Taq Master Mix (Novagen). PCR reactions consisted of a total of 30 cycles of amplification. The PCR product was desalted and cleaned of unincorporated primers and dNTPs using QIAquick PCR Purification Kit (Qiagen) and restricted with enzymes AVR II and Bam HI.

A) Forward primer

AAGGATCCACGCTGTGCCTGGGACATC

AA: spacer;

GGATCC: BamHI restriction site;

ACGCTGTGCCTGGGACATC: 3' end of the HA gene excluding the signal peptide

B) Reverse primer

TT**CCTAGG**CACCAGCTGAACACCGTTGATCTGGAACCG

TT**:** spacer;

CCTAGG: Avr II restriction site;

CCTAGGCACCAG: thrombin protease cleavage site

CTGAACACCGTTGATCTGGAACCG: 5’end excluding the gene sequence of the transmembrane and cytoplasmatic domain.

Fig.2 Primer design

7.2) Insert and plasmid restriction digestion and ligation: The HA insert and the PacGp67 transfer vector were separately digested with AVR II and Bam HI restriction enzymes at 37°C/2hours using a thermal cycler. Between digestions and after the final digestion the reactions were purified using MinElute Reaction Cleanup kit (Qiagen). Ligation of the insert to the vector was performed with using T4 ligase 100 Weiss Units (Promega), LigaFast™ Rapid DNA Ligation buffer (Promega) at the insert: vector ratio of 5 or 10:1. The PacGp67 vector and a protocol to clone H3 were kindly provided by Rebecca DuBois at the Saint Jude Children’s Hospital. The vector includes features such as his/strep tags, thrombin digestion site, and foldon that are not included in the transfer vector commercially sold by Becton Dickinson.

ATGCTACTAGTAAATCAGTCACACCAAGGCTTCAATAAGGAACACACAAGCAAGATGGTAAG

CGCTATTGTTTTATATGTGCTTTTGGCGGCGGCGGCGCATTCTGCCTTTGCGGCGgatcttggatc

cACGCTGTGCCTGGGACATCATGCAGTACCAAACGGAACGTTAGTGAAAACAATCACGGATG

ACCAAATTGAAGTGACTAATGCTACTGAGCTGGTTCAGAGTTCCTCAACAGGTAGAATATGC

AACAGTCCTCACCAAATCCTTGATGGGGAAAATTGCACACTGATAGATGCTCTATTGGGAGA

CCCTCATTGTGATGACTTCCAAAACAAGGAATGGGACCTTTTTGTTGAACGAAGCACAGCCT

ACAGCAACTGTTACCCTTATTATGTGCCGGATTATGCCTCCCTTAGGTCACTAGTTGCCTCAT

CCGGCACCCTGGAATTTACCCAAGAAAGCTTCAATTGGACTGGAGTTGCTCAAGATGGATCA

AGCTATGCTTGCAGAAGGAAATCTGTTAACAGTTTCTTTAGTAGATTGAATTGGTTGCATAAT

TTGAATTACAAATATCCAGCGCTGAACGTAACTATGCCAAACAATGACAAATTTGACAAATTG

TACATTTGGGGGGTTCACCACCCGGGTACGGACAGGGACCAAACCAACCTATACATTCAAG

CATCAGGGAGAGTTACAGTCTCCACCAAAAGAAGCCAACAAACTGTAATCCCGAATATCGGG

TCTAGACCCTGGGTAAGGGGTGTCTCCAGCATAATAAGCATCTATTGGACAATAGTAAAACC

GGGAGACATACTTTTGATTAACAGCACAGGGAATCTAATTGCCCCTCGGGGTTACTTCAAAA

TACAAAGTGGGAAAAGCTCAATAATGAGGTCAGATGCACCAATTGGCAACTGCAATTCTGAA

TGCATTACTCCAAATGGAAGCATTCCCAATGACAAACCTTTTCAAAATGTAAACAGGATCACA

TATGGGGCCTGTCCCAGATATGTTAAGCAAAACACTCTGAAATTGGCAACAGGAATGCGGAA

TGTACCAGAGAAACAAACTAGAGGCATATTTGGCGCAATCGCAGGTTTCATAGAAAATGGTT

GGGAGGGGATGGTGGACGGTTGGTACGGTTTCAGGCATCAAAATTCTGAAGGCACAGGACA

AGCAGCAGATCTTAAAAGCACTCAAGCAGCAATCAACCAAATCACCGGGAAACTAAATAGAG

TAATCAAGAAAACGAACGAGAAATTCCATCAGATCGAAAAAGAATTCTCAGAAGTAGAAGGG

AGAATTCAGGACTTAGAGAAATACGTTGAAGACACTAAAATAGATCTCTGGTCTTACAACGC

GGAGCTTCTTGTTGCCCTGGAGAACCAACATACAATTGATTTAACTGATTCAGAAATGAACAA

ACTGTTCGAAAGAACAAGAAAGCAACTGCGGGAAAATGCTGAGGACATGGGCAATGGTTGC

TTCAAAATATACCACAAATGTGACAATGCCTGCATAGGATCAATCAGAAATGGAACTTATGAC

CATGATGTATACAGAAACGAGGCATTAAACAATCGGTTCCAGATCAACGGTGTTCAGCTGGT

GCCTAGGGGCTCCCCTGGTTCCGGCTACATTCCTGAGGCTCCTCGTGACGGACAGGCTTAC

GTTCGCAAGGACGGTGAGTGGGTTCTGCTCTCCACCTTTCTGGGTCATCACCACCATCATCA

CGGAGGCTCCTGGTCCCATCCTCAGTTTGAGAAG

Fig 3: *DNA sequence the multiple cloning site of Pacgp67-B plasmid containing the H3 insert and plasmid features*. **Yellow:** Plasmid strong Gp67 secretion signal; **Pink:** AVR II restriction site completing Trombin cleavage site; **Red:** restriction site of Bam HI; **Dark Blue**: Foldon; **Light Blue:** Trombin cleavage site; Green **and Purple**: purification tags; and **Grey:** H3 insert

The foldon feature included in the plasmid mimics the transmembrane domain and helps the expressed protein to maintain its correct folding and three dimensional structures. The plasmid carrying the insert was transformed into one-shot chemically competent E. coli (Invitrogen) and positive clones were selected for sequencing**.** After completion and confirmation of the correct HA and plasmid feature sequences the plasmid was used to perform co-transfection with linear DNA in insect cells.

A/swine/Minnesota/SG-00235/2007(H3N2) hemagglutinin segment 5'to 3'

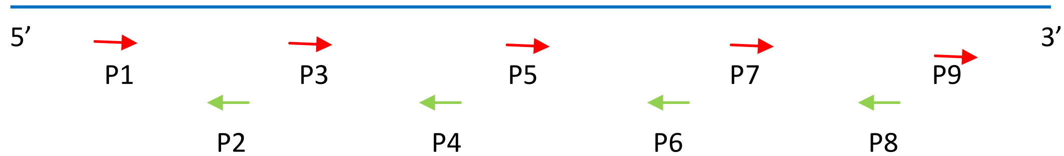

Fig 4: *Sequencing strategy*. A total of 9 primers (P1 to P9) were used to sequence the insert in the plasmid. The forward and reverse primers were distant 200 nucleotides from each other. The forward and forward and reverse and reverse primers were distant 300 nucleotides from each other.

7.3) Production of recombinant protein: Recombinant H3 was expressed in a baculovirus expression system using protocols and vectors established by our collaborators at St. Jude Center for Excellence in Influenza Research and Surveillance (SJCEIRS). This system is based on the generation of recombinant baculovirus carrying the H3 gene incorporated in its genome. Briefly, to create rBaculovirus, linear DNA (BD BaculoGold) and a transfer plasmid PACgp67-B (BD BaculoGold) carrying the H3 gene were co-transfected in *Spodoptera frugiperda* insect cells (Invitrogen). First, linear DNA was mixed with transfer plasmid in the ratio of 1:10 and incubated for 15 minutes (mixture1). After 15 minutes 30µl of Sf-900 serum-free medium Complete 1X (Novagen) was added to mixture1 and homogenized by pipetting resulting in mixture 2. After homogenization 150 µl of serum free medium containing 50 µl of insect gene juice reagent (Novagen) was added to the mixture 2 and the new medium, insect gene juice, linear DNA, and plasmid (mixture 3) was incubated at room temperature for 25 minutes. After the incubation period mixture 3 became turbid and 750 µl of cell culture medium (mixture 4) was added to it. To start the transfection of sf9 cells with linear DNA and plasmid, 1 ml of mixture 4 was added to 70% confluent sf9 monolayers. The monolayers were incubated for a maximum of 6 hours at 27°C and then to stop transfection 3 ml of serum free media with antibiotic-antimycotic (Invitrogen) agents was added to each flask. Monolayers that could not reach 90% confluency by the end of the third day were considered to have the first stock of recombinant virus (P1) and this supernatant was harvested. To amplify P1 (which should have about 10^2 pfu/ml) 1 ml of the

supernatant harvest from flasks with less than 90% confluence was added to t-75 flasks containing 70% confluent sf9 monolayers and these flasks were incubated for 5 days. By the end of the fifth day the supernatant of monolayers that could not reach 90-100% confluency were harvested and contained passage 2 (P2) recombinant baculovirus. The viral titer of P2 was about 10^6 $TCID_{50}$ and when amplified to P3, a 10^9 $TCID_{50}$ was achieved. Before proceeding with the amplification of P3, protein precipitation was performed in the supernatant of P2 infected flasks and samples were analyzed by Western blot. After identification of positive bands using the anti-histag antibody, P2 was amplified to P3 and used to infect 300 ml of $2x10^6$ sf9 cells/ml to express larger quantities of protein.

7.4) Protein expression and purification: With the confirmation that the rBaculovirus was producing the H3 hemagglutinin protein expression was conducted by incubating 300 ml of $2x10^6$ sf9 cells/ml 3 days at 27°C with MOI 10 of virus. First 300 ml of 90-100% viable sf9 cells with less than 10 passages were seeded into roller flasks at a concentration of $2x10^6$ cells/ml. The flasks were incubated at 27°C in a roller flask incubator with constant rotation. Next day the cells were infected with P3 viral titer 10^9 $TCID_{50}$ MOI 10 and incubated for 3 days. On the third day supernatant of infected monolayers was harvested and centrifuged 1500 rpm/15 min. Cell pellets were discarded and complete protease inhibitor cocktail tablets (Roche) added to the supernatant. Protein purification was performed at 4°C in supernatant using 2 ml of cobalt resin (PIERCE Scientifics) following the batch method protocol suggested by PIERCE Scientifics. Washing steps were performed 5 times and elution of the protein was performed using Gravity-flow Columns (PIERCE Scientifics). Recombinant H3 was eluted in two fractions. Previous experiments done to standardize the best MOI to infect sf9 monolayers in roller flasks showed that a total of 26.5 µg/ml of rHA could be purified from $2x10^8$ sf9 cells infected with MOI 10. The amount of protein expressed can vary depending on the cell passage number and quality of the viral stock used in the infections.

7.5) Indirect H3 and M1 ELISA

Recombinant H3 or M1 proteins diluted in carbonate buffer pH 9.6 were used to coat high binding Costar strips (Corning). The plates were incubated overnight at 4°C with a total of 100 ng/well of recombinant protein. Before use the plates were washed three times with 1X PBST (G Biosciences) using plate washer (Fisher Scientifics) and blocked 2 hours at room temperature with 300µl/well of 1X PBST with 5% non fat dry milk (NFDM) 0.05% Tween 20, pH9.6. After blocking, plates were washed three with 1X PBS and 100 µl of pig sera diluted 1:200 was added to each well. Sera were diluted in 1X PBST with 5% NFDM and all samples were tested in triplicates. After addition of the test samples plates were incubated at 37°C for 30 minutes. Following incubation plates were washed three times with 1X PBST and 100µl of pig IgG-Fc antibody HRP conjugated (Bethyl laboratories), at a dilution of 1:50,000, was added to each well. Plates were incubated and 37°C for 30 min and washed three times with 1X PBST. To develop the assay 100 µl of TMB Peroxidase Substrate (KPL) was added to each well and plates were incubated at room temperature for 8 minutes. To stop the reaction100µl of 1N HCl was added to each well. Plates were read at 450nm using SpectraMax M2 Microplate reader (Molecular Devices).

Plates containing the rH3 had the following controls:

A) Primary antibody or test sera and TMB substrate;

B) Secondary antibody and TMB substrate;

C) Pig sera positive to Pfizer Vaccine strain H3N2 cluster IV, secondary antibody, and TMB substrate (Positive control);

D) Gnotobiotic influenza negative pig sera, secondary antibody, and TMB substrate (Negative control).

The H3 protein used to coat the plates was more than 90% pure confirmed by SDS PAGE. The amino acid sequence of the protein was determined and it was confirmed to have the same sequence H3 as A/swine/Minnesota/SG235 2007(H3N2) by mass spectrometry.

The M1 protein used in the ELISA was recombinantly expressed in *Escherichia coli* and purchased at Bioclone Inc, San Diego CA. The DNA sequence used to express the protein came from A/Wisconsin/629-DO1414/2009(H1N1) and the protein represents amino acid 21 to 252. The N terminus of the M1 protein was fused to an HIS tag and the molecular weight is approximately 28 kDa. The purity level of the expressed protein was confirmed to be more than 90% by SDS-PAGE.

The controls used in the M1 ELISA were:

A) Primary antibody or test sera and TMB substrate;

B) Secondary antibody and TMB substrate;

C) Sera from animals 14 days post infection with A/Sw/IA/00239/04(H1N1), secondary antibody, and TMB substrate (Positive control);

D) Gnotobiotic pig serum (negative control), secondary antibody, and TMB substrate.

According to reports of Thacker and colleagues antibody response against M1 protein was shown to be delayed in infected and vaccinated pigs[18]. Based on this information, and in the fact the pig sera positive to the Pfizer Vaccine used as positive control in H3 ELISA did not reacted in the M1 ELISA, serum samples from study 2 were screened in M1 ELISA to evaluate reactivity. As serum samples from this study corresponded to 14 days post infection some samples reacted with OD values higher than 1 in the M1 ELISA. The serum sample that showed highest reactivity in the assay, reaching OD values of 1.7, was used as a positive control in all M1 ELISA screenings.

7.6) NucleoProtein ELISA

The commercial “FlockCheck” or Multi Screen Avian Influenza Virus antibody test kit (IDEXX Laboratories, Maine, USA) was used in the screening of the samples. The assay was previously validated for use with pig sera by Zimmerman and his group and also by the University of Minnesota Veterinary Diagnostic Laboratory. The cut-off value adopted in our study was 0.6 OD value according to Zimmerman and his group[17] . Samples with OD values higher than 0.6 were classified as negative and below 0.6 classified as positive. The assay was performed in a 96 well plate coated with avian influenza nucleoprotein. As controls 100µl of avian influenza positive and negative chicken sera were added in duplicates to the plates. The pig sera tested was diluted 1:10 in sample diluent buffer, and 100µl of the diluted sera was added in triplicates to the plates. Plates were incubated 1 hour at room temperature and then washed 4 times with 1X washing concentrate using an automated washer, 4Mk2 (Fisher Scientifics). A total of 100µl per well of anti avian influenza Horseradish Peroxidase conjugate was added to each well and plates were incubated for 30 min at room temperature. After incubation plates were washed 4 times with 1X washing concentrate using plate washer, and 100µl of TMB substrate was subsequently added to each well. Plates were incubated for 15 minutes at room temperature and addition of 100µl of stop solution stopped the reaction. Plates were read at 650 nm using SpectraMax M2 Microplate reader (Molecular Devices).

8) Serum samples

A total of 433 serum samples were taken from three different longitudinal studies **(Figure 5)** and screened using ELISA H3, M1 and NP. Study 1 had a total of 129 samples, study 2 had 90 samples, and study 3 had 214 samples. In study 1, groups of animals were vaccinated with homologous vaccine containing A/swine/Colorado/00294/2004 (H3N2), or one of three heterologous inactivated vaccines containing H3N2 and H1N1 SIV subtypes. The animals were vaccinated at two times points: day 0 and day 14. The heterologous vaccines were from a commercial source and contained H3N2 viruses from cluster I. At day 28 the animals were challenged with a cluster III A/swine/Colorado/00294/2004(H3N2) virus and euthanized at day 35.

Serum samples were collected at each time point and screened in H3, M1 and NP ELISA. Serum samples collected before vaccination at day 0 were considered as baseline values. In study 2, animals were vaccinated at day 0 and 14 with a heterologous trivalent vaccine containing A/Swine/Missouri/069/05(H3N2), A/swine/North Carolina/031/05 (H1N1), and A/swine/Iowa/110600/00 (H1N1), or with a homologous vaccine containing A/swine/Iowa/00239/04 (H1N1). The heterologous vaccine used in the study was from Pfizer and contained a cluster IV H3N2, an H1N1 strain representing Delta H1 group, and a second H1N1 strain representing Gamma H1 group [47]. Animals were challenged at day 30 and euthanized at day 43. Only serum samples from day 43 were available for screening using H3, M1, and NP ELISA. Hemagglutination inhibition titers against vaccine and challenge strains were available for the 43-day time point. Serum samples from study 3 represented animals vaccinated at day 0 and 21 with a commercial experimental vaccine containing H1N1 and H3N2 strains. Serum samples from days 21, 35, and 56 were screened using ELISA H3, M1 and NP and HI titers were also available for each time point of the study. The name of the SIV strains included in the experimental vaccine of study 3 could not be disclosed due to confidentiality polices from Newport laboratories.

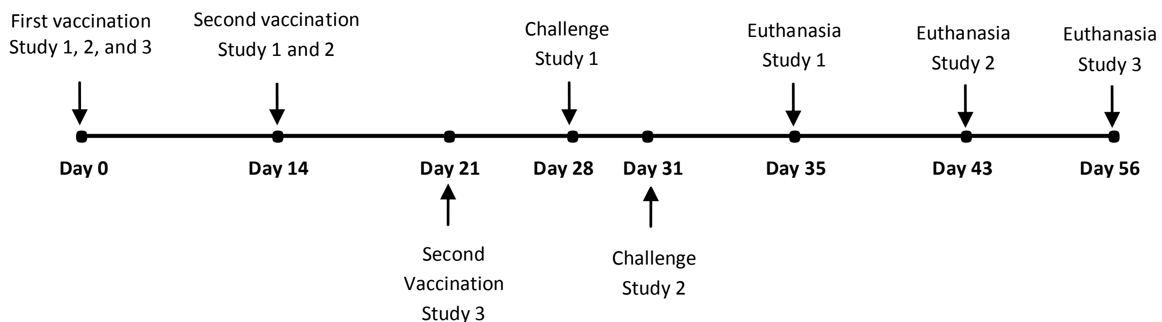

Figure 5. *Summary of studies 1, 2, and 3*. Study 1 had serum samples from day 0, 14, 28, and 35 screened by ELISA H3, M1, and NP. Study 2 had serum samples from day 43 screened by HI, ELISA H3, M1 and NP. Study 3 had serum samples from day 21, 35, and 56 tested by HI, ELISA H3, M1, and NP.

9) Statistical analysis

Of a total of 433 serum samples available from the three studies, 384 samples were screened using NP ELISA, 304 samples were screened using hemagglutination inhibition assay, and all 433 samples were screened using H3 and M1 ELISAs.

9.1.1) Association of serum ELISA tests and HI H3 results

The association of the serum ELISA results for H3, M1, and NP with the results of the HI-H3 were studied using a linear regression, where the results of the serum ELISA tests were used as dependent variables and the results of the HI-H3 as independent variable. Models also included the study source of the data, and the 2-way interaction source of the data and HI-H3 test results. Test results of the serum ELISAs and HI-H3 were transformed using logarithm (base 10) to accomplish the linear association and homoscedasticity assumptions. Negative results of the HI-H3 test, usually reported as “0,” were changed to “1”and all serum ELISA test results with negative value were changed to 0.0001. Model assumptions were finally evaluated using the residual plot. Statistical significance was declared at $P \leq 0.05$ and all models were fitted using Proc Mixed of SAS (SAS/STAT v.9.2 SAS Institute Inc, Cary, NC).

9.1.2) Association of serum ELISA tests and HI-H1 results

A linear model was used to study the association of the results of the 3 different serum ELISA tests (H3, M1 and NP) and the results of the HI-H1. Models included serum ELISA test results as dependent variables and the results of the HI-H1 as independent variable. Hemagglutination inhibition of the 5 different strains of H1 included on the study (Study 2 A/swine/North Carolina/031/05 (H1N1), Study 2 A/swine/Iowa/110600/00 (H1N1), Study 2 A/swine/Iowa/110600/00 (H1N1), and Study 3 H1N1, and the 2-way interaction of HI-H1 results and the H1 strains were included in all models as covariates. Test results of the serum ELISAs and HI H1 were transformed using $\log_{10}$ to accomplish the linear association and homoscedasticity assumptions. Negative results of the HI-H3 test reported as “0” were changed to “1”and all serum ELISA test results with negative value were changed to 0.0001 to accomplish

real numbers from log transformed data. Model assumptions were finally evaluated using the residual plot. Statistical significance was declared at $P \leq 0.05$ and all models were fitted using Proc Mixed of SAS (SAS/STAT v.9.2 SAS Institute Inc, Cary, NC).

9.1.3) Association among serum ELISA NP, M1 and H3

Three linear regression models were used to explore the association between ELISA M1 and NP, M1 and H3, and NP and H3. Models included ELISA M1 results explained by ELISA NP results, ELISA M1 results explained by ELISA H3 results, and ELISA NP results explained by ELISA H3 results. The study source of the data was included in all models as explanatory variable and the 2-way interaction between source of study and serum ELISA test results were also included in the models if significant. Statistical significance was declared at $P \leq 0.05$ and all models were fitted using Proc Mixed of SAS (SAS/STAT v.9.2 SAS Institute Inc, Cary, NC). ELISA M1, NP and H3 results were transformed using logarithm (base 10) to accomplish the linear association and homoscedasticity assumptions of the linear models. Negative values on the serum ELISA results were changed to 0.0001.

10) Phylogenetic analysis

A phylogenetic tree was generated to evaluate the relative evolutionary position of the H3 hemagglutinin DNA sequences of SIV used in infection or vaccination of animals in study 1, 2 and 3, and the hemagglutinin sequence of the cluster IV hemagglutinin used in ELISA screenings. The tree was build using neighbor joining, 1000 bootstraps, and Molecular Evolutionary Genetics analysis program (MEGA) version 5 **(Figure 17)**[48]. The numbers represented in the nodes of the tree represent the percent of times sequences clustered together. The sequences were uploaded from the National Center for Biotechnology Information (NCBI) and were chosen according to Christopher Olsen's paper describing H3N2 triple reassortants SIV from cluster IV [49]. Based on Olsen's work we included the same hemagglutinin sequences to represent Clusters IV, III, II and I. Cluster IV H3 were represented by NCBI accession numbers GI 94404591, GI 94404583,GI 94494587, GI 94494593,GI 94404589, GI 94404585,GI 84626335,

GI 56159972; Cluster III H3 represented by accession numbers GI9988669, GI 9988671, GI 9988673, GI 9988667; Cluster II H3 by accession number GI 9988677; and Cluster I H3 by accession numbers GI 5764322, GI 9887146, GI9887129, GI 8871639, GI 110632007, GI5764320, GI 5764318, GI9988675. Other sequences included in the tree had accession numbers: GI99093049, GI12231891, GI15004991, GI 5764316, GI 1160596, GI 2114485, GI348123, GI 60694, GI 3885871, GI 3885863, GI 3885859, GI3885857, GI 460239, GI146216778,, GI 324410, GI 221273, GI 324091, GI324413, GI 324409, GI 14846308, GI61927932, GI 92918926, GI2271216,GI2275432,GI18139815,. The root for our phylogenetic three is A/England/939/1969(H3N2) instead of A/Bayern/95 (H1N1).

11) Receiver Operating Characteristic (ROC) analysis

11.1) ROC analysis ELISA H3 and M1

ROC analysis was performed taking into account vaccinated or challenged animals as true positives, and baseline animals from study 1 and negative controls from study 2 and 3 as true negatives. The curves were generated for each study individually and using combined data from the three studies. SAS (SAS/STAT v.9.2 SAS Institute Inc, Cary, NC) was used to perform the analysis.

Cohen's kappa coefficients were generated using the cut value of 0.15 for H3 ELISA and 0.6 for the NP ELISA. Due to very low sensitivity of M1 ELISA a cut off value for this assay was not determined. The analysis was performed using proc Freq in SAS (SAS/STAT v.9.2 SAS Institute Inc, Cary, NC)

12) Serological profile

The serological profile of each study was assessed using a repeated measurement linear model for the ELISA H3, M1, and NP results explained by time. A different model was used for each study. The working correlation structure among different time points was modeled using compound symmetry and evaluated using the Akaike's information criterion[50]. The mean reactivity of samples at each time point was graphically represented including their 95%

confidence intervals. The models were evaluated using Proc Mixed in SAS (SAS/STAT v.9.2 SAS Institute Inc, Cary, NC).

13) Results

13.1) Association between HI-H3 and ELISAs

ELISA H3 versus HI-H3

Results of ELISA H3 were positively associated with HI titers against H3N2 strains in study 2 and 3 meaning that an increase in HI titers corresponded with an increase in ELISA OD values. The interaction between HI-H3 and study was significant (P<0.01), indicating that the magnitude of association between ELISA H3 and HI-H3 was different between study 2 and 3. An increase in 1% on the HI-H3 was associated with an increase of 0.79% (SE=0.08) on ELISA H3 OD values in study 2, and an increase of 0.49% (SE=0.03) on ELISA H3 OD values in study 3, respectively. The linear model explained 51% of the variation (r^2= 0.508) of ELISA H3 results. Cohen's Kappa coefficient was 0.48 for combined data from studies 2 and 3, 0.59 within study 2, and 0.43 within study 3.

ELISA NP versus HI-H3

The model predicted negative association between HI-H3 titers and NP-ELISA OD values (P<0.01). As NP-ELISA is a competitive assay the increase in HI titers was associated with an increase in positive values in NP-ELISA. As previously mentioned, the cut-off value of 0.6 was adopted in the classification of positive and negative samples and the serum samples with OD values below 0.6 were classified as positive and serum samples with OD values above this threshold were considered as negative.

The interaction between HI-H3 and study was significant (P<0.05), showing that the magnitude of association between the assays was different between study 2 and 3. A 1% increase on HI H3 titers was associated with a decrease of 0.7% (SE=0.04) in NP-ELISA OD values in study 2, and a decrease of 0.2% (SE=0.02) in NP-ELISA OD values in study 3, respectively. The linear model

explained 43% of the variation (r^2= 0.425) in NP-ELISA results. Cohen's Kappa coefficient was 0.31 for combined data from studies 2 and 3, 0.14 within study 2, and 0.39 within study 3.

ELISA M1 versus HI-H3

Results of ELISA M1 were positively associated with HI H3 results ($P<0.01$) meaning that an increase in HI titers corresponded to an increase in ELISA M1 OD values. The model predicted that the magnitude of association between HI-H3 titers and study was not significant meaning that study source did not impact in the magnitude of association. In both studies, an increase of 1% in HI H3 titers corresponds to an increase of 0.41% (SE=0.06) in ELISA M1 OD values. The determination coefficient in this model was 0.15 (r^2= 0.151), indicating that the models explained only 15% of variations in ELISA M1 OD values.

13.2) Association between HI-H1 and ELISAs

ELISA H3 versus HI-H1

There was a significant positive linear association between H3 ELISA results and HI titers against A/swine/Iowa/110600/00 (H1N1) from study 2, and against the H1N1 from study 3 ($P<0.01$). The association between ELISA-H3 results and HI titers against A/swine/North Carolina/031/05 (H1N1) and the challenge A/swine/Iowa/00239/04 (H1N1) from study 2 was not statistically significant. The magnitude of association between the HI-H1 titers and ELISA OD values was also different between H1N1 strains from study 2 and 3. The slopes for the linear association were: 0.51 (SE= 0.08) for A/swine/Iowa/110600/00 (H1N1), 0.41 (SE= 0.04) for the H1N1 from study 3, 0.16 (SE=0.1) for A/swine/North Carolina/031/05 (H1N1), and 0.1 (SE=0.08) for the challenge virus A/swine/Iowa/00239/04 (H1N1). The linear model explained 24% of the variation (r^2= 0.2428) in ELISA H3 results. In study 2, Cohen's Kappa coefficient of agreement were 0.45 between ELISA H3 and Hi titers against A/swine/Iowa/110600/00, 0.06 for ELISA H3 and HI titers against A/swine/North Carolina/031/05 , and "zero" for ELISA H3 and HI titers against the challenge virus. In study 3 Cohen's Kappa coefficient was 0.3 for agreement between ELISA H3 and the H1N1 strain used in the experimental vaccine.

ELISA NP versus HI H1

A linear negative association between NP-ELISA and HI-H1 results was observed ($P<0.01$). Although the association was negative, higher HI titers were associated with higher positive values in NP-ELISA. The magnitude of association between the assays was different depending on the H1N1 analyzed ($P<0.01$). It was estimated that an increase in 1% in HI-H1 titers was associated with a decrease in NP-ELISA OD values of 0.14% (SE= 0.03) for A/swine/Iowa/110600/00 (H1N1), 0.26% (SE=0.03) for the challenge A/swine/Iowa/00239/04 (H1N1), 0.3% (SE=0.04) for A/swine/North Carolina/031/05 (H1N1), and 0.19% (SE=0.02) for the H1N1 strain used in study3. This model explained 47% (r^2= 0.474) of the variation of NP-ELISA results. In study 2, Cohen's Kappa coefficient was 0.23 between NP-ELISA and A/swine/Iowa/110600/00 (H1N1), 0.52 between NP-ELISA and A/swine/North Carolina/031/05, and 0.39 between NP-ELISA and the challenge virus.

ELISA M1 versus HI-H1

There was a positive linear association between ELISA M1 and HI-H1 test results ($P<0.01$). The magnitude of association was not modified by the different H1N1 strains from each study. For both studies an increase of 1% on the HI-H1 titers was associated with an increase on 0.38% (SE=0.06) on ELISA M1 OD values. The model explained only 13% (r^2= 0.125) of the variation in ELISA M1 results. Due to the very low sensitivity calculated for M1 ELISA in the ROC analysis, a cut-off point for this assay was not defined. Without a cut-off Cohen's kappa coefficient values were not calculated to any of ELISA M1 associations.

13.3) Association between ELISAs

ELISA M1 versus ELISA H3

There was a positive linear association between ELISA M1 and ELISA H3 results ($P<0.01$). The magnitude of association was not modified by study source. An increase of 1% in ELISA H3 OD values was associated with an increase of 0.66% (SE=0.07) in ELISA M1 OD values. The model predicted 23% of the variance in ELISA M1 results (r^2= 0.23).

ELISA M1 versus ELISA NP

There was a negative linear association between ELISA M1 and NP results ($p<0.01$) meaning that an increase in ELISA M1 OD values was associated with an increase in positive values in NP-ELISA . The study source did not change the magnitude of association, but ELISA M1 results were different within studies ($P<0.01$). An increase in 1% in NP-ELISA OD values was associated with a decrease of 1.26% (SE=0.16) in ELISA M1 OD values. Results of ELISA M1 were 63% lower in study 2 compared to study 1 and 3 ($P<0.01$). ELISA M1 results were the same between study 1 and 3. After linear transformation, the model explained 19% of the variance of the ELISA M1 results ($r^2= 0.197$).

NP-ELISA versus ELISA H3

The results of NP-ELISA and ELISA H3 were negatively associated and the magnitude of association was different for each study ($P<0.01$). The strongest association between ELISA H3 and NP was observed in study 3 with a decrease of 0.36% (SE=0.02) in NP-ELISA OD values for each 1% increase in ELISA H3 OD values. Study 1 had a decrease of 0.26% (SE=0.05) in NP-ELISA OD values for each 1% increase in ELISA H3 OD values and study 2 had a decrease in 0.07% (SE=0.04) in NP-ELISA OD values for each 1% increase on the ELISA H3 OD values. The model explained 51% of the variation of NP-ELISA results ($r^2= 0.513$). Cohen's Kappa coefficient was 0.35 for combined data from studies 1, 2, and 3; 0.25 within study 1, 0.14 within study 2, and 0.3 within study 3.

14) Receiver Operating Characteristic (ROC) analyses and cut-off points

14.1) ELISA H3

In order to maximize sensitivity, and specificity, a cut-off value of 0.15 OD_{450} was chosen to represent the combined data from studies 1, 2, and 3. At this cut-off point H3 ELISA sensitivity was 56.33% and specificity was 100% **(Fig 6 and Table 1)**. The ROC curve representing all studies was generated excluding data from serum samples at day 56 from study 3. At day 56, the reactivity of serum samples in the H3 ELISA was very low **(Fig.14, Study 3, and 56 days).** As

these values were impacting the sensitivity calculated by ROC, ELISA H3 OD values at day 56 were considered as outliers and eliminated from the analysis. At the cut-off of 0.15, exclusion of ELISA data at day 56 resulted in an increase in sensitivity from 48.07% to 56.33% when considering data from all studies, and from 49% to 68% when considering data from study 3 (data not shown). The inclusion or exclusion of OD values from serum samples at day 56 did not impact specificity which was 100% in both cases. Analyzing the cut-off values for individual studies, the best cut-off value was 0.1 for data from studies 1 and 3. In study 1 **(Fig. 7 and Table 2)** 0.1 cut-off was associated with 64.86% sensitivity and 100%specificity, and in study 3 **(Fig. 9 and Table 4)** it was associated with 70% sensitivity and 100% specificity. In study 2, the best cut-off value was 0.15 associated with 32% sensitivity and 100% specificity **(Fig.8 and Table 3)**. The final cut-off point of 0.15 was chosen to represent H3 ELISA based of ROC analysis of data from studies 1, 2, and 3 together. This value was further used in the construction of 2x2 tables and Kappa coefficient of agreement.

Although ROC curves were generated to determine the cut-off OD values for H3 and M1 ELISAs figure 6 to 9 for the H3 ELISA, and figures 10 to 13 for M1 ELISA are not representing ROC curves. These figures represent the impact of different cut off OD values (X axis) in the sensitivity and specificity of the assay (Y axis). As the cut off OD value of 0.1 established for H3 ELISAs is lower than the cut-off OD values for the commercial IDEXX H3N2 ELISA[46], which is 0.3-0.39 for suspect animals, and higher than 0.4 for positive animals, we decided to show the impact of higher cut off OD vales in the H3 and M1 ELISAs. Although the cut-off of 0.1 works well in the classification of positive and negative when using our set of serum samples, a higher cut-off value offers a comfortable distance between the OD readings of a positive and negative serum sample. This distance excludes the possibility that serum samples that for some reason show higher background reactivity are classified as positive although they are negative. The cut off of 0.1 works fine in the classification of animals in a very controlled population which was not exposed to common pathogens in swine herds such as PRRS, *Mycoplasma hyopneumoniae, Haemophilus parasuis* and others. The performance of the assay in serum samples from animals in the field needs to be explored.

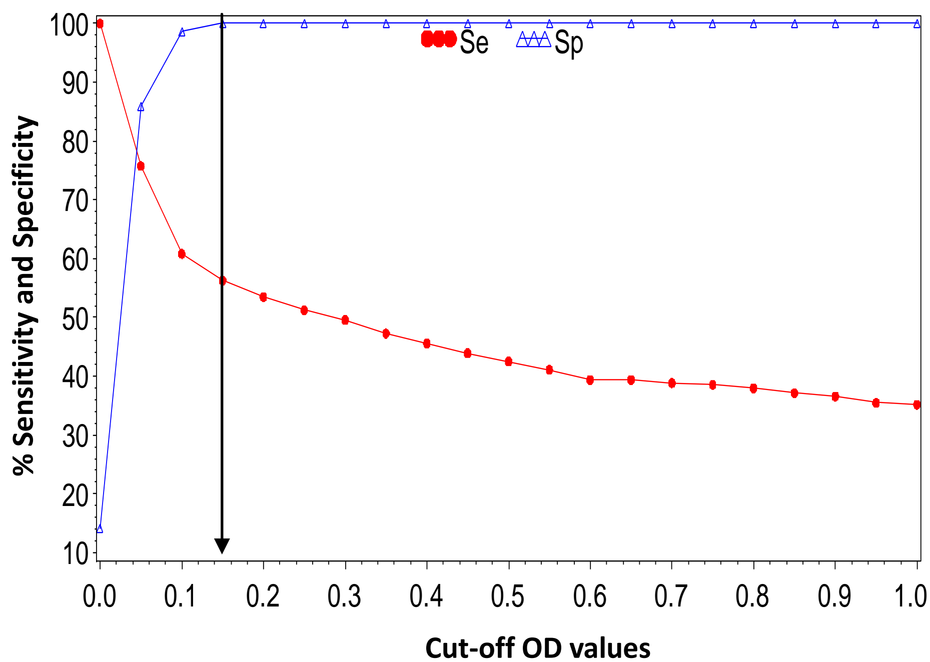

Figure 6. *Representation of the impact of varying cut-off values in the sensitivity and specificity of H3 ELISA from studies 1, 2, and 3.* The Y axis represents the percent of sensitivity and specificity and the X axis represent the different cut-off OD values. The arrow corresponds to the cut-off OD value of0.15 where specificity is 100% and sensitivity is 56.33%.

Cut-Off OD	True Positive	False Positive	Sensitivity	Specificity
0.00	100.000	85.9155	100.000	14.085
0. 05	75.775	14.0845	75.775	85.915
0.10	60.845	1.4085	60.845	98.592
0.15	**56.338**	**0.0000**	**56.338**	**100.000**
0.20	53.521	0.0000	53.521	100.000
0.25	51.268	0.0000	51.268	100.000

Table 1. Sensitivity and specificity estimates at different cut-off values. Values were taken from the ROC analyses of H3 ELISA data from study 1, 2 and 3.

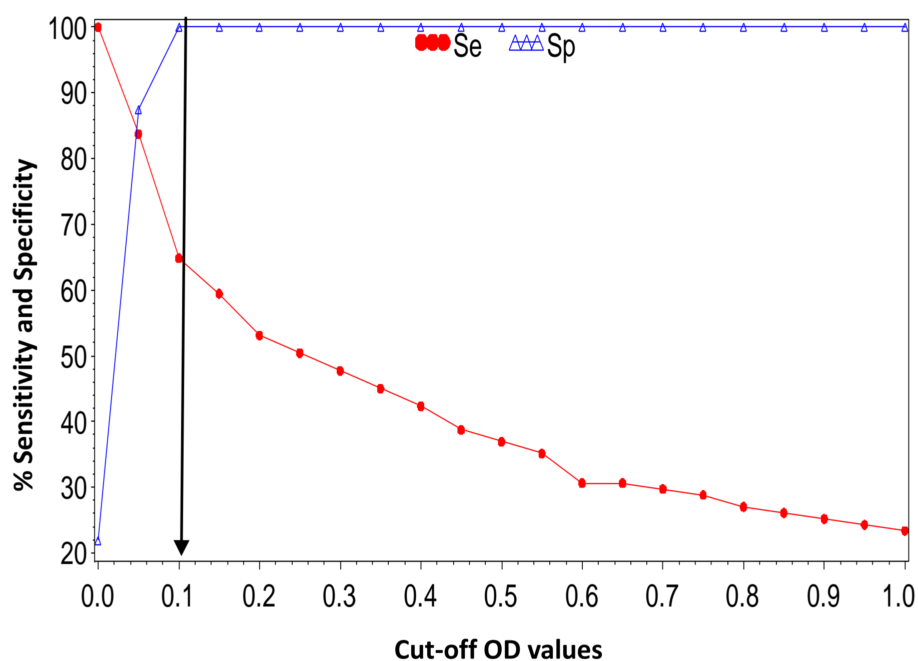

Figure 7. Representation of the impact of different cut-off values in the sensitivity and specificity of H3 ELISA from study 1. The Y axis represents the percent of sensitivity and specificity and the X axis represent the different cut-off OD values. The arrow corresponds to the cut-off OD value of 0.1 where specificity is 100% and sensitivity is 64.86%.

Cut-Off OD	True Positive	False Positive	Sensitivity	Specificity
0.00	100.000	78.125	100.000	21.875
0. 05	83.784	12.500	83.784	87.500
0.10	**64.865**	**0.000**	**64.865**	**100.000**
0.15	59.459	0.0000	59.459	100.000
0.20	53.153	0.0000	53.153	100.000
0.25	50.450	0.0000	50.450	100.000

Table 2. Sensitivity and specificity estimates at different cut-off values. Values were taken from ROC analyses of H3 ELISA data from study 1.

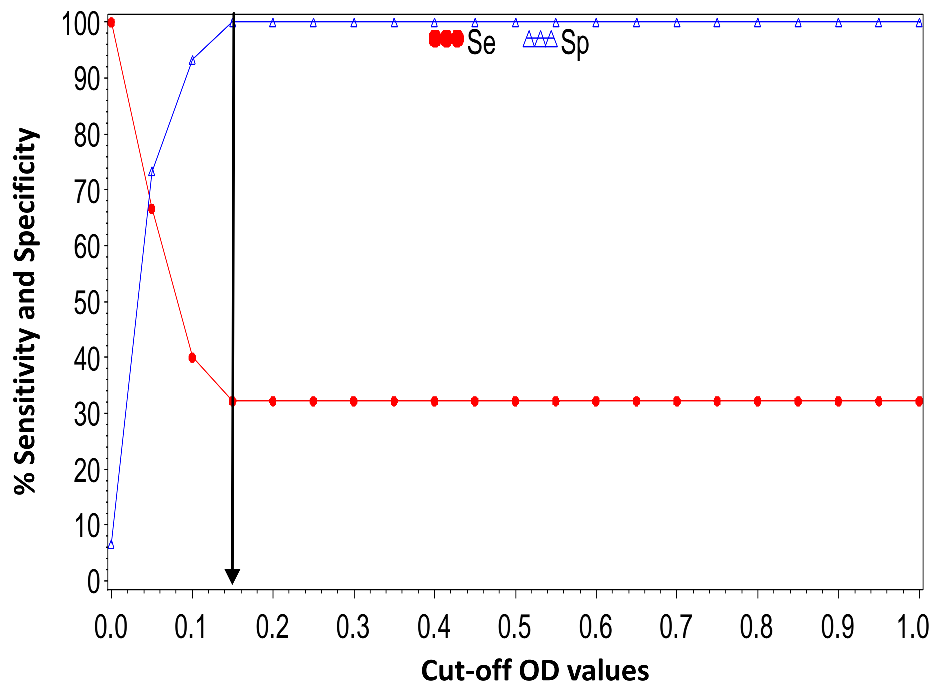

Figure 8. Representation of the impact of different cut-off values in the sensitivity and specificity of H3 ELISA from study 2. The Y axis represents the percent of sensitivity and specificity and the X axis represent the different cut-off OD values. The arrow corresponds to the cut-off OD value of 0.15 where specificity is 100% and sensitivity is 32.22%.

Cut-Off OD	True Positive	False Positive	Sensitivity	Specificity
0.00	100.000	93.3333	100.000	6.667
0. 05	66.667	26.6667	66.667	73.333
0.10	40.000	6.6667	40.000	93.333
0.15	**32.222**	**0.0000**	**32.222**	**100.000**
0.20	32.222	0.0000	32.222	100.000
0.25	32.222	0.0000	32.222	100.000

Table 3. Sensitivity and specificity estimates at different cut-off values. Values are taken from ROC ROC analyses of H3 ELISA data from study 2

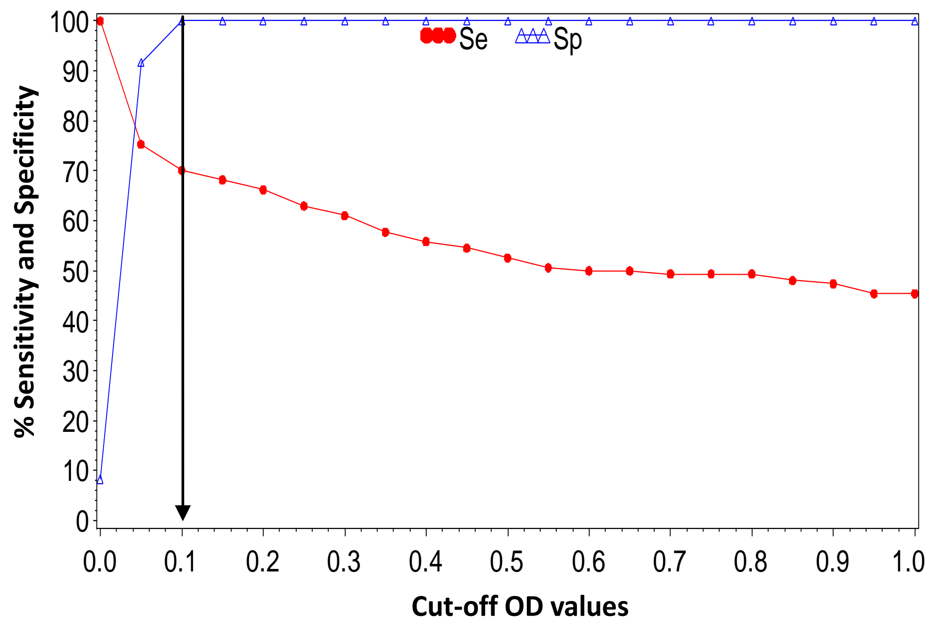

Figure 9. Representation of the impact of different cut-off values in the sensitivity and specificity of H3 ELISA from study 3. The Y axis represents the percent of sensitivity and specificity and the X axis represent the different cut-off OD values. The arrow corresponds to the cut-off OD value of 0.1 where specificity is 100% and sensitivity is 70%.

Cut-Off OD	True Positive	False Positive	Sensitivity	Specificity
0.00	100.000	91.6667	100.000	8.333
0. 05	75.325	8.3333	75.325	91.667
0.10	**70.130**	**0.0000**	**70.130**	**100.000**
0.15	68.182	0.0000	68.182	100.000
0.20	66.234	0.0000	66.234	100.000
0.25	62.987	0.0000	62.987	100.000

Table 4. Sensitivity and specificity estimates at different cut-off values. Values are taken from ROC ROC analyses of H3 ELISA data from study 3 (day 56 data not included).

14.2) ELISA M1

Overall, ROC analysis using M1 ELISA OD values described very low sensitivity but high specificity. At the cut-off point of 0.1 ROC curves generated using combined M1 ELISA OD values from study 1, 2, and 3 show sensitivity of 21% and specificity of 100% **(Fig 10 and Table 5)**. When this was reanalyzed for individual studies, sensitivity was 17% and specificity was100% for study 1 **(Fig 11 and Table 6);** while those for study 2 were 13.3% and 100% **(Figure 12 and Table 7);** and for study 3, 26% and 100%, respectively **(Figure 13 and table 8)**. Although serum samples from study 3 day 56 also showed very low reactivity in M1 ELISA **(fig. study 3)**, exclusion of these samples from the analysis did not improve the overall sensitivity of the test. As a cut–off value was not established for ELISA M1, Cohen's Kappa coefficient of agreement was not calculated between ELISAs or between ELISA M1 and HI titers.

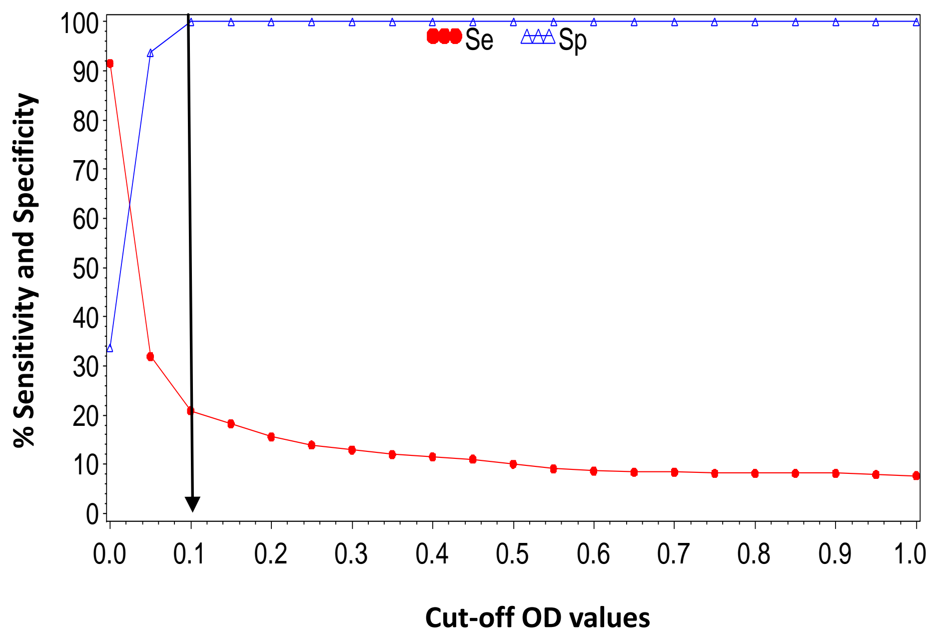

Figure 10. Representation of the impact of different cut-off values in the sensitivity and specificity of M1 ELISA from studies 1, 2, and 3. The Y axis represents the percent of sensitivity and specificity and the X axis represent the different cut-off OD values. The arrow corresponds to the cut-off OD value of 0.1 where specificity is 100% and sensitivity is 21%.

Cut-Off OD	True Positive	False Positive	Sensitivity	Specificity
0.00	91.5865	66.25	91.5865	33.75
0. 05	31.9712	6.25	31.9712	93.75
0.10	**20.9135**	**0.00**	**20.9135**	**100.00**
0.15	18.2692	0.00	18.2692	100.00
0.20	15.6250	0.00	15.6250	100.00
0.25	13.9423	0.00	13.9423	100.00

Table 5. Sensitivity and specificity estimates at different cut-off values. Values are taken from ROC ROC analyses of H3 ELISA data from studies 1, 2, and 3.

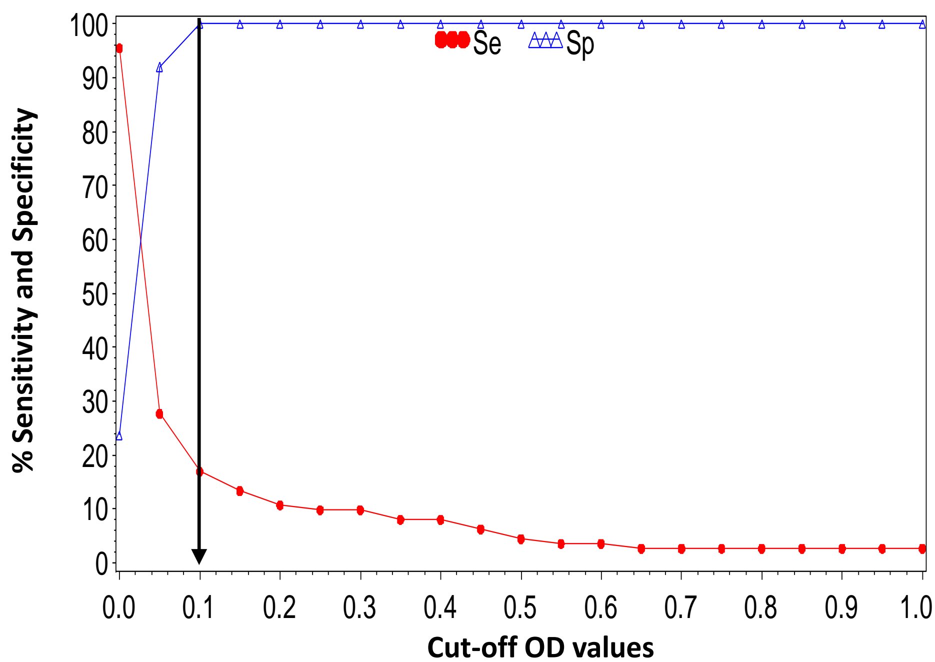

Figure 11. Representation of the impact of different cut-off values in the sensitivity and specificity of M1 ELISA from study 1. The Y axis represents the percent of sensitivity and specificity and the X axis represent the different cut-off OD values. The arrow corresponds to the cut-off OD value of 0.1 where specificity is 100% and sensitivity is 17%.

Cut-Off OD	True Positive	False Positive	Sensitivity	Specificity
0.00	95.5357	76.3158	95.5357	23.684
0. 05	27.6786	7.8947	27.6786	92.105
0.10	**16.9643**	**0.0000**	**16.9643**	**100.00**
0.15	13.3929	0.0000	13.3929	100.000
0.20	10.7143	0.0000	10.7143	100.000
0.25	9.8214	0.0000	9.8214	100.000

Table 6. Sensitivity and specificity estimates at different cut-off values. Values are taken from ROC ROC analyses of M1 ELISA data from study 1.

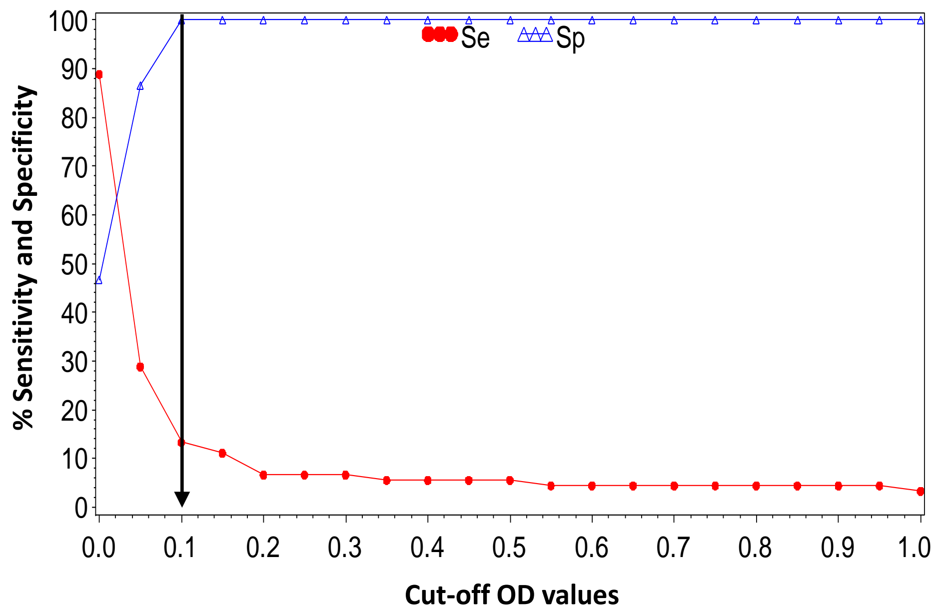

Figure 12. Representation of the impact of different cut-off values in the sensitivity and specificity of M1 ELISA from study 2. The Y axis represents the percent of sensitivity and specificity and the X axis represent the different cut-off OD values. The arrow corresponds to the cut-off OD value of 0.1 where specificity is 100% and sensitivity is 13.3%.

Cut-Off OD	True Positive	False Positive	Sensitivity	Specificity
0.00	88.8889	53.3333	88.8889	46.667
0. 05	28.8889	13.3333	28.8889	86.667
0.10	**13.3333**	**0.0000**	**13.3333**	**100.000**
0.15	11.1111	0.0000	11.1111	100.000
0.20	6.6667	0.0000	6.6667	100.000
0.25	6.6667	0.0000	6.6667	100.000

Table 7. Sensitivity and specificity estimates at different cut-off values. Values are taken from ROC analysis of M1 ELISA OD values from study 2.

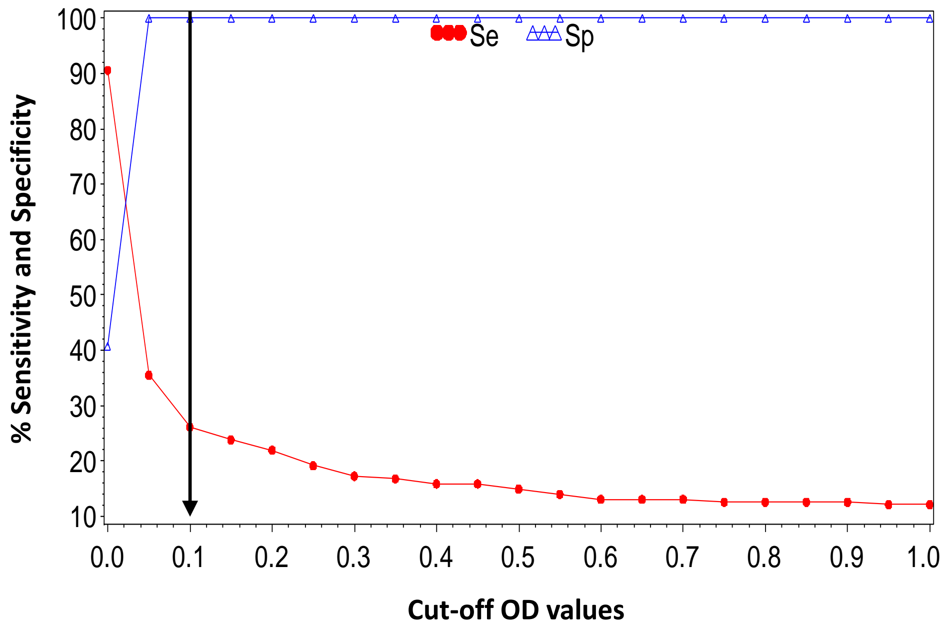

Figure 13. Representation of the impact of different cut-off values in the sensitivity and specificity of M1 ELISA from study 3. The Y axis represents the percent of sensitivity and specificity and the X axis represent the different cut-off OD values. The arrow corresponds to the cut-off OD value of 0.1 where specificity is 100% and sensitivity is 26%.

Cut-Off OD	True Positive	False Positive	Sensitivity	Specificity
0.00	90.6542	59.2593	90.6542	40.741
0. 05	35.5140	0.0000	35.5140	100.000
0.10	**26.1682**	**0.0000**	**26.1682**	**100.000**
0.15	23.8318	0.0000	23.8318	100.000
0.20	21.9626	0.0000	21.9626	100.000
0.25	19.1589	0.0000	19.1589	100.000

Table 8. Sensitivity and specificity estimates at different cut-off values. Values are taken from ROC analyses of M1 ELISA OD values from study 3.

15) Serological profile characterization

15.1.1) Serological profile characterization by H3 ELISA

Using 0.15 as a cut-off value for H3 ELISA, serum samples from baseline animals in study 1 **(Figure 14)** showed very low reactivity at the time of first vaccination (day 0) followed by an increase in reactivity of serum samples by the time of the second vaccination (day 14). There was no increase in reactivity of serum samples after booster (day28) and OD values at this time point were significantly lower compared with OD values at day 14 with some samples being classified as negative. There was a slight increase in reactivity of samples post challenge (day 35), but the level of reactivity of samples was still lower at this time point compared to day 14. At the same cut-off value of 0.15, serum samples from study 2 represented a mix of positive and negative values having OD values ranging between 0.1 and approximately 0.3. In study 3, at the time of second vaccination (day 21), serum samples also represented a mix of positive and negative values with OD values varying between 0.1 and 0.25. At day 35, following booster at day 21, serum samples showed a peak in reactivity having OD values between 0.35 and 0.7. At day 56 the reactivity of the samples was very low showing OD values close to zero.

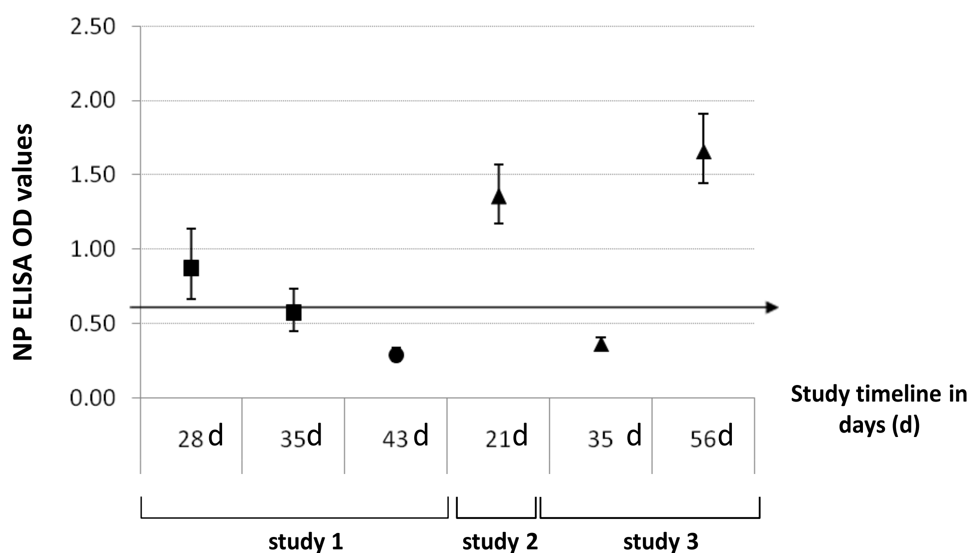

Figure 14: Serological profile characterized by screening of serum samples from study 1, 2 and 3 using H3 ELISA. The Y axis represents OD values and the X axis represents the time line of individual studies according to screening in H3 ELISA. The cut off OD value of 0.15 is being represented by the horizontal arrow. Reactivity of serum samples in OD values are being represented by squares in study 1, circle in study 2, and triangles in study 3.

15.1.2) Serological profile characterized by M1 ELISA

Overall, the reactivity of serum samples from all studies was very low when screened using M1 ELISA **(Figure 15)**. In study 1 there was no significant difference in reactivity between serum samples from baseline animals (day 0) and animals at the time of second vaccination (day 14). There was a slight increase in reactivity following booster at day 28, and no significant difference in reactivity of serum samples between day 28 and euthanasia at day 35. In study 2 serum samples showed low reactivity with maximum OD values around 0.05. In study 3, at the time of second vaccination (day 21) serum samples showed low reactivity reaching maximum OD values between 0.05 and 0.1. Following booster at day 21, serum samples at day 35 showed the highest reactivity level within study 3 having OD values between 0.1 and 0.25. The reactivity of serum

samples at day 56 dropped significantly compared with day 35 showing OD values close to zero. Since OD values were low for M1 ELISA, a serological profile could not be developed.

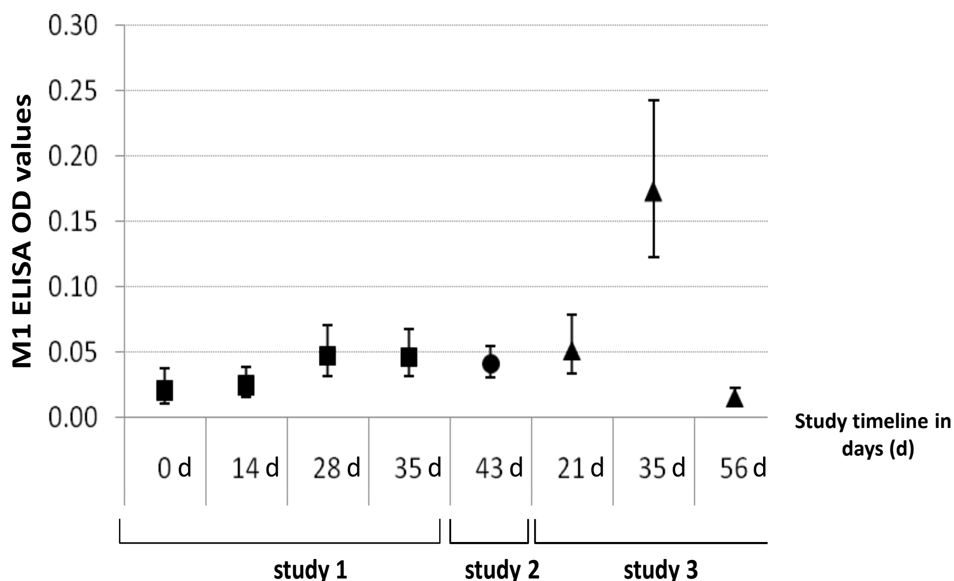

Figure 15: Serological profile characterized by screening of serum samples from study 1, 2 and 3 using M1 ELISA. The Y axis represents OD values and the X axis represents the time line of individual studies according to screening in M1 ELISA. Reactivity of serum samples in OD values are being represented by squares in study 1, circle in study 2, and triangles in study 3. .

15.1.3) Serological profile characterized by NP ELISA

The cut-off point for the NP ELISA was adopted as 0.6 with positive samples having OD values lower than 0.6 and negative values higher than 0.6. In study 1 **(Figure 16)** screening with NP ELISA was only applied to serum samples at day 28 (challenge) and day 35 (euthanasia). As the cut-off of 0.6 was already validated for serological screening involving pig serum samples [17], ROC curves were not generated for NP ELISA and baseline animals from study 1 were not tested in the assay. Although serum samples at day 28 correspond to animals after booster all samples tested at this time point were classified as negative by NP ELISA. At day 35, post challenge, part of the animals was still classified as negative, but there was an increase in the proportion of

positive animals identified in the assay. In study 2 all serum samples at day 43 were classified as positive. In study 3, all serum samples from the time of second vaccination (day 21) tested negative, but an increase in reactivity was seen at day 35 with all samples being classified as positive. At day 56, in agreement with ELISA H3 serological profile, serum samples were all classified as negative having high OD values.

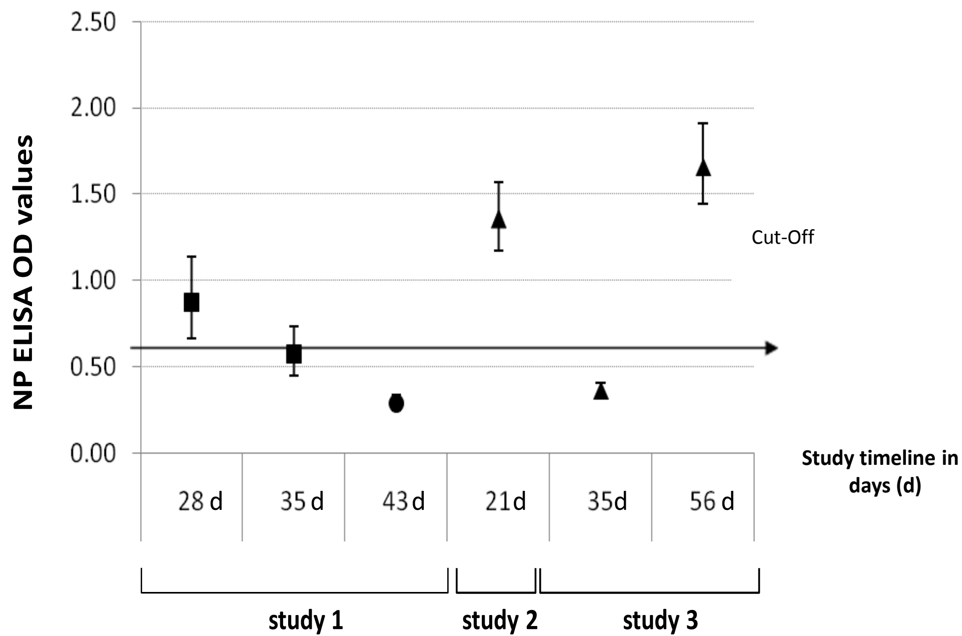

Figure 16: Serological profile characterized by screening of serum samples from study 1, 2 and 3 using NP ELISA. The Y axis represents OD values and the X axis represent the time line of individual studies according to screening in NP ELISA. The cut off OD value of 0.6 is being represented by the horizontal arrow. Reactivity of serum samples in OD values are being represented by squares in study 1, circle in study 2, and triangles in study 3.

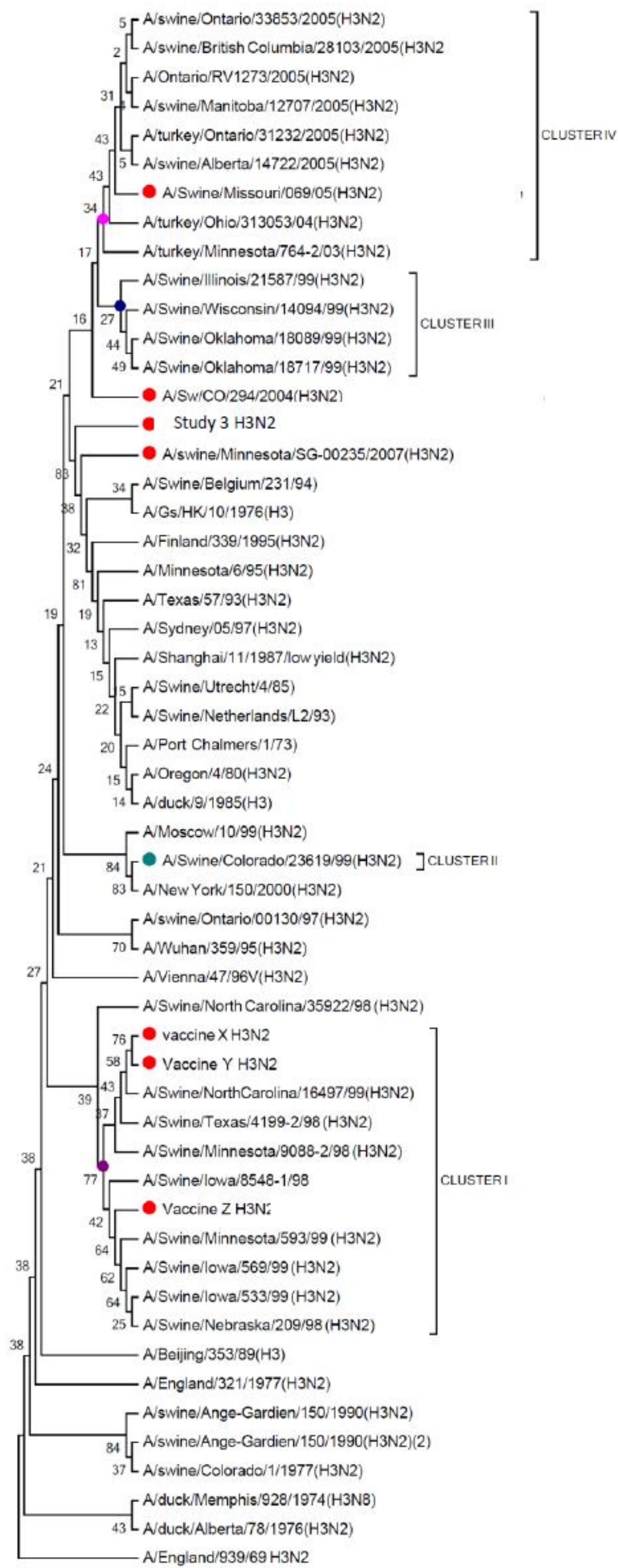

Figure 17: Phylogenetic analysis of hemagglutinin gene sequences of H3N2 viruses used in vaccination or challenge of animals in studies 1, 2, and 3, and in the expression of the rH3. **Red circle:** isolates used in studies 1, 2 and 3. **Purple circle:** demarcation of cluster I. **Green circle:** demarcation of cluster II. **Blue circle:** demarcation of cluster III. **Pink circle:** demarcation of cluster IV.

16) Discussion

16.1) ROC analysis of H3 and M1 ELISAs

Receiver operating characteristic (ROC) curves generated for H3 and M1 ELISAs showed that both assays are highly specific, but sensitivity of H3 ELISA is higher than sensitivity of M1 ELISA. One of the possible explanations for the differences in the sensitivity of H3 ELISA between studies might be related with the level of antigenic similarities between the hemagglutinin of strains involved in vaccination/challenge and the cluster IV hemagglutinin used as antigen. In study 1, the hemagglutinin gene of the H3N2 strain included in the heterologous vaccines X, Y, and Z and the hemagglutinin gene from the challenge virus share respectively 92.4 %, 92.4%, 92.5%, and 94.4% similarity with H3 hemagglutinin in the H3 ELISA. Although the levels of cross reactivity between two strains tended to decrease with the increase in genetic differences between their hemagglutinin genes [51], the sensitivity of H3 ELISA in study 1 was almost identical in sensitivity to that in study 3 and higher than sensitivity in study 2. The higher sensitivity from study 1 suggests that there is cross reactivity between antibodies against cluster I and III hemagglutinins and the cluster IV hemagglutinin used in the assay. Vaccination and challenge of pigs with H3N2 from different clusters might have resulted in a diverse pool of antibodies increasing the likelihood of recognition of epitopes in the surface of the cluster IV hemagglutinin used in the assay. It is also possible that antibodies against only one type of hemagglutinin (cluster I or cluster III) are cross reacting with the cluster IV H3 in the ELISA. Although animals from study 2 were immunized with a commercial vaccine containing a cluster IV H3N2 virus, the sensitivity for the ELISA was low. As the ROC analysis was performed using the true status or exposure (vaccination or challenge) as the gold standard, all vaccinated pigs, including the ones vaccinated and challenged with H1N1 (homologous group) were included in the analysis. Inclusion of serum samples from the homologous group could have increased the rate of false negatives in the analysis decreasing the sensitivity for H3 ELISA calculate for study2. In agreement with the high specificity calculated for the assay, and the serotype specific nature of H3 ELISA, all sera from the homologous group were classified as negative at the cut-off of 0.15.

In study 3, the hemagglutinin gene of the H3N2 in the experimental vaccine matched 97.5% of the hemagglutinin sequence of the H3 used in the ELISA. Although it is not possible to determine antigenic similarities from genetic data alone, high genetic similarities between strains are highly correlated with increased levels of cross reactive antibodies between these strains. In addition, phylogenetic analysis involving isolates from all studies show that the hemagglutinin gene of the H3N2 virus in the vaccine of study 3 and the hemagglutinin of A/swine/Minnesota/SG-00235/2007(H3N2) cluster close to each other **(Figure 17)**. It is known that closely related nucleotide sequences in phylogenetic analysis generally belong to the same antigenic cluster [52], which supports the finding that higher sensitivity seen in study 3 is likely due to antigenic similarities.

One limiting factor during the ROC analysis from study 2 and 3 was the absence of baseline samples in the serological screenings. To overcome this limitation, the negative controls of the plates were considered as representing the true negative population. Although negative controls were obtained with the use of negative sera from gnotobiotic pigs, they are not representing the background reactivity of Specific Pathogen Free (SPF) pigs used in study 2 and 3. Considering that the OD values of baseline SPF pigs from study 1 are varying between 0.03 to 0.08 in H3 ELISA, and 0.01 to 0.03 in M1 ELISA, the OD values of zero to 0.04 obtained with gnotobiotic pigs serum samples are very close to values of SPF pigs.

As the use of diagnostic tools in swine herds are not meant to diagnose the infectious state of an individual animal, but to represent the picture of the circulation of an infectious agent in a population, an alternative way to analyze our data would be decreasing the specificity to gain in sensitivity. Gain in sensitivity would increase the chance of detecting antibody response against influenza when the virus is infecting a low number of animals. Although our data was generated considering 100% specificity, high specificity still very useful to evaluate how much of that population still susceptible to the virus and to estimate when the virus was introduced into a swine herds.

16.2) Serological profile in H3 ELISA

The antibody profile resultant from screening with H3 ELISA demonstrated that the seroconversion of pigs was not the same between studies **(Figure 14)**. In study 1, the lack of HI data for each time point makes it difficult to predict if antibody titers actually decreased after booster, or if other factors contributed with this phenomenon. As the serological profile for study 2 is only being represented by one time point it is difficult to analyze the performance of the test following vaccination and booster. Despite this, the mixture of positive and negative samples identified at day 43 is expected because serum samples are being represented by the homologous and heterologous group. In study 3, contrary to what was seen in study 1, there was an increase in serum reactivity following booster and a decrease in reactivity by the end of the study. The phenomenon might be explained by the fact the vaccination of a naïve population in study 1 stimulated an antibody pool against Cluster I and III hemagglutinins which was able to cross react non specifically with epitopes in the surface of the cluster IV hemagglutinin. With the second vaccination of animals in study 1 antibodies went through the process of affinity maturation and reacted more specifically with overlapping epitopes in the surface of the Cluster IV hemagglutinin used in the assay. In this case the reactivity of antibodies at the time point of 28 days in study 1 reflects the real cross reactivity between antibodies against cluster I and III hemagglutinin and Cluster IV hemagglutinin. In study 3, as it is very likely that the hemagglutinin of the vaccinal virus and the hemagglutinin used in the assay are share higher level of antigenic similarities, the same phenomenon was not observed. In addition to antigenic mismatches between vaccine strains and the HA in the assay, adjuvant type and antigenic dose in the vaccines might have contributed with the differences observed between studies [18]. As information about antigenic dose and adjuvant type were not available for any of the studies, no conclusions were drawn regarding these variables.

16.3) Serological profile in M1 ELISA

The antibody profile generated using M1 ELISA **(Figure 15)** illustrates that neither vaccination, nor vaccination followed by challenge seems to stimulate high levels of anti M1 antibodies in the pigs. In agreement with reports by Thacker and her group describing delayed antibody response against the M1 protein, our study also shows that the levels of anti M1 antibodies started to increase 14 to 20 days post booster in study 1 and 3 [18]. Contrary to her reports describing higher OD values from serum samples from pigs boostered and challenged, our studies show no increase in reactivity in serum samples from pigs boostered and challenged. Interestingly, in study 1 antibody levels were higher following booster compared with booster followed by challenge. Although animals from study 2 are being represented by serum samples post booster and challenge, the lack of information about previous time points difficult the prediction of an antibody profile for this study. Thacker and her group also suggest a lack of correlation between serotype or antigenic dose in the induction of anti M1 antibodies during vaccination or challenge. Although effect of dose was not tested in our study, there were higher levels of antibodies induced after booster in study 3 compared with study 1 which could be correlated with vaccinal dose. In study 3, the extremely low levels of anti M1 antibodies detected at day 56 in M1 ELISA were also observed in the serological profiles produced with H3 and NP-ELISAs. This repeated observation in serological profiles described by H3, M1, and NP ELISA indicates that at the time point of 56 days circulating antibody levels had likely waned.

Contrary to H3 ELISA, screening with M1 ELISA more accurately represents the levels of anti M1 antibodies at each time point since this protein is highly conserved across lineages

16.4) Serological profile in NP-ELISA

In contrast to reports by Zimmerman and his group describing identification of anti NP antibodies 14 days after first vaccination and more consistently after booster [17], we started detect these antibodies only after booster or challenge **(Figure 17).** The detection of positive serum samples seven days post challenge in study 1 is in agreement with Zimmerman reports describing

identification of anti NP antibodies early in the infection. Although it was not performed screening in serum samples at the time of first and second vaccination in study 1, the high sensitivity and specificity of NP-ELISA excludes the possibility of seroconversion before challenge because all ‘samples were classified as negative at day 28. Having said that, it is unlike that seroconversion happened before day 28. In study 2, although all animals were classified as positive 14 days post challenge, the lack of serum samples to previous time points make it difficult to evaluate if there was seroconvesion before that.

During replication of influenza in the host there is an increase in production of NP protein resulting in induction of higher levels of anti NP antibodies [17]. This observation suggests that induction of anti NP antibodies is dose dependent and that levels of anti NP antibodies in vaccinated pigs might be different depending on the antigenic dose included in the vaccine. This could be one possible explanation for the differences in detection of anti NP antibodies post first vaccination between studies 1, and 3, and Zimmerman’s study. Further studies exploring the correlation between vaccine dose and NP antibody response needs to be performed in order to confirm this supposition. Differences in the adjuvants used in the vaccination could also be one of the explanations for the differences observed between our study and Zimmerman’s study.

16.5) Association between HI-H3 and ELISA assays

The low agreement between the H3 ELISA and HI titers might be related with the fact that, contrary to the strain used in the ELISA assay, the strain used in the HI assay was an exact match to those used in the vaccination or challenge of pigs in study 1, 2, and 3. Even though the similarity levels between the hemagglutinin gene of strains in vaccines of study 2 and 3 were respectively 98% and 97.5% compared to that used in the ELISA, small genetic differences between strains might have resulted in antigenic differences which impacted cross reactivity in the ELISA. The low agreement between ELISA H3 and HI results signifies that no assumptions regarding protective antibody levels in vaccinated or challenged pigs could be made by analyzing reactivity in ELISA.

The low agreement between HI H3 and ELISA NP might be related to the fact antibodies detected in these assay are directed against two different proteins. As these proteins have different antibody profiles correlated with them, and vaccinated animals were shown to have a delayed antibody response against NP proteins, screening of samples at time point of first vaccination and booster might have lowered the agreement between HI and ELISA.

16.6) Association between HI-H1 and ELISA assays

The agreement values between HI-H1 titers and H3 ELISA were low independent of H1N1 strain analyzed. The low agreements were anticipated because cross reactivity between anti H1 antibodies and H3 hemagglutinin are expected to be low. Although kappa values between HI-H1 and H3 ELISA assays were higher when analyzing A/swine/Iowa/110600/00 (H1N1) from study 2, and the H1 strain from study 3, these values don't reflect higher cross reactivity between anti H1 antibodies and cluster IV hemagglutinin. As pigs from study 2 and 3 were immunized with vaccines containing H1N1 and H3N2 viruses, serum samples from these animals had high titers of anti H3N2 antibodies. Such antibodies reacted in H3 ELISA, at the same time they had high HI-H1 titers associated with them. In study 2, the difference in agreement between the two H1N1 strains was probably because some samples which tested positive in the ELISA had lower HI titers against A/swine/North Carolina/031/05 (H1N1) compared to A/swine/Iowa/110600/00 (H1N1). It is possible that the immunogenicity between strains might have been higher for A/swine/Iowa/110600/00 (H1N1) compared to A/swine/North Carolina/031/05 (H1N1). In study 3, as all animals were vaccinated with a bivalent vaccine containing H3N2 and H1N1 serotypes, the agreement value between HI-H1 and H3 ELISA was expected to be higher compared with study 2.

16.7) Association between ELISA assays

The delayed immune response of vaccinated pigs against the NP protein might have lowered the agreement between H3 ELISA and M1 ELISA at the time point of challenge in study 1, and booster in study 3. In study 2, although all animals were classified as positive in the NP ELISA, screening of serum samples from the homologous group in the H3 ELISA lowered the agreement

between assays. This discrepancy in the classification of the same serum sample in study 2 highlighted the complementary role of NP and H3 ELISA when used in parallel. As NP ELISA can identify exposure to the virus when the history of vaccination of the animals is known, but cannot distinguish the subtype infecting the animals, the parallel use of serotype specific ELISA becomes convenient in the fast identification of SIV circulating in the herd. In vaccinated herds where there is no vaccination of pigs at the finishing site, the presence of anti NP antibodies are usually due to infection, as maternal immunity decreases 60 days after colostrum intake [53]. Although there is the potential of using NP-ELISA in the differentiation between infected and vaccinated animals, the fact vaccination induces anti NP antibodies excludes the use of this assay as the preferred method for determination of herd status. As NS1 is only expressed during the infection of the virus, the use of this protein may be a better option in case determination of herd status is desired [34].

As a cut-off point was not defined for the M1 ELISA, an agreement value between NP and M1 ELISA was not generated, and NP-ELISA could not be used as a reference for the M1 ELISA. Despite that, the agreement between assays is predicted to be low as reactivity of samples in the M1 ELISA was low in all studies.

One of the goals when using a cluster IV H3 hemagglutinin in ELISA assays was to represent the dominant H3 cluster currently circulating in swine-herds. The serological screening of serum samples from study 1, 2, and 3 was unique because they represented influenza viruses antigenically distinct and distantly related to each other. Thus, these samples are mimicking or representing the high diversity of viruses circulating in the field.

The sensitivity obtained for the H3 ELISA in this study cannot be translated to performance of ELISAs based on cluster IV H3N2 whole virus. Both assays measure antibodies against the hemagglutinin, but ELISAs based on whole viruses also detect antibodies against the NA and M2 surface proteins. Based on the fact HI shows high sensitivity using reference strains, it is very likely that the update of strains used in commercial ELISAs would also result in better sensitivity of the assay.

In summary, hemagglutination inhibition continues to be the best assay in the identification of serotype specific conversion in swine-herds. Although HI assays can only be performed in certified laboratories, sometimes show low reproducible results between laboratories, is susceptible to variation in results due to type of RBC used in the assay, virus titer, personnel, and show asymmetrical results in cross-HI tests, the low agreement between H3 ELISA and HI titers excludes the possibility of using only ELISA as the preferred test in the screening of pigs. Serotype specific ELISA can still be used in the rapid screening of serological conversion post vaccination. However, the total of immunized animals in a group, antibody titers, and potential protection against infection can only be assessed with HI assays. NP and M1 specific antibody responses were detected in pigs vaccinated against influenza. This finding excludes the use of NP or M1 ELISAs in the differentiation between vaccinated and infected animals. NP ELISA stills a useful assay to detect infection in pigs in experimental settings where the animals are known to be negative and are subsequently challenged with different SIV subtypes. M1 protein seems to induce a non homogeneous antibody profile in infected and vaccinated pigs. Further studies to understand the immunogenicity of this protein still have to be conducted.

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