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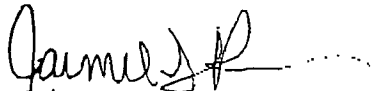

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Yucatan Miniature Swine as an Animal Model for Dengue-1 Disease

Master of Science Thesis

Jaimie S. Robinson

Abstract

Currently there is no animal model for dengue disease. Non-human primates and mice have been useful for vaccine efficacy studies, but they do not reproduce human disease. Yucatan Miniature swine were examined as a novel animal species in which to study dengue virus infection, resultant immune responses, protective efficacy of the immune responses, and signs of illness with the aim of identifying an animal model for dengue disease.

Ten Yucatan Miniature swine were used for this study. The animals were divided into three groups: Group 1 (N=4), Group 2 (N=4), and Group 3 (N=2). On study day 0, Groups 1 and 2 were injected via subcutaneous route with $1x10^5$ or $1x10^7$ plaque forming units (PFU) of Dengue-1 Western Pacific 1974 virus (WP74), respectively. Blood samples were collected on study days 0 through 14, 29, 86, and 176. Sera from study day 0 through 14 specimens were assayed for viremia by cell culture. All specimens were assayed for anti-dengue IgM antibody. Study day 0, 14, 29, 86, and 176 specimens were assayed for anti-dengue IgG antibody and WP74 neutralizing antibodies. On all blood sampling days, the animals were monitored for signs of illness. Group 1 animals averaged 3.75 days viremia, and Group 2 animals averaged 6.75 days of viremia. All Group 2 animals developed IgM, IgG, and neutralizing antibodies, while the antibody responses in two of the animals from Group 1 were barely detectable. No signs or symptoms of disease were seen in any of the animals.

On study day 176, all animals (Groups 1 through 3) were challenged with $1x10^7$ PFU of WP74. The animals were observed for signs of illness, and blood

samples were collected on study days 176 through 186, 190, 197, and 204. Sera from study day 176 thorough 186 were assayed for viremia. Group 3 animals averaged 5 days of viremia compared to 1 day of viremia for Groups 1 and 2, a reduction in length of viremia of 80%. All specimens were assayed for dengue IgM antibody. Group 3 animals developed IgM responses equal in magnitude to those of the responders of Groups 1 and 2 following the primary WP74 infection. The IgM responses of Groups 1 and 2 were of lower magnitude than that for Group 3. Study day 176, 190, 197, and 204 specimens were assayed for anti-dengue IgG antibody and WP74 neutralizing antibodies. Group 3 animals developed IgG and neutralizing antibody responses equal in magnitude to those of the responders of Groups 1 and 2 following primary WP74 infection, while all Group 1 and 2 animals developed elevated responses. Group 3 animals showed no signs of illness. On study days 180 through 186, five animals from Groups 1 and 2 developed extensive maculopapular rashes. Biopsies of the rashes revealed abnormal pathological findings that are consistent with those from humans experiencing dengue-related rashes. These findings support the use of Yucatan miniature swine as an animal model for dengue virus infection since the immune responses and pathological characteristics are similar to that which is seen in humans.

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Introduction

Emerging Diseases

An emerging disease is one that is either newly recognized or one that has a shift in its normal infection pattern. Examples of a change in infection pattern are: 1) an organism undergoing evolution to be able to infect new species; 2) a change in the disease presentation that is caused by an organism; and 3) either reappearance of an organism after a long period of decline in its prevalence or a introduction of a disease to an area in which this organism was not previously found. The field of study in emerging diseases is very diverse and complicated.

Dengue

Dengue as an Emerging Infectious Disease

Dengue or dengue-like viruses are believed to have been present since the third to fourth century common era (C.E.), and sporadic epidemics of dengue-like disease have been documented in Asian and central American countries from the late eighteenth through the early twentieth centuries, as reviewed by Gubler[1]. Craig and Ashburn demonstrated that dengue was caused by an agent that was less than 0.05 microns in diameter in 1907[2]. Hotta identified dengue to be a virus in 1952 through serial passage in suckling mouse brain[3]. Despite dengue's presence for hundreds of years throughout many regions of the world, recent factors have occurred to have dengue classified as an emerging infectious disease worldwide. These factors are the emergence of the disease presentation of dengue

hemorrhagic fever/dengue shock syndrome (DHF/DSS) and the drastic increase in incidence of dengue associated disease worldwide.

Increase in the incidence of dengue worldwide is greatly affected by the vector as well as the available susceptible population. There are currently 2.5 billion people living in dengue endemic areas, and approximately 100 million cases of dengue occur annually[1]. The number of cases of dengue worldwide is increasing at a dangerous rate; in many countries such as the Philippines and Singapore, the incidence rate of dengue infections has doubled yearly over the past several years[4]. Dengue is endemic in the tropical areas of the world as pictured in Figure 1. In areas where dengue is endemic, the increased population contributes to the increased number of mosquitoes present as the number of artificial habitats available increase with human waste in the form of non-biodegradable plastic containers[5]. Air travel also has greatly impacted the spread of dengue in a few ways. The first way is that travelers leave dengue endemic areas after having been infected with dengue and then travel to areas where there are dengue competent vectors which results in the spread of disease in these areas, as was seen in Hawaii in 2001[6]. Another way that air travel has affected incidence of dengue is that infected mosquitoes can catch a ride on a plane and then spread into a new area[7]. Annually there are an average of 20 cases of confirmed imported dengue disease in the United States[8].

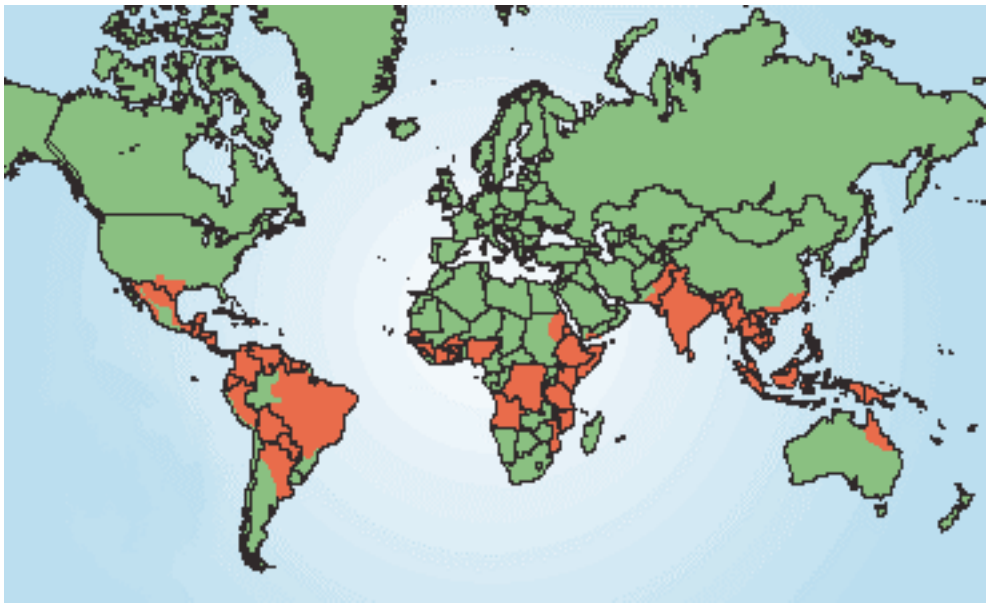

Figure 1: Dengue Endemic Areas[9]

Worldwide distribution of dengue endemic areas is marked in orange on this map as of 2004.

Spread of dengue competent vectors, particularly *Aedes(A.) aegypti and A. albopictus,* worldwide is ongoing[5]. This spread has allowed for the distribution of virus to new susceptible populations. The increase in vector range along with the decrease in vector control programs in most countries worldwide has had a great effect on the incidence of dengue infections and occurrence of DHF. The most dramatic example of the effect of vector control is shown in South America. In South America, there was a widespread vector control program during the 1950's to the early 1970's during which *A. aegypti* was nearly eradicated. When the program ended, *A. aegypti* returned to its previous range and with it dengue virus transmission[10].

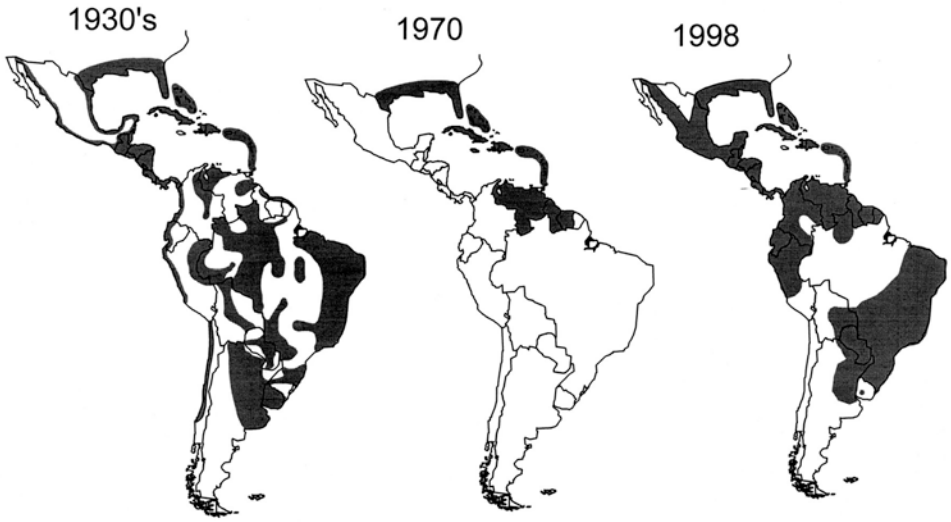

Figure 2: *Aedes aegypti* Distribution in South and Central America[1]

Aedes aegypti distribution is indicated in black, This vector was nearly eradicated from its range in South America prior to the 1970's but with the cease of the vector control program, the mosquito and dengue disease returned to this area and spread to new areas of this continent.

In addition to an increase in vector range, the vectorial capacity of the insects is on the rise as the infection rates for *A. aegypti* and *A. albopictus,* a secondary vector for dengue, have increased 100 fold from the 1960's to 1998.[11] This increased vectorial capacity indicates that the virus is being selected for in mosquitoes to be more efficient or that more people are infected and able to infect more mosquitoes.

In the 1950's, presentation of the first cases of DHF began to appear and have been occurring at an increasing rate ever since[12]. The appearance of DHF coincided with the development of hyperendemnicity, multiple serotypes of virus circulating at the same time in the same place, in many countries of Southeast

Asia[1]. DHF is often correlated with secondary dengue infections, but mechanisms for progression to severe disease have not been elucidated.

Dengue Virus Properties

Dengue is a member of the Family Flaviviridae, which is characterized by a single-stranded, positive sense genome, approximatly ten thousand nucleotides in length. The polycistronic genome encodes 10 proteins, which make up the virus including 3 structural; capsid (C), pre-membrane (prM), and envelope (E) and 7 non-structural proteins; NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5. There are 4 serotypes of dengue viruses[1]. Each serotype is antigenically unique. The serotypes were first separated by differences in reactivity with sera, and as genome sequencing became available, it became clear that these differences were from genetically conserved regions within each serotype of virus[13, 14].

Dengue viruses are arthropod borne. There are several cycles in which dengue viruses are transmitted[1]. These cycles are the forest, rural, and urban cycles. In the forest cycle, virus is transmitted among non-human primates and humans are infected incidentally to this cycle. A human infected this way may enter either the rural or urban cycles in which virus can further be propagated into the population.

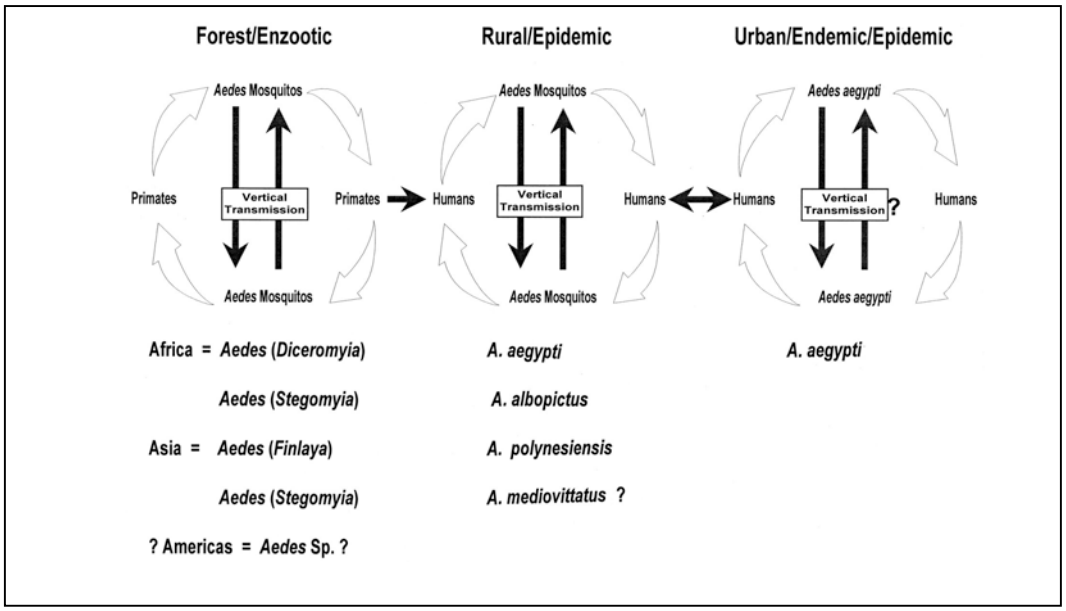

Figure 3:Dengue Infection Cycles[1]

Dengue viruses are transmitted by several vectors, with a principal vector of *A. aegypti*. Viruses move can move from the jungle, sylvatic cycle, with no problem. With the vast majority of cases occurring in the urban cycle.

Transmission from mosquito to human is usually in the amount of 2 to 3 $\log_{10}$ PFU of virus[15], and the virus replicates in humans up to titers of 9 $\log_{10}$ PFU/ml of blood. Humans typically have an incubation period of 3 to 21 days before virus can be isolated from the blood, and they then experience 5 days of viremia on average[2, 16]. For a mosquito to become infected, a certain amount of virus must be ingested during the blood meal. The amount of virus required varies between mosquito species and is also affected by the strain of virus[15, 17]. The average amount of virus needed to infect the *A. aegypti* mosquito is 10 to 100 PFU of virus[17]. During the period from viremia to convalescence, there are several presentations of dengue that are found in infected humans.

Dengue Disease Presentations

There are three main categories of disease found in humans who are infected with dengue: these are asymptomatic infections, dengue fever (DF), and DHF. The presentations in the latter two vary from individual to individual. Many people have asymptomatic primary infections with dengue, as determined by seroprevalence in a population where the people do not recall experiencing a dengue-like illness. A classical DF case is what is commonly referred to as "break bone fever" due to the severe bone and muscle pain associated with the infection. Other common signs and symptoms of DF are retro-orbital pain, fever, rash, nausea, and vomiting[2]. A DHF case is defined by the WHO as a person who has a high continuous fever for 2 to 7 days, a hemorrhagic diathesis, hepatomegaly, thrombocytopenia (platelets less than or equal to 100,000 mm^{-3}) with hemoconcentration (hematocrit increased by 20% or more), and shock. A positive tourniquet test is a strong predictor for DHF. Once one has been classified as having DHF, the grade of disease is then determined. DHF cases are classified into 4 grades of disease presentation. These grades are separated by the following characteristics:

- Grade I: Fever accompanied by non-specific constitutional symptoms; the only hemorrhagic manifestation is a positive tourniquet test and/or easy bruising.
- Grade II: Spontaneous bleeding in addition to the manifestations of Grade I patients, usually in the forms of skin or other hemorrhages.
- Grade III: Circulatory failure manifested by a rapid, weak pulse and narrowing of pulse pressure or hypotension, with presence of cold, clammy skin and restlessness.

- Grade IV: Profound shock with undetectable blood pressure or pulse[18].

One of the main questions posed to dengue researchers is why individuals progress to DHF. The elucidation of these reasons is problematic for several reasons: 1) one cannot ethically conduct research on an individual who is experiencing hemorrhage, 2) there is currently no published animal model for dengue disease, 3) the progression to severe dengue disease varies among individuals. Despite these limitations, there are several theories as to the development of severe disease in some individuals as compared to others.

Theories to Development of Severe Disease

Two theories of dengue disease severity are the antibody dependant enhancement (ADE) theory and the integral hypothesis.

The theory of antibody dependant enhancement is dependant on an individual having pre-existing antibodies to dengue, acquired in the form of maternal antibodies or from a prior exposure to dengue virus, typically of a non-homologous serotype. It is postulated that the pre-existing antibodies bind to the infecting virus in a manner that is does not neutralize virus infection of cells. This can occur by antibodies binding to epitopes that are not required for viral entry; non-neutralizing antibodies, or neutralizing antibodies that are in sufficiently low concentration such that they are unable to neutralize viral replication. The virus-antibody complex then encounters cells bearing Fc receptors. The Fc receptor expressing cells then allow viral entry into these cells at a rate faster than the virus could normally enter permissive cells. The virus then replicates to a higher level

in this individual, which results in greater disease[16, 19, 20]. This theory is corroborated by positive correlations between viral load and/or expression of higher levels of viral antigens and DHF[16].

The integral hypothesis theory of DHF progression is not mutually exclusive from the ADE theory. The integral hypothesis states that no one factor alone causes development of severe disease, rather that multiple factors are involved. The factors hypothesized to be involved fall into 3 major groups: epidemiological risk factors, individual risk factors, and viral factors as pictured in figure 4[21]. Each of the factors outlined have individually been shown to be correlates for severe disease[21]. Epidemiological risk factors are based on mosquito and human population densities as discussed above.

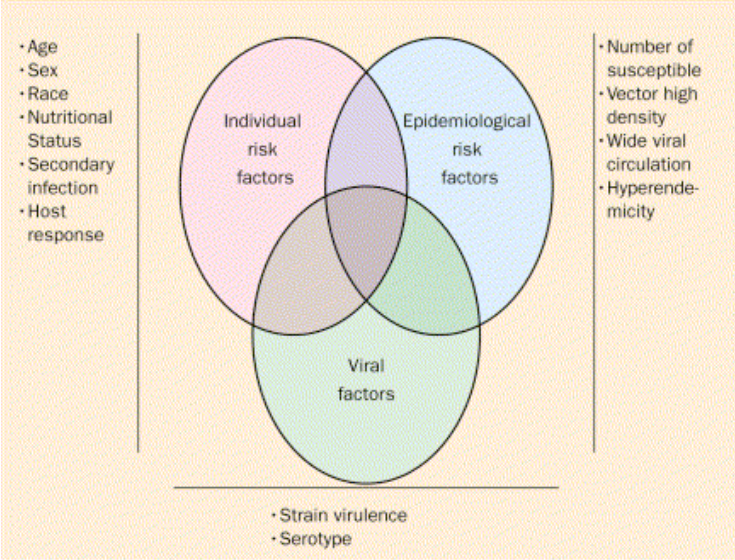

Figure 4: Integral Hypothesis of Severe Dengue Disease[21]

The integral hypothesis of dengue disease progression states that several factors are involved in the development of severe dengue disease.

Individual risk factors thought to be involved in progression to severe dengue disease are diverse; however, it is believed that one's immune response to dengue is integral in development of severe disease. Some of the immune mediated factors believed to be involved are cross-reactive antibodies, immune complex formation, proinflammatory cytokine production, and lysis of bystander cells by dengue reactive T-cells[22].

Cross-reactive antibodies have been correlated with the development of severe dengue disease. These antibodies are developed against E and NS1 proteins of dengue viruses. Antibodies that are developed against E protein of dengue have been shown to cross-react with plasminogen[23, 24, 25]. The presence of these antibodies is correlated with more severe disease and are often associated with secondary dengue infections. The antibodies are present in the serum of infected individuals during viremia but appear to be transient as they decline during convalescence. These antibodies that cross-react with plasminogen bind near the site of binding for α2-antiplasmin, which regulates plasmin activity[26]. This binding is postulated to alter hemostasis, which may lead to the hemorrhagic manifestations that are seen with DHF. Antibodies against NS1 protein of dengue have been shown to cross-react with endothelial cells and extracellular matrix proteins[27]. This interaction has been shown *in vitro* to mediate apoptosis in these cells via several mechanisms and is thought to mediate hemorrhagic leakage by apoptosis of vessel endothelia.

Immune complex formation has been correlated with the development of DHF. Immune complexes are antibody-antigen complexes, which have been

postulated to have a major impact on hemorrhage during secondary dengue infections. When one experiences a secondary dengue infection, an anamnestic response ensues and the antibodies bind to dengue antigen. These dengue antigen-antibody complexes are found readily in the sera of patients with DHF. These complexes are thought to play a large role in complement activation that is associated with DHF infections. The complement components C3a and C5a, which are released during complement fixation, are known anaphalatoxins that may play a major role in mediating vascular permeability and vasolidation leading to hemorrhage during DHF[28, 29, 30].

Production of proinflammatory cytokines has also been correlated with development of DHF. In patients who develop DHF, elevated levels of sTNFR80, IFN-γ, sCD8, sIL2R, and TNF-α develop prior to defervescence and severe manifestations of disease[31]. Increased levels of IFN-γ and TNF-α have been shown to mediate a change in monolayer permeability of endothelial cell culture, which suggests a possible role in progression of DHF by mediating capillary leakage[32].

Lysis of bystander cells is another mechanism of immunopathogenesis thought to be involved in development of dengue disease severity. Bystander cell lysis is a process by which previously activated $CD4^+$ cytotoxic T-lymphocytes (CTLs) respond to infected cells against which they are activated but subsequently lyse uninfected bystander cells. This action is thought to be a main factor involved in the liver damage of dengue infected individuals as dengue specific $CD4^+$ CTLs respond to infected Kupfer cells[33] and lyse nearby

hepatocytes[34] resulting in hepatomegaly, a common symptom in dengue infections.

There are several viral factors postulated to be involved in dengue disease severity and progression to DHF[35]. A few of these factors are specific changes in expressed dengue antigens and changes in the genome that affect replication efficiency. Changes in dengue antigens that are thought to play a role in viral severity occur in PrM, E, NS1, NS2A, NS3, and NS5. These correlations between viral changes were made based on disease severity compared to viral sequence in several samples from humans who were naturally infected[36, 37]. Genome structural differences that are thought to affect severity of disease fall in both the 5' and 3' non-translated regions (NTR) of dengue viruses. Changes in these areas are thought to alter the efficiency with which the virus is able to replicate and thus affect viral titer and the severity of disease[35, 37, 38, 39]

Immune Response to Dengue in Humans

When humans are experimentally infected with dengue viruses, they begin to develop both IgM and IgG antibodies to dengue two days after the first virus positive specimen with all people developing an IgM response by the fifth day post infection. The IgM response lasts for 50 days as measured by ELISA. The IgG response appears in all individuals within two weeks of first sign of dengue infection[40]. A portion of the antibodies that are developed to dengue in these two isotypes will neutralize dengue viruses *in vitro* to high titers. These titers will reach a peak in intensity, then wane to a level that is maintained for the life of the individual[41]. After a dengue infection with one serotype, there are often cross-

neutralizing antibodies that will neutralize other serotypes of dengue up to 6 months post infection[42]. Humans will also develop T-cell memory to a dengue infection.

Animal Models

Animal Models for Dengue

An animal model for disease is defined by Webster's as:

> an animal sufficiently like humans in its anatomy, physiology, or response to a pathogen to be used in medical research in order to obtain results that can be extrapolated to human medicine; *also* **:** a pathological or physiological condition that occurs in such an animal and is similar (as in its pathology or physiology) to a human condition[43].

An ideal animal model for dengue would have the following characteristics: 1) similar in anatomy and/or physiology to humans, 2) sufficient in size such that multiple blood samplings can be taken in a small period of time, 3) widely available and relatively inexpensive, 4) susceptible to infection that can be measured, 5) show signs and/or symptoms of disease, 6) susceptibility to viremia/disease symptoms is not age dependant, 7) develop immune responses to infection, and 8) vaccines for dengue are efficacious in these animals[44].

Currently there are 2 animal models for dengue virus infection, these are murine and non-human primate models. Each model presents its own benefits and problems. Neither of these are a model for dengue disease and are therefore not ideal models for use in dengue virus pathogenesis research.

Murine models for dengue disease fall into two groups; these are suckling and immunocompromised models. In suckling mouse models for dengue, virus is

inoculated intercranially, and the mice develop encephalitis. These animals are immunocompetent, but the animals become refractory to infection at 6 weeks of age. This change in host infectability prevents the use of mice as challenge models for vaccine efficacy or study of disease progression associated with secondary dengue infections. Immunocompromised murine models for dengue include SCID and AG129 mice. When inoculated with virus these mice develop encephalitis. These mice can be used as challenge models in vaccine studies as they do not become refractory to infection at a certain age. The limitation of these models, however, is that the animals are immunocompromised and correlations of disease cannot easily be made to humans who are immunocompetent. Both of the murine models are models for dengue encephalitis, which is a very rare form of dengue disease that is highly debated as to its origin and currently not a priority in the field of dengue research[45, 46].

There are three non-human primate models that are currently used in dengue studies: *Aotus nancymae, Rhesus macaque, and Cynomologous macaque*[47, 48, 49]. Non-human primates develop an asymptomatic dengue virus infection, but have 2 to 5 days of detectable viremia as determined by cell culture isolation[47]. The primates also develop antibodies against dengue viruses and can be used for testing vaccine efficacy. Efficacy of vaccines is measured as a protection from subsequent viremia following challenge with homologous serotypes of virus. Protection from viremia is considered either a complete lack of viremia or reduction in days of viremia compared to that which is seen in animals that were not immunized.

Yucatan Miniature Swine

Yucatan Miniature swine were chosen as a potential model for dengue disease for many reasons including their physiological and immunological similarities to humans, as well as their susceptibility to many viruses of the Family Flaviviridae.

Physiology

Swine in general are desirable as models for human disease because of their anatomical and physiological similarities to humans. Swine are used as models for research on the following systems: cardiovascular, pulmonary, gastrointestinal, renal, immunologic, metabolic, embryonic, neonatal, and integumentary[44, 50, 51]. Because dengue viruses are known to affect these systems in humans, it is of great benefit that the normal structure and function of these systems is widely studied in this model so that changes due to dengue can be readily identified in the pig.

Yucatan Miniature swine are a special breed of pig that were created for their size and specific physiologic characteristics. They are important models used in the fields of integumentary, cardiology, and pulmonary research[44]. These swine are used as models of skin disease[50] due to their anatomical similarities to humans. This is important when looking at a model for dengue, as dengue viruses are transmitted by arthropods and the skin is the site of the initial encounter with dengue. Similarities in the dermal structure are also very important because the most common symptom associated with dengue infections is a rash, and

comparisons between the rash of humans and that of swine can be readily made. When looking for an animal model for a disease that has manifestations of hemorrhage, swine are a perfect candidate, as they are susceptible to von Willebrand's disease[51]. The animals are used to study this phenomenon that is very similar to one of the manifestations of DHF and mechanisms of hemorrhage are widely known for this animal[52].

Immune System

When swine are acutely infected with virus, they will develop antibodies to the virus. The IgM isotype response typically is detectable on Day 2 post infection, peaks on Days 7 to 9, and then wanes. The IgG response to virus is measurable, by ELISA, starting on Day 4 post infection. It peaks at Day 14, and then is maintained at least out to Day 28[53, 54, 55]. When swine are rechallenged with homologous virus, they have a boosting in the level of IgG as compared to the response to the primary infection[54]. When swine are secondarily inoculated with homologous viruses, they are protected from secondary viremia completely or by reduction in either virus titer in the blood or days of viremia[56]. These responses are very similar to what is seen in humans with, which also makes swine a suitable immunologic model for humans acutely infected with virus.

Susceptibility to Flaviviruses

Swine are susceptible to many members of the Family Flaviviridae. These include Japanese Encephalitis virus, Yellow Fever virus, Murray Valley Encephalitis virus, and Kunjin virus. Swine are one of the main reservoirs for

Japanese Encephalitis[57]. When swine are acutely infected with the flaviviruses mentioned above, they experience 1 to 3 days of viremia as measured by cell culture isolation and RT-PCR[53, 58]. Both immortalized and primary cells from swine are susceptible to dengue virus infection[59, 60].

The physiological similarity of swine to humans combined with the susceptibility of swine to many members of the Family Flaviviridae make swine a suitable choice for testing as a possible model for dengue infection and disease. These factors led to the development of the hypothesis that:

Sus scrofa, strain Yucatan Miniature pig, is susceptible to dengue infection and will present a model for dengue disease.

Materials and Methods

Animals

Ten Yucatan Miniature pigs were obtained from Sinclair Research Center (Columbia, Missouri). The animals were 13-week old females and weighed approximately 10 kilograms. Each animal had a vascular access port surgically implanted 2 weeks prior to shipment. The animals were housed at the AALAC certified Walter Reed Army Institute of Research animal facility, Silver Spring, Maryland and were cared for following the guidelines set forth by The Animal Welfare Act (7 U.S. Code Section 2131 et. seq.), DOD Directive Number 3216.1, Army Regulation 70-18/SECNAVINST 3900.38B, and the *Guide for the Care and Use of Laboratory Animals.*

Cell Culture

Vero76 (# CRL-1587) and C636 (#CRL-1660) cell lines were obtained from ATCC (Manassas, Virginia). The Vero76 cells were propagated in minimal essential media (EMEM) (Cellgro #10-010-CV) supplemented with heat inactivated (30 minutes at 56° C) 5% (V/V) fetal bovine serum (FBS) (Tissue Culture Biologicals #101) and 1% antibiotic-antimycotic (Cellgro # 30-004-CI). This media is referred to as 5% EMEM. Virus isolation medias are EMEM with 1% antibiotic-antimycotic and either 0% or 2% (V/V) FBS and are referred to as 0% EMEM or 2% EMEM, respectively.

Raji B cells that are stably transfected with DC-SIGN were obtained from Timothy Burgess (Naval Medical Research Center [NMRC]). These cells are referred to as SIGN cells, and they were propagated in RPMI 1640 (Cellgro #10-040-CV) media containing 10% (V/V) heat inactivated FBS, 1% penicillin/streptomycin (Gibco # 15140-122), and 1% L-glutamine (Cellgro # 25-005-CI). This media is referred to as complete SIGN media.

Virus

The virus used in this experiment is a dengue-1 isolate that was first isolated from a dengue-1-infected individual on Nauru Island in 1974 called Western Pacific 1974(WP74)[61].

Virus that was used for injection was propagated in the following way. Parent virus stock was obtained from Dr. Eckels (Walter Reed Army Institute of Research). T-162 cell culture flasks, which were 25% confluent (approximately $5x10^5$ Vero76 cells per flask), were inoculated with $5x10^3$ plaque forming units (PFU) of WP74, a multiplicity of infection (MOI) of 0.01, in 5 milliliters (mL) 2% EMEM and incubated for 1 hour at 37°C and 5% CO_2. Thirty-five mL of 2% EMEM was added to each flask, and the flasks were then incubated at 37°C and 5% CO_2 for 48 hours. The media was then aspirated, and 35 mL 0% EMEM was added. The flasks were incubated at 37°C and 5% CO_2 for 48 hours, at which time the media was collected and stored at 4°C. For the following 8 days, at 48 hour intervals, the media was collected and stored at 4°C, 35 mL of 0% EMEM was added to the flasks, and the flasks were incubated at 37°C and 5% CO_2. When all samples were pooled, the virus was concentrated using the Filtron Ultrasette

Tangential Flow Device (Filtron #05100C70) per the manufacturer's instructions. After virus was concentrated, 20% (V/V) heat inactivated FBS was added, virus was aliquotted into 0.1 mL volumes, and frozen to -80°C.

Animal Infection

The pigs were divided into 3 groups. Group 1 (N=4) was inoculated with $1x10^5$ PFU of WP74, Group 2 (N=4) with $1x10^7$ PFU of WP74, and Group 3 (N=2) with EMEM only on day 0 of this study. On study day 176, all animals were inoculated with $1x10^7$ PFU of WP74. All innocula were injected subcutaneously in the left rear ham of the animal in a volume of 1 mL using 25 gauge needles and 3 mL syringes.

Blood Sample Collection

On days 0, 14, 21, 29, 176, 190, 197, and 204, 9 mL of blood was collected from each animal. On days 1 through 13, 86, and 177 through 186, 2 mL of blood was collected. On study days 183, 185, and 186 an additional 3mL of blood was taken for hematologic analysis and was submitted to Combat Casualty Care, NMRC, Silver Spring, Maryland. The samples were collected at approximately the same time each day.

Blood was collected on days 0 through 14, 21, and 29 through the vascular access port. The port was accessed using a 20 gauge Huber needle, 1 and ½ inches in length, attached to a three-way stopcock with a 5 and a 10 mL syringe on the 2 luer lock ends. The catheters were locked using a 1:1 solution of 50% Dextrose (Sigma # D-9559) and 4% Trisodium Citrate solution (Sigma #S5770).

The Huber needle was inserted through the skin over the right shoulder blade of the animal into the port. The locking solution along with 1 mL of blood was drawn into the 5 mL syringe. The stopcock was then turned to the 10 mL syringe, and the blood sample was then taken. The 10 mL syringe of normal saline (0.9% NaCl) (NS) was then used to replace the 5 mL syringe, and 9 mL of NS was infused to wash the catheter. A 5 mL syringe containing the locking solution was used to replace the 10 mL NS syringe and 4 mL of the locking solution was injected into the port. The last milliliter of locking solution was infused during withdrawal of the needle to fill the hub of the port.

Blood samples were taken from all animals, on days 86, 176 through 186, 190, 197, and 204 via the *cranial vena cava*. The injection site was cleaned using isopropanol soaked gauze. A 20 gauge, 1 ½ inch needle was used for phlebotomy. The needle was inserted at the "notch" between the upper ribs and the sternum. The needle was inserted at a 45-degree angle and was aimed at the animals left shoulder. Backpressure was put on the syringe, which was inserted until a flash of blood was seen, and then the blood sample was collected. The needle was then removed upon the same path of entry, and pressure was held on the injection site for 1 minute to prevent hematoma formation. The blood from the syringe was collected in a serum separator tube (SST) (BD Bioscienses #367381).

On all days, serum was prepared from 2 mL of blood, using a SST following the manufacturers instructions, and stored at -80°C. On the days in which 9 mL of blood was collected, an additional 7 mL of blood was separated into plasma and peripheral blood mononuclear cells (PBMC) using 8 mL Cell

Preparation Tubes (CPT)(BD Biosciences #362761) following the manufacturer's instructions. The plasma was stored at -80°C, and the PBMCs were stored in liquid nitrogen.

Skin Sample Collection and Processing

On day 183, 4 or 8 mm skin punch biopsies were taken from all animals. Biopsies were taken from each animal in the area most affected by the skin pathology. In animals that were not experiencing a rash, biopsies were collected from the same areas as the animals that were experiencing a rash. The skin samples were divided into 2 sections; one was placed directly into formalin and the other into NS. The NS samples were transferred into Tissue-Tek® Optimal Cutting Temperature (OCT) (Sakura Finektek, Torrance, California) and after 4 hours at room temperature, the samples were frozen at -80°C. All samples were taken to the NMRC Department of Pathology for processing. The formalin-fixed samples were processed, stained with hematoxylin and eosin, and examined using light microscopy at 10 and 40 times magnification.

Vero Cell Culture Isolation

Vero76 cells were seeded at a concentration of $3x10^5$ per T-25 flask, in 5 mL 5% EMEM and incubated at 37°C and 5% CO_2 for 48 hours. The media was decanted and 80 microliters (μL) of sera plus 0.5 mL of 2% EMEM was added to each flask. The flasks were incubated for 1 hour at 37°C, after which 4.4 mL of 2% EMEM was added to each flask and incubated at 37°C and 5% CO_2 for 10 days. The cells were then scraped from the flasks using cell scrapers (Fisher

Scientific, #08-773-2), and transferred into one 15 mL conical tube per flask. The cells were pelleted by centrifugation in a Sorvall RT 6000D centrifuge using a H-1000B rotor at 1200 RPM for 5 minutes. The supernatants were decanted, and the cells were washed by resuspending the cell pellet into 10 mL of phosphate buffered saline (PBS). The cells were pelleted by centrifugation as above. The cell washing process was repeated twice and the cell pellets were resuspended in 4 mL of PBS. Ten microliter aliquots of the cells were spotted into duplicate wells of 24 well slides. The slides were then air-dried and fixed by submersing the slide in acetone for 10 minutes.

The 24 well slides were analyzed as previously described by Wulff[62]. Briefly, 10 μL of a 1:200 dilution of mouse antibody 7E11 (NS1-specific) was added to each well of the slides. The slides were incubated in a humidity chamber at 37°C for 1 hour. The slides were washed 3 times by soaking them in PBS for 5 minutes per wash. A 1:400 dilution of horseradish peroxidase labeled goat anti-Mouse IgG (KPL #02-18-15) and was made in a 1:1 (V/V) solution of 0.1% (W/V) Evans blue, and PBS and 10 μL was added to all wells. The slides were returned to the humidity chamber for 1 hour at 37°C. The slides were washed 3 times by soaking them in PBS for 5 minutes per wash. Slides were then blotted dry, and Vectashield (Vector Laboratories #H-1000) was added along with a cover slip. Slides were analyzed using a fluorescence microscope at a wavelength of 490 nanometers (nm). The level of infection is represented as follows: 0 = no cells infected, 1 =5-25 % of cells infected, 2 = 25-50% of cells infected, and 3 = 50-100% of cells infected. This assay was completed in duplicate flasks. When

the level of infection between the flasks differed the average of the 2 numbers was taken, resulting in a value ending in 0.5.

SIGN Cell Virus Isolation

Following the protocol of LCDR Jeffrey Tjaden (Virus Diseases Department, NMRC), $6x10^4$ SIGN cells were added per well into a 96-well round bottom culture plate in a volume of 30 μL. Ten microliters of test serum plus 0.2 μL of complete SIGN media, was added to duplicate wells and incubated at 37°C for 1 hour. The plates were then centrifuged at 1200 RPM in a H1000D rotor on the Sorvall RT6000D centrifuge for 5 minutes. The cells were then washed twice by decanting the media, resuspending the cells in 0.2 mL RPMI 1640, and centrifuged as described above. Post washing, 0.2 mL complete SIGN media was added to the wells and incubated for 96 hours at 37°C and 5% CO_2. At 96 hours post infection, cells were pelleted by centrifugation as described above. Supernatants from the cells were decanted, cells were resuspended by light Vortexing, and 0.2 mL of PBS was added per well to wash the cells. Eighty microliters of Cytofix/Cytoperm (BD Biosciences #554714) was added to each well to fix the cells, which were then incubated for 15 minutes at 4°C. One hundred twenty microliters of Permwash (BD Biosciences # 51-2091KZ) was added to each well, cells were centrifuged as described above, supernatants were decanted, and this step was repeated 2 times. Fifty microliters of a 1:200 (V/V) dilution of FITC-conjugated 2H2 in Permwash was added to each well of the plate, which was incubated for 15 minutes at 4°C. Plates were treated as described in the previous step, and 150 μL of Permwash was added to each well. Samples

were then analyzed using the FACScan flow cytometer. The machine was first calibrated by running an uninfected control sample to set the gates for determination of the forward and side scatter characteristics of the cell population, as well as to determine the level of background fluorescence in the cells along the FITC wavelength. Cells that were infected with dengue were then run on the machine to confirm that the infected cells would conform to the same forward and side scatter characteristics as the uninfected, and that they do emit fluorescence that fall into the positive field of the dot plot. The Cytex AMS plate reader(Cytex, Freemont, California) was then attached to the flow cytometer, and plates were read after resuspension of cells with slight Vortexing. Output from the assay was analyzed using FlowJo software(FlowJO, Ashland, Oregon). The main population was gated as live cells. These cells were then gated by separation into positive and negative along the FITC output. So, the output of this assay is percentage of FITC positive cells. A positive value for this assay is 0.88, which has been subtracted from the reported values.

ELISA Antigen

ELISA antigen was made as described by Dittmar[63]. For positive antigen, T-162 cell culture flasks, which were 25% confluent (approximately $5x10^5$ Vero cells per flask), were inoculated with $5x10^3$ PFU WP74, a MOI of 0.01, in 5 mL 2% EMEM, and incubated for 1 hour at 37°C and 5% CO_2. Negative antigen was made as above, but no virus was added to the flasks. Thirty-five milliliters of 2% EMEM was added to each flask, and the flasks were then incubated at 37°C and 5% CO_2 for 48 hours. The media was then aspirated, and 35 mL 0% EMEM was

added. The flasks were incubated at 37°C and 5% CO_2 for 48 hours, at which time the media was collected and stored at 4°C. For the following 8 days at 48-hour intervals, the media was collected and stored at 4°C, 35 mL of 0% EMEM was added to the flasks, and the flasks were incubated at 37°C and 5% CO_2. The stored supernatants, which contain virus, were clarified by centrifugation at 6000 RPM for 10 minutes on the Sorvall RC5C centrifuge using the GSA rotor. The supernatants were then pooled, and PEG8000 (Fisher Scientific #P156-500) and NaCl (JT-Baker # 3624-01) were added to concentrations of 7% (W/V) and 2.3 % (W/V), respectively. This mixture was placed at 4°C and stirred for 15 hours. The solution was centrifuged on the same centrifuge and rotor for 30 minutes at 8000 RPM. The supernatants were decanted from the pellet of virus. The residual liquid was removed from the centrifuge bottles by inverting them for 5 minutes followed by blotting the bottle rims with a paper towel. The virus pellet was then resuspended in 100 mL of PBS, which was aliquotted in 0.5 mL volumes and stored at -20°C.

IgG ELISA

Since no swine anti-dengue IgG antibodies were commercially available for use as positive control antibody for the IgG ELISA, swine anti-dengue sera were needed for ELISA calibration. Plasma from 4 Yucatan Miniature swine, which had been immunized with a DNA vaccine that expresses the dengue-1 WP74 prM and E genes, was used as dengue-1 positive swine sera. The pool was made from plasma that was collected 72 days post-vaccination, and it contained

WP74 neutralizing activity with titers between 1:320 and 1:640 by the SIGN neutralization assay.

One hundred microliters of antigen was coated onto plates in dilutions of 1:20 to 1:160 in carbonate-bicarbonate buffer (Sigma # C-3041), alternating plate rows with positive and negative antigen dilutions. The plates were incubated at 4°C for 15 hours. The plates were washed 5 times with wash buffer (1X PBS and 0.1% (V/V) Tween 20 (Sigma # P1379)). Blocking solution, 0.2 mL (5% (W/V) Skim milk in 1x PBS , pH 7.4), was added to all wells, and the plate was incubated at 37°C for 1 hour. The plates were again washed 5 times in wash buffer. Positive sera (0.1 mL per well) was added at a 1:100 dilution in dilution buffer (5% (W/V) Skim milk in 1x PBS (pH 7.4) and 0.1% (V/V) Tween-20) for 1 hour at 37°C. The plates were then washed 5 times with wash buffer. Horseradish peroxidase-conjugated goat anti-pig IgG (ICLLaboratories #GGHL-5P) was diluted in dilution buffer to concentrations between 1:2000 and 1:7000. One hundred microliters of the dilutions were added to the plate wells in duplicate rows and the plates were incubated at 37°C for 1 hour. The plates were washed 5 times with wash buffer and 2 times with PBS. ABTS (KPL # 50-66-06), which was previously equilibrated to 37°C, was added at a volume of 0.1 mL per well, and the plates were incubated at 37°C for 30 minutes, after which the optical density of the wells was quantified using a Molecular Devices VMax Kinetic Microplate reader at 405 nm. The data obtained indicated that the optimal dilution of antigen is 1:20 and 1:2000 for the conjugate. For test samples, the samples were treated as above with antigen and conjugated antibody at the

optimum concentrations. Test sera from study days 0,14, 21, 29, 86, 176, 190, 197, and 204 were diluted to 1:100 (V/V) in dilution buffer and analyzed using the optimal parameters. A positive value for this assay is 3 standard deviations from the mean of the negative samples, a value of 0.13, which has been subtracted from the reported values.

IgM ELISA

Swine anti-dengue IgM antibodies are also not commercially available. Day 5-9 pooled sera from group 2 animals were used to calibrate this IgM ELISA. These samples were chosen because it has been demonstrated that IgM responses to a viral infection in swine peak between days 5 and 9[53, 54]. The parameters of the dengue-1 IgM ELISA were determined as described for the IgG ELISA using the same test range of concentrations of antigen and conjugate. The conjugate antibody used for this assay was goat anti-pig IgM (ICLLaboratories # GM5P) that was Horseradish peroxidase-conjugated. The optimal antigen and conjugate dilutions were determined to be 1:20 and 1:3000, respectively. Test samples diluted 1:100 in dilution buffer from all days in which blood was collected were analyzed using this assay. A positive for this assay is 3 standard deviations from the mean of the negative value, a value of 0.91, which has been subtracted from the reported values.

Plaque Reduction Neutralization Test (PRNT)

The Vero cell PRNT assay as described by Russell was followed[64]. Confluent Vero76 cells were split using trypsin (Cellgro # 25-051-CI), counted

using trypan blue, and diluted to $1.3x10^5$ cells per mL in 5% EMEM. Three milliliters of these cells were added to each well of a 6-well plate. The plates were placed in a 5% CO_2 incubator at 37°C for 48 hours. Sera were heat inactivated at 56°C for 30 minutes. Sera were then serially diluted in 4-fold dilutions from 1:5 to 1:5120 in a final volume of 0.225 mL 2% EMEM using a MEGABLOCK microtiter plate. WP74 was diluted to a concentration of 356 PFU/mL, and 0.225 mL was added to each well of the titer plate minus the cell sheath control wells. A positive control serum of anti-dengue-1 mouse hyper-immune ascites fluid was diluted as described for the test sera. The negative control serum was from a dengue-negative swine serum that was diluted similarly. The microtiter plate was incubated at 37°C for 30 minutes. After this incubation, the 6-well plates were removed from the 37°C incubator along with the serum/virus mixture. The media was aspirated from the wells of the plates and 0.2 mL of the serum/virus mixture was added to duplicate wells on the plates. The plates were returned to the incubator at 37°C for one hour. After the incubation, the mixture was aspirated from the wells, at which point the first overlay was added to the wells in a volume of 3 mL per well. The first overlay consisted of a 1:1 mixture of the following: component 1 is a 3% (W/V) low melting point agarose solution that was microwaved to bring the agarose into solution and then placed at 42°C for 1 hour, and component 2 was 2X EMEM, 10% (V/V) FBS and 1% (V/V) antibiotic-antimycotic (Cellgro # 30-004-CI). Component 2 was maintained at room temperature, and the components were mixed just prior to addition to plates. The overlay was allowed to solidify for 20 minutes, after which the plates were

returned to the incubator at 37°C and 5% CO_2 for 4 days. On the fourth day, the second overlay was added to the plates, which was allowed to solidify for 20 minutes. The second overlay was a solution of 1% (W/V) low melting point agarose that was microwaved to bring the agarose into solution and placed at 42°C for 1 hour. Prior to addition to the plates, neutral red (Sigma # N2889) was added to a concentration of 4% (V/V) in total solution. The plates were then returned to the incubator for 24 hours at which point the plaques were counted. The data were analyzed using SPSS and PROBIT analysis to determine the reciprocal serum dilution at which 50% of the virus was inhibited from infecting the Vero cells and thus forming plaques.

SIGN Neutralization Assay

Sera collected on study days 0, 14, 21, 29, and 86 for each animal were heat inactivated at 56°C for 30 minutes. The serum was then serially diluted in 2 fold dilutions from 1:5 to 1:640 and 30 μL was added to 96 well culture plates. WP74 was diluted to a concentration of $8x10^4$ PFU/mL in SIGN media, and 30 μL was added to the sera in the 96-well culture plates. Positive controls for this assay consisted of addition of 30 μL of virus and 30 μL of media and a 10-fold dilution of the virus added to the same amount of media. The negative control for this assay consisted of serially diluted sera plus complete SIGN media in the place of the virus. All samples were incubated for 30 minutes at 37°C and 5% CO_2. During the incubation period, the SIGN cells were transferred from their growth flask and placed into a 50 mL conical tube. The cells were collected by centrifugation at 1200 RPM on the Sorvall RT 6000D centrifuge using the H-

1000B rotor for 5 minutes. After centrifugation, media were decanted from the cells, and the cells were resuspended in complete SIGN media to a volume of 10 mL. Cells were counted using trypan blue and adjusted to a concentration of $2x10^6$ cells per milliliter in complete SIGN media. When the 30-minute incubation period was finished, 60 μL of the cells were added to the virus/serum mixture. The plates were placed back in the 37°C and 5% CO_2 incubator for 24 hours. Cells were then fixed and stained as described for the SIGN cell isolation assay. The virus positive cells yielded approximately 20% of cells infected, and output of this assay is determined as reduction of percent cells infected. Probit analysis was used on the data to determine the serum dilution at which 50% of infection was neutralized.

Results

Primary Dengue-1 Infection

Primary WP74 Inoculation

To determine if Yucatan Miniature swine are susceptible to WP74 infection, 10 animals were divided into 3 groups: Group 1 (N=4), Group 2 (N=4), and Group 3 (N=2). Group 1 was inoculated with $1x10^5$ PFU of WP74 while Group 2 was inoculated with $1x10^7$ PFU of the same virus, and Group 3 was the control group, which was inoculated with virus diluent. Blood samples were collected from Groups 1 and 2 on study days 0 through 14, 21, 29, 86, and 176. Blood samples were collected from Group 3 animals on all days except days 1 through 13. Viremia, signs, symptoms, and immune responses were monitored daily.

Viremia Determination

Viremia was determined in animals using serum samples from days 0 through 14. Vero cell culture and SIGN cell isolation were used to determine viremia in Group 1 and 2 animals.

By Vero cell culture isolation, animals in Group 1 had between 1 and 7 days of viremia with an average of 3.75 days of viremia for the group, while Group 2 animals experienced between 3 and 9 days of viremia with an average viremia of 6.75 days (Table 1). The viremia occurred between days 1 and 10 post inoculation, demonstrating an active infection.

		Group 1				Group 2			
	Animal	5595	5596	5597	5604	5598	5600	5609	5612
Study Day									
Day 0		0	0	0	0	0	0	0	0
Day 1		3	0	0	1	2	1	2	0
Day 2		0	0	0	0	0	0	1	1
Day 3		1	0	0	3	2	1	2	2
Day 4		1	1	1	1	1	1	0	1
Day 5		1	0	0	1	1	1	1	0
Day 6		0	0	0	0	1	1	0.5	0
Day 7		1	0	0	0.5	1	1.5	0	0
Day 8		1.5	0	0	1	1	1	0	0
Day 9		1	0	0	0	1	1	1	0
Day 10		0	0	0	0	1	1	0	0
Day 11		0	0	0	0	0	0	0	0
Day 12		0	0	0	0	0	0	0	0
Day 13		0	0	0	0	0	0	0	0
Day 14		0	0	0	0	0	0	0	0
Group Average Days					3.8				6.8

Table 1: Primary WP74 Infection, Vero Cell Culture Isolation

Data in this Table are presented in levels of infection as follows: 0 = no cells infected, 1 =5-25 % of cells infected, 2 = 25-50% of cells infected, and 3 = 50-100% of cells infected. This assay was completed in duplicate; when the level of infection between the flasks differed, the average of the 2 numbers was taken, resulting in a value ending in .5.

Using SIGN cell isolation, animals in Group 1 experienced an average of 6.75 days of viremia with the range being 1 to 13 days of viremia. Animals in Group 2 had an average of 12.75 days of isolation with a range of 11 to 14 days of viremia. Virus was isolated using this technique between days 1 and 14 (Table 2).

		Group 1				Group 2			
	Animal	5595	5596	5597	5604	5598	5600	5609	5612
Study Day									
Day 0		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Day 1		6.54	0.00	0.00	3.19	1.39	0.36	1.90	3.50
Day 2		5.16	0.00	0.00	2.24	3.21	0.54	2.19	6.32
Day 3		3.94	0.00	0.00	5.21	0.51	0.57	2.27	6.05
Day 4		3.59	0.00	0.00	1.67	0.14	0.44	2.37	6.25
Day 5		3.64	0.00	0.00	0.96	0.22	0.00	1.47	3.95
Day 6		4.36	0.00	0.00	0.31	0.19	0.15	0.46	0.46
Day 7		3.84	0.11	0.00	0.00	0.00	0.09	0.32	0.64
Day 8		4.01	0.00	0.00	0.25	0.00	0.07	0.42	7.21
Day 9		2.94	0.00	0.00	0.23	2.65	0.17	0.46	0.15
Day 10		2.64	0.00	0.00	0.24	0.24	0.13	0.21	0.05
Day 11		1.69	0.00	0.00	1.07	2.00	0.04	0.37	0.08
Day 12		0.21	0.00	0.00	0.85	2.93	0.13	0.17	0.23
Day 13		0.56	0.00	0.24	0.39	0.73	0.00	2.52	0.12
Day 14		0.00	0.00	0.34	0.85	0.03	0.00	0.17	0.09
Group Average Days					6.75				12.75

Table 2: Primary WP74 Infection, SIGN Cell Isolation

Data in this Table are presented as percent of live cells that were infected with dengue virus. The background level of fluorescence plus 3 standard deviations from the mean of the background fluorescence in the cells has been subtracted from the values presented in this Table.

Isotypic Antibody Response

Anti-dengue-1 isotype-specific antibody responses were determined. Specimens from study days 0 through 14, 21, 29, 86, and 176 were analyzed using IgM ELISA, and specimens from study days 0, 14, 21, 29, 86, and 176 were analyzed by IgG ELISA.

Fifty percent of Group 1 animals developed measurable dengue-1 IgM antibodies (Table 3). IgM levels in these animals peaked between days 11 and 12 post infection and waned until day 21, at which point they are no longer detectable (Figure 5). The animals, which did not develop a measurable response,

were those that developed only one day of viremia as determined by Vero cell culture isolation and one to two days by SIGN cell isolation. All Group 2 animals developed dengue-1 IgM antibodies (Table 3), which peaked between days 6 and 7, at which point they began to wane; by day 21 titers were no longer detectable (Figure 5).

		Group 1				Group 2				Group 3	
	Animal	5595	5596	5597	5604	5598	5600	5609	5612	5594	5611
Study Day											
Day 0		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Day 1		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	ND	ND
Day 2		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	ND	ND
Day 3		0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.00	ND	ND
Day 4		0.41	0.00	0.00	0.13	0.15	0.12	0.39	0.14	ND	ND
Day 5		0.51	0.00	0.00	0.22	0.86	0.66	1.22	0.82	ND	ND
Day 6		0.58	0.00	0.00	0.19	1.27	0.71	1.42	0.82	ND	ND
Day 7		0.59	0.00	0.00	0.44	1.30	0.99	1.63	0.71	ND	ND
Day 8		0.72	0.00	0.00	0.33	1.50	0.86	0.77	0.47	ND	ND
Day 9		0.98	0.00	0.00	0.49	1.38	0.50	1.23	0.43	ND	ND
Day 10		0.41	0.00	0.00	0.00	1.18	0.49	0.11	0.27	ND	ND
Day 11		1.10	0.00	0.00	0.50	1.10	0.40	0.28	0.15	ND	ND
Day 12		1.01	0.00	0.00	0.50	1.10	0.26	0.61	0.29	ND	ND
Day 13		1.09	0.00	0.00	0.00	0.59	0.29	0.54	0.07	ND	ND
Day 14		0.79	0.00	0.00	0.27	0.48	0.07	0.36	0.00	0.00	0.00
Day 21		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00
Day 29		0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Day 86		0.00	0.00	0.00	0.00	0.03	0.00	0.00	0.00	0.00	0.00
Day 176		0.00	0.25	0.00	0.18	0.00	0.00	0.00	0.00	0.00	0.00

Table 3: Primary WP74 Infection, IgM Response

The data presented in this table are OD values at 405 nm. The background level of activity in negative serum plus 3 standard deviations from the mean of negative samples has been subtracted from all values.

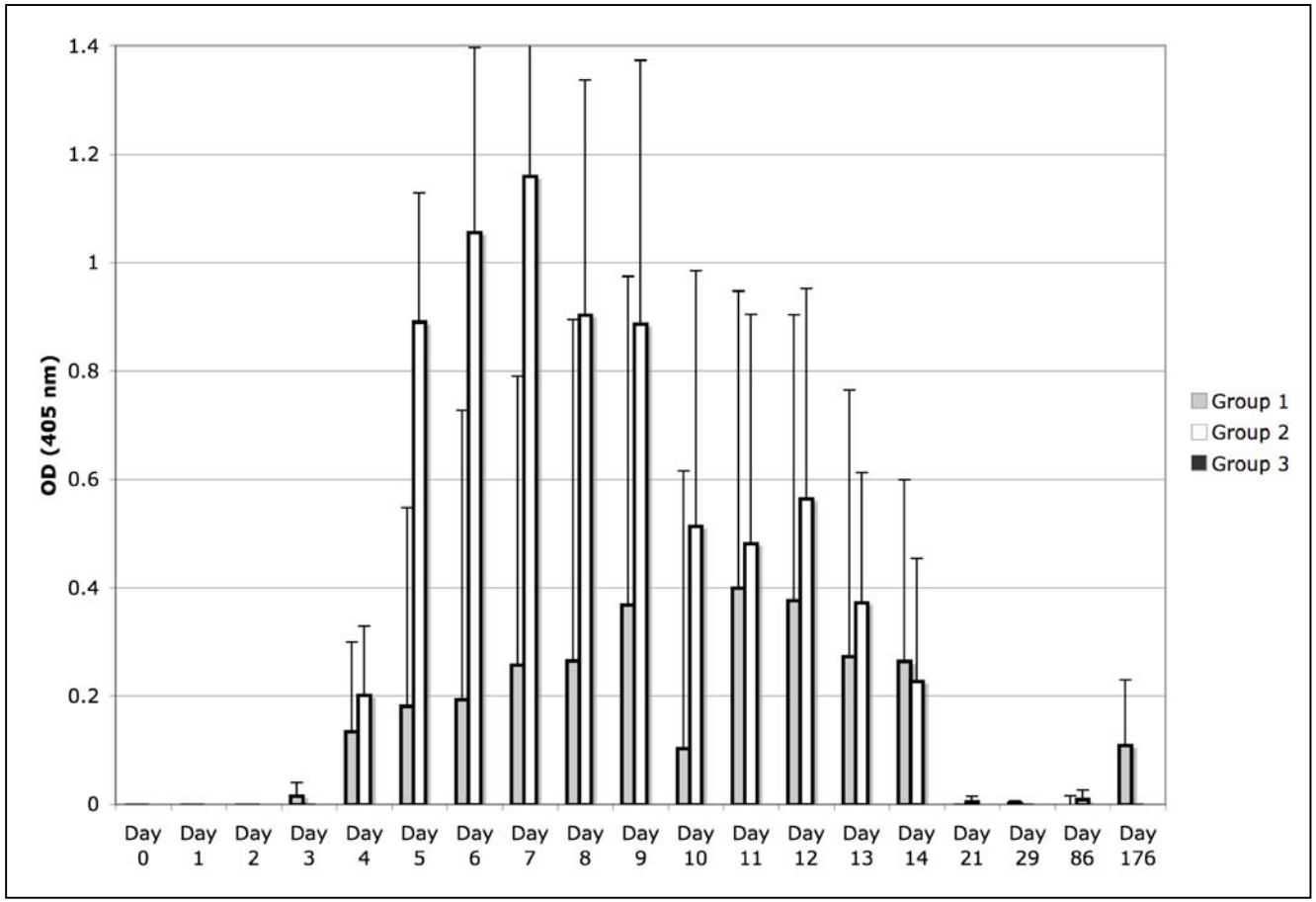

Figure 1: Group IgM Response, Primary WP74 Infection

Group mean responses are presented for observation of group trends. Error bars are 1 standard deviation from the group mean for that value.

IgG responses were detected in all animals that developed IgM responses and also had greater than 2 days of viremia using virus isolation techniques (Table 4). The IgG responses peaked at day 14, which then waned until day 29, and were maintained (Figure 4).

		Group 1				Group 2				Group 3	
	Animal	5595	5596	5597	5604	5598	5600	5609	5612	5594	5611
Study Day											
Day 0		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Day 14		0.49	0.00	0.00	0.09	0.39	0.37	0.79	0.44	0.00	0.00
Day 21		0.18	0.00	0.00	0.05	0.35	0.32	0.61	0.36	0.00	0.00
Day 29		0.17	0.00	0.00	0.06	0.39	0.30	0.55	0.22	0.00	0.00
Day 86		0.14	0.00	0.00	0.16	0.38	0.35	0.38	0.21	0.00	0.00
Day 176		0.31	0.20	0.00	0.10	0.26	0.12	0.16	0.34	0.00	0.00

Table 1: Primary WP74 Infection, IgG Response

The data presented in this table are OD values at 405 nm. The background level of activity in negative serum plus 3 standard deviations from the mean of negative samples has been subtracted from all values.

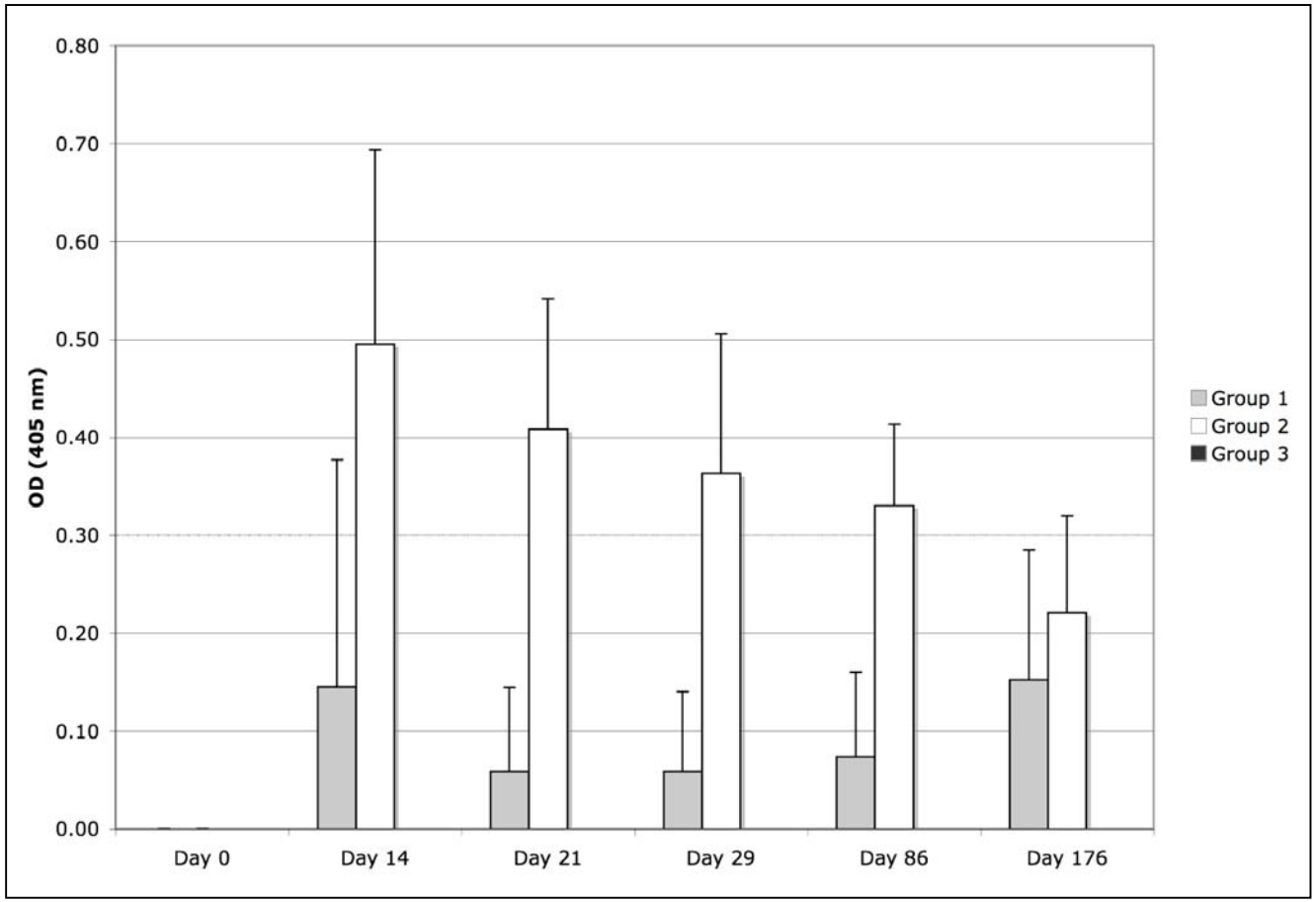

Figure 1: Group IgG Response, Primary WP74 Infection

Group mean responses are presented for observation of group trends. Error bars are 1 standard deviation from the group mean for that value.

Serum Neutralization Levels

Virus neutralizing serum antibodies were measured using Vero PRNT and SIGN neutralization assays. Serum samples from study days 0, 14. 21, 29, 86, and 176 (PRNT only) were tested for virus neutralization activity.

The results of the PRNT mirror those of the isotype ELISA (Table 5). However, the two Group 1 animals (5595 and 5604) that developed measurable IgM and IgG titers developed higher serum neutralizing antibodies than seen in the animals from Group 2, all of which developed serum neutralizing antibodies. The most likely cause of the higher titer is the later peak in IgM titer found in Group 1 animals. Neutralizing antibodies, as measured by Vero PRNT, peaked on day 14, then waned slightly, and were maintained for at least 6 months. (Figure 7)

	Study Day	Day 0	Day 14	Day 21	Day 29	Day 86	Day 176
	Animal						
Group 1	5595	0	90	27	29	26	49
	5596	0	0	0	0	0	<10
	5597	0	<10	<10	<10	0	0
	5604	0	191	78	22	<10	45
Group 2	5598	0	49	20	20	37	30
	5600	0	46	22	19	16	35
	5609	0	48	16	12	11	21
	5612	0	11	<10	<10	17	33
Group 3	5594	0	0	0	0	0	0
	5611	0	0	0	0	0	0
	Group 1 Mean	0	71	28	13	8	26
	Group 2 Mean	0	39	16	15	20	30
	Group 3 Mean	0	0	0	0	0	0

Table 1: Primary WP74 Infection, Reciprocal Vero $PRNT_{50}$ Titer

Data in this table are presented as the reciprocal titer at which 50 percent of plaques are neutralized by serum. The lowest dilution that was tested was 1:10. So samples that had neutralization activity, but the titer was calculated to be lower than 1:10, are presented as <10 in this table. Samples with no neutralization activity are presented as 0. Average values were calculated using predicted values from Probit analysis when the titer was less than 1:10.

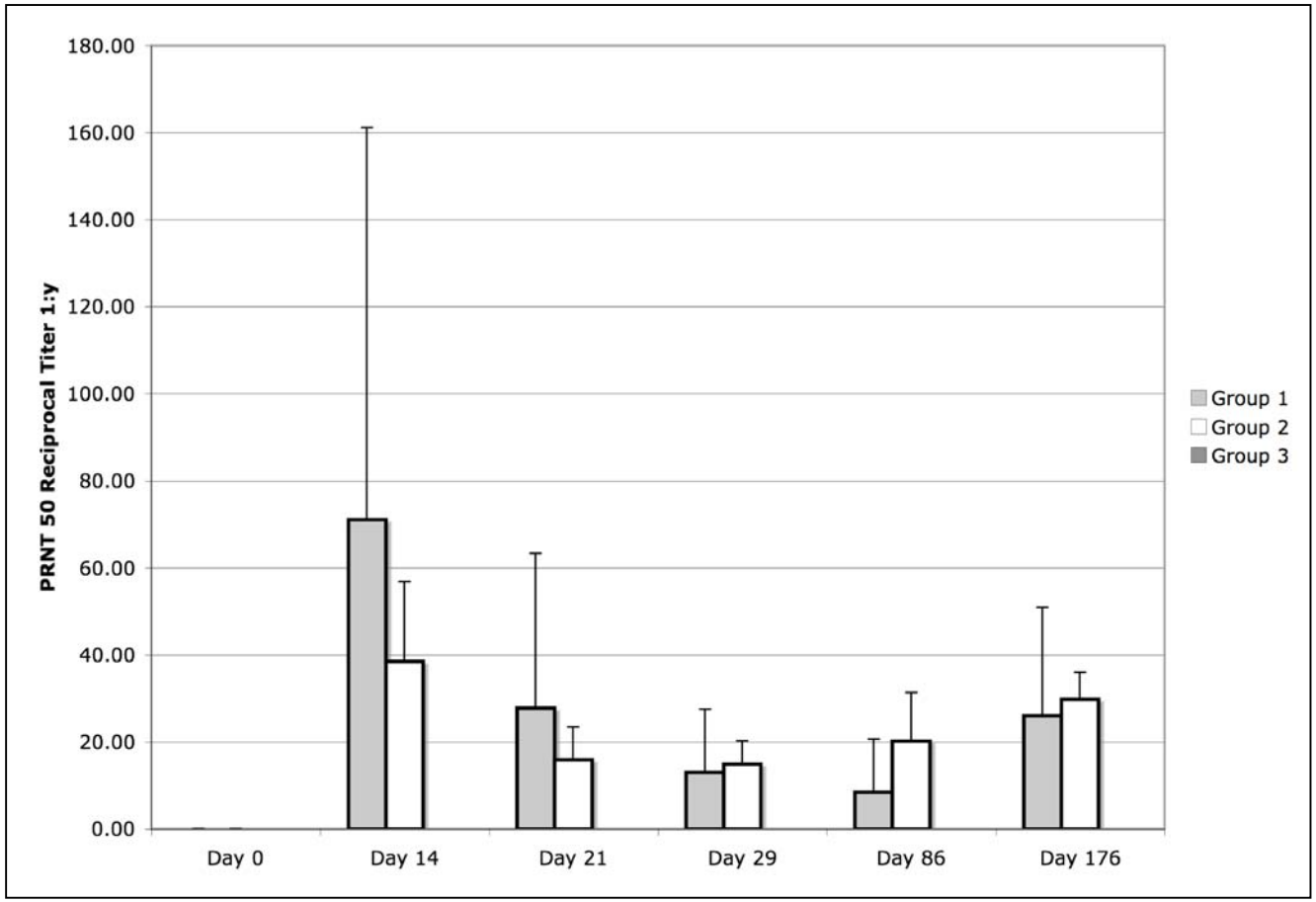

Figure 1: Group Reciprocal Vero $PRNT_{50}$ Titer, Primary WP74 Infection

Values presented are group means with error bars representing 1 standard deviation from the group mean.

The SIGN neutralization assay results parallel those of the PRNT assay (Table 6). All animals that developed PRNT responses also showed virus neutralizing antibody responses by this assay. The time course of WP74 SIGN neutralizing activity is shown in Figure 8. Neutralizing activity is maximal at day 14 for Group 1 animals 5595 and 5604. All Group 2 animals developed measurable neutralization activity by day 14, which remained constant through day 86.

	Study Day	Day 0	Day 14	Day 21	Day 29	Day 86
	Animal					
Group 1	5595	0	159	62	38	50
	5596	0	0	0	0	0
	5597	0	<10	0	0	<10
	5604	0	60	<10	0	10
Group 2	5598	0	15	<10	<10	31
	5600	0	11	<10	<10	17
	5609	0	15	10	<10	13
	5612	0	15	<10	<10	17
Group 3	5594	ND	ND	ND	ND	ND
	5611	ND	ND	ND	ND	ND
	Group 1 Mean	0	55	16	10	16
	Group 2 Mean	0	14	6	6	19

Table 1: Primary WP74 Infection, Reciprocal SIGN Neutralization Titer

Data in this table are presented as the reciprocal titer at which 50 percent of infection of cells is neutralized by serum. The lowest dilution that was tested was 1:10. So samples that had neutralization activity, but the titer was calculated to be lower than 1:10, are presented as <10 in this table. Samples with no neutralization activity are presented as 0. Average values were calculated using predicted values from Probit analysis when the titer was less than 1:10.

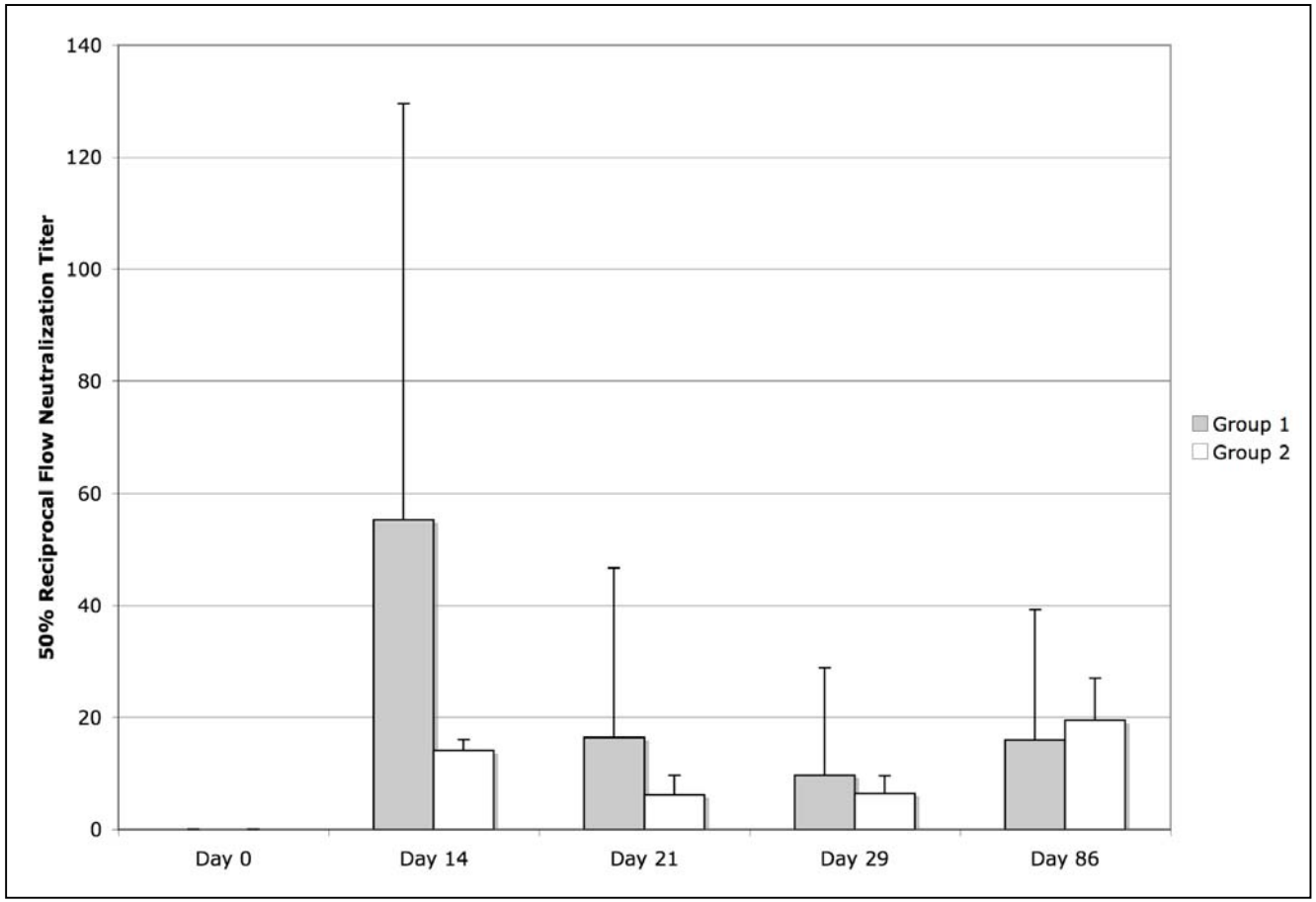

Figure 1: Group Reciprocal SIGN Neutralization Titer, Primary WP74 Infection

Values presented are group means with error bars of 1 standard deviation from the group mean.

Signs and Symptoms

The animals were examined for disease signs and symptoms on all blood sampling days. No signs or symptoms of dengue illness were noted for any of the animals.

Secondary Homologous Dengue-1 Infection

Secondary Homologous WP74 Inoculation

To determine if the immune response mediated by a primary WP74 infection is protective against a secondary homologous WP74 challenge, all animals (Groups 1 through 3) were inoculated with $1x10^7$ PFU of WP74 on day 176 post primary infection. Viremia, signs, symptoms, and immune responses were monitored daily. A reduction in viremia of the immune animals, Groups 1 and 2, compared to the control animals, Group 3, is a measure of protective immunity.

Viremia Determination

Serum samples from all animals (Groups 1 through 3) on study days 176 through 186 were used for viremia determination. Viremia was determined using Vero cell culture isolation and SIGN cell isolation.

Using Vero cell culture isolation, Group 3 control animals had between 4 and 6 days of viremia with an average of 5 days. (Table 7) These results present a similar duration of viremia to what was observed in the Group 2 animals upon primary WP74 infection. The animals in Groups 1 and 2 experienced only 1 day of viremia on day 177. This reduction of viremia from 5 days to 1 day represents an 80% reduction in the viremia period.

		Group 1				Group 2				Group 3	
	Animal	5595	5596	5597	5604	5598	5600	5609	5612	5594	5611
Study Day											
Day 176		0	0	0	0	0	0	0	0	0	0
Day 177		1	1	1	1	1	0.5	1	0.5	1	1.5
Day 178		0	0	0	0	0	0	0	0	1	1
Day 179		0	0	0	0	0	0	0	0	1	0
Day 180		0	0	0	0	0	0	0	0	1	1
Day 181		0	0	0	0	0	0	0	0	1	1
Day 182		0	0	0	0	0	0	0	0	1	0
Day 183		0	0	0	0	0	0	0	0	0	0
Day 184		0	0	0	0	0	0	0	0	0	0
Day 185		0	0	0	0	0	0	0	0	0	0
Day 186		0	0	0	0	0	0	0	0	0	0
Group Average Days					1				0.75		5

Table 1: Secondary Homologous WP74 Challenge, Vero Cell Culture Isolation

Data in this table are presented in levels of infection as follows: 0 = no cells infected, 1 =5-25 % of cells infected, 2 = 25-50% of cells infected, and 3 = 50-100% of cells infected. This assay was completed in duplicate flasks, when the level of infection between the flasks differed, the average of the 2 numbers was taken, resulting in a value ending in .5.

Surprisingly, the SIGN cell isolation did not yield similar results to the Vero cell isolation. The Group 3 animals did not develop 11 to 14 days of viremia as did the Group 2 animals during a primary WP74 infection. One animal developed 0 days of viremia and the other 6 days of viremia (Table 8) for the group average of 3 days of viremia. Only one of the Group 1 animals had 2 days of viremia.

		Group 1				Group 2				Group 3	
	Animal	5595	5596	5597	5604	5598	5600	5609	5612	5594	5611
Study Day											
Day 176		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Day 177		0.14	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.74	0.00
Day 178		0.38	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.71	0.00
Day 179		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.30	0.00
Day 180		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.18	0.00
Day 181		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.56	0.00
Day 182		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.49	0.00
Day 183		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Day 184		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Day 185		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.21	0.00
Day 186		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00
Group Average Days					0.25				0		3

Table 2: Secondary Homologous WP74 Challenge, SIGN Cell Isolation

Data in this table are presented as percent of live cells that were infected with dengue virus. The background level of fluorescence plus 3 standard deviations from the mean background fluorescence in the cells has been subtracted from the values presented in this table.

Isotypic Antibody Response

Anti-dengue-1 isotype specific antibody responses were determined. Study day specimens 0, 176 through 186, 190, 197, and 204 were examined for anti-dengue IgM antibodies. Study day specimens 0, 176, 190, 197, and 204 were examined for IgG antibodies.

The IgM responses in the secondarily challenged swine varied within individuals (Table 10). The responses from group 3 animals were similar in magnitude to Group 1 and 2 animals after a primary WP74 infection. At the group level, a similar trend is seen in all 3 groups with the responses in Groups 1 and 2 being to a lower response level (Figure 9).

		Group 1				Group 2				Group 3	
	Animal	5595	5596	5597	5604	5598	5600	5609	5612	5594	5611
Study Day											
Day 0		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Day 176		0.00	0.25	0.00	0.18	0.00	0.00	0.00	0.00	0.00	0.00
Day 177		0.00	0.18	0.00	0.20	0.00	0.00	0.00	0.00	0.00	0.00
Day 178		0.00	0.11	0.00	0.24	0.00	0.00	0.00	0.00	0.00	0.00
Day 179		0.00	0.00	0.00	0.18	0.00	0.00	0.00	0.00	0.00	0.00
Day 180		0.54	0.05	0.09	0.37	0.00	0.00	0.00	0.92	0.00	0.91
Day 181		1.08	0.25	0.81	0.30	0.24	0.00	0.00	1.20	0.31	1.08
Day 182		1.12	0.18	0.83	0.31	0.44	0.00	0.00	1.31	0.97	1.17
Day 183		1.25	0.03	0.78	0.39	0.41	0.00	0.00	1.32	1.22	1.26
Day 184		1.15	0.00	0.61	0.39	0.25	0.00	0.00	1.37	1.15	1.17
Day 185		1.12	0.00	0.57	0.41	0.22	0.00	0.00	1.31	1.10	1.23
Day 186		1.17	0.00	0.26	0.45	0.00	0.00	0.00	1.35	0.81	1.28
Day 190		0.55	0.00	0.00	0.00	0.00	0.00	0.00	1.03	0.00	0.65
Day 197		0.88	0.00	0.00	0.00	0.00	0.00	0.00	0.88	0.00	0.00
Day 204		0.57	0.00	0.00	0.00	0.00	0.00	0.00	0.35	0.00	0.00

Table 3: Secondary Homologous WP74 Challenge, IgM Response

The data presented in this table are OD values at 405 nm. The background level of activity in negative serum plus 3 standard deviations from the mean of negative samples has been subtracted from all values.

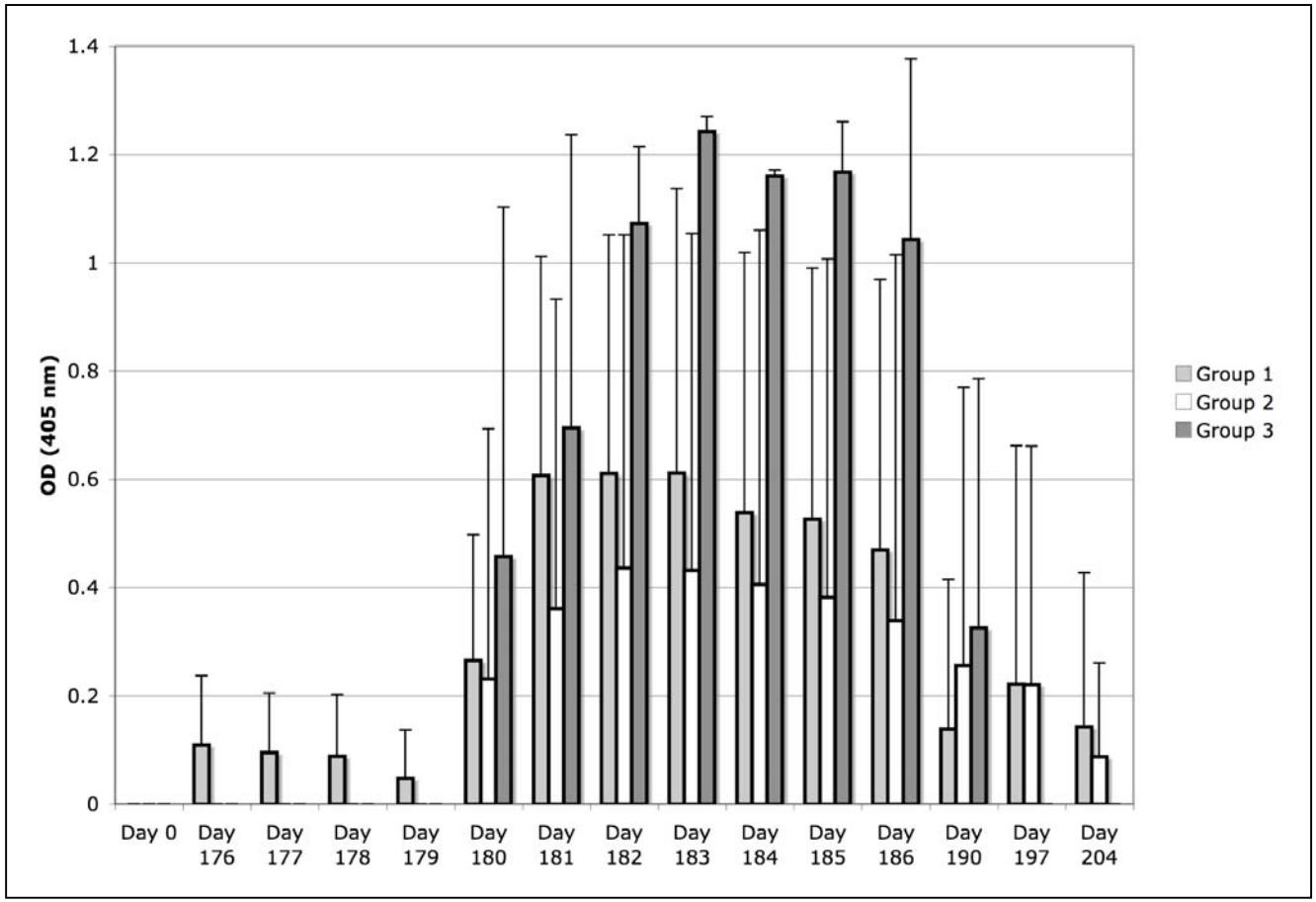

Figure 1: Group IgM Response, Secondary Homologous WP74 Challenge

Group mean responses are presented for observation of group trends. Error bars are 1 standard deviation from the group mean for that value.

The IgG response of Group 3 animals is identical to that which was seen for IgG positive animals from Groups 1 and 2 after primary infection with WP74. The animals in Groups 1 and 2 developed IgG levels that were boosted as compared to their primary response (Table 10). These levels peaked on day 190 and were maintained at a high level out to day 204 (day 28 post challenge).

		Group 1				Group 2				Group 3	
	Animal	5595	5596	5597	5604	5598	5600	5609	5612	5594	5611
Study Day											
Day 0		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Day 176		0.31	0.20	0.00	0.10	0.26	0.12	0.16	0.34	0.00	0.00
Day 190		0.26	0.81	0.74	1.20	1.94	1.99	1.42	2.01	1.08	1.14
Day 197		1.60	0.78	0.72	1.12	1.89	1.95	1.38	1.78	0.97	1.28
Day 204		1.55	0.83	0.36	1.17	1.77	1.82	1.32	1.91	1.04	1.22

(Figure 10)

Table 1: Secondary Homologous WP74 Challenge, IgG Response

The data presented in this table are OD values at 405 nm. The background level of activity in negative serum plus 3 standard deviations from the mean of negative samples has been subtracted from all values.

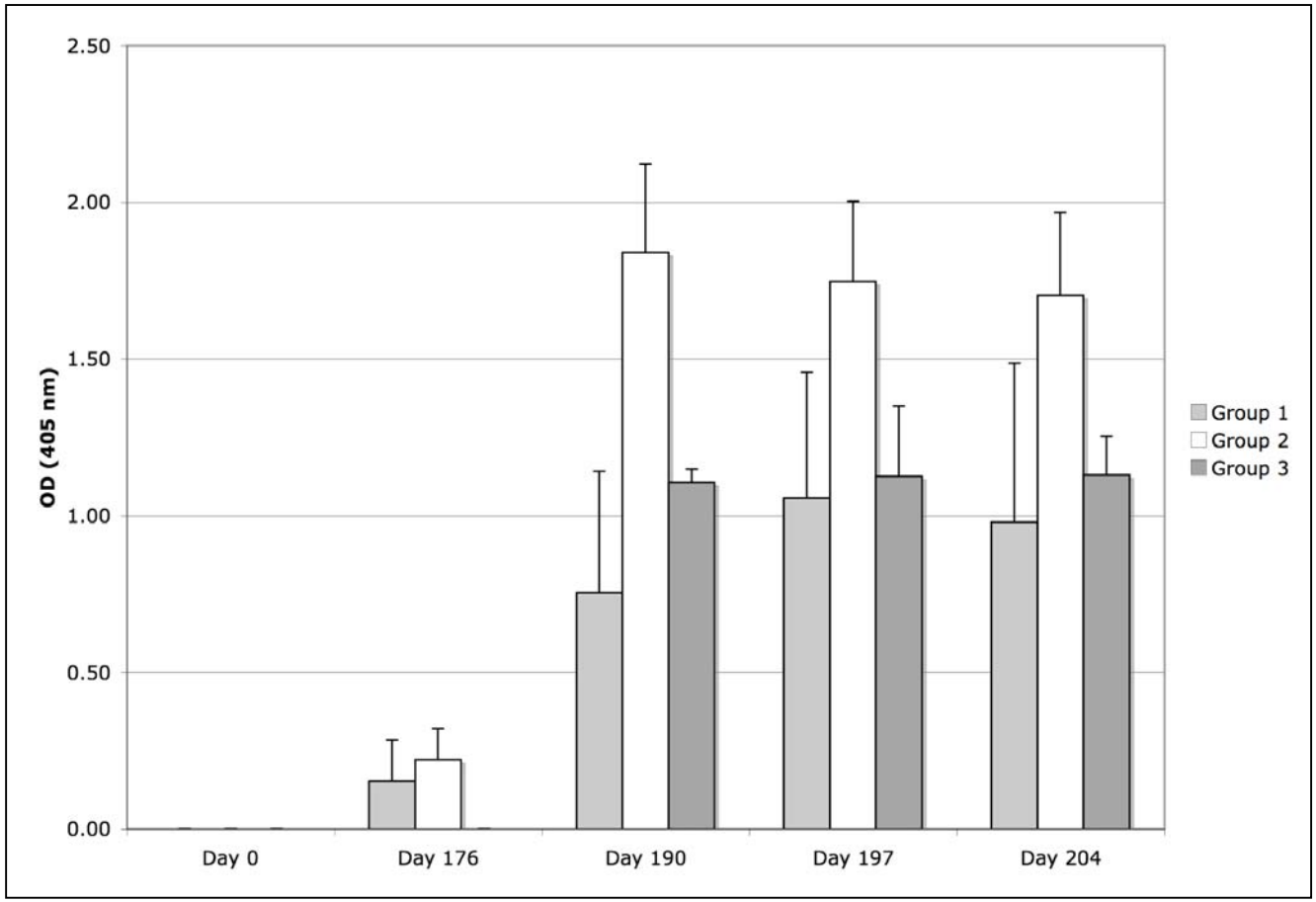

Figure 1: Group IgG Response, Secondary Homologous WP74 Challenge

Group mean responses are presented for observation of group trends. Error bars are 1 standard deviation from the group mean for that value.

Serum Neutralization Levels

Virus neutralizing serum antibodies were measured via Vero PRNT using samples from study days 0, 176, 190, 197, and 204.

Post challenge, Group 1 and 2 animals developed neutralizing antibody levels that were significantly boosted (p= 0.004-0.023), with a peak on day 21 post infection, as compared to levels seen in primary infection (Figure 11). Group 3 animals presented responses that mirrored the response of Group 2 animals post a primary WP74 infection that is characterized by a peak in titer on day 14 post challenge with a titer less than 1:200 (Table 11).

	Study Day	Day 0	Day 176	Day 190	Day 197	Day 204
	Animal					
Group 1	5595	0	49	1356	2627	499
	5596	0	<10	137	413	84
	5597	0	0	211	317	160
	5604	0	45	209	468	339
Group 2	5598	0	30	1005	2129	706
	5600	0	35	308	1387	254
	5609	0	21	199	615	194
	5612	0	33	770	2657	614
Group 3	5594	0	0	60	46	50
	5611	0	0	133	75	69
	Group 1 Mean	0	26	478	956	271
	Group 2 Mean	0	30	571	1697	442
	Group 3 Mean	0	0	97	61	59

Table 1: Secondary Homologous WP74 Challenge, Reciprocal Vero $PRNT_{50}$ Titer

Data in this table are presented as the reciprocal titer at which 50 percent of plaques are neutralized by serum. The lowest dilution that was tested was 1:10. So samples that had neutralization activity but the titer was calculated to be lower than 1:10, are presented as <10 in this table. Samples with no neutralization activity are presented as 0. Average values were calculated using predicted values from Probit analysis when the titer was less than 1:10.

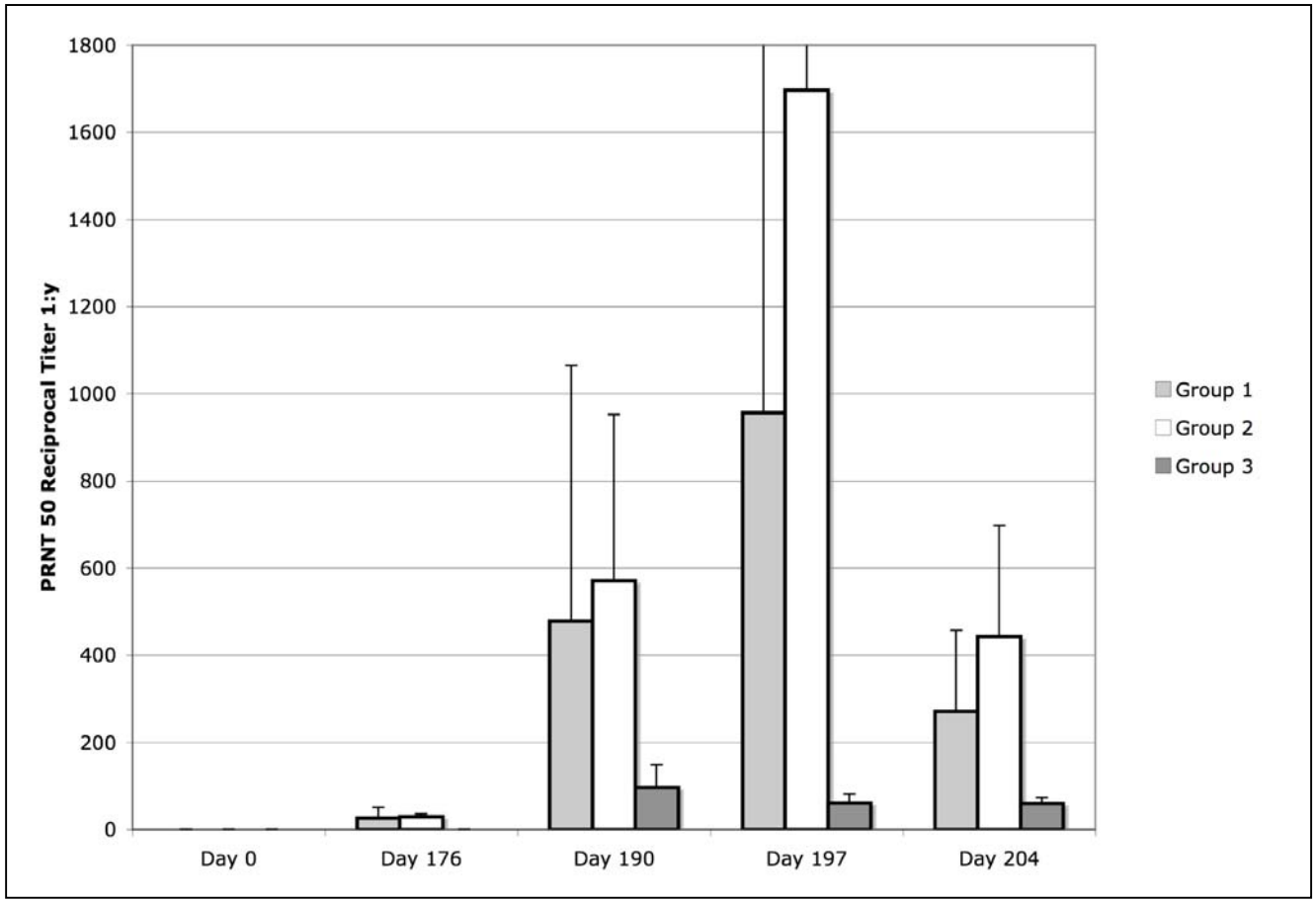

Figure 1: Group Reciprocal Vero $PRNT_{50}$ Titer, Secondary Homologous WP74 Challenge

Values presented are group means with error bars representing 1 standard deviation from the group mean.

Signs and Symptoms

All animals were examined for disease signs and symptoms on all blood sampling days. No changes in temperature were observed but, on the fourth day post inoculation, 5 animals developed a rash. Two of the animals were from Group 1 and the other three animals were from Group 2. All animals that developed a rash were experiencing their secondary dengue inoculation and also had developed IgM, IgG, and dengue-1 neutralizing antibodies to their primary WP74 infection. The rashes did not originate at the inoculation site, were maculopapular in nature, and spread over the entire body of the animals. The rash was 4 to 7 days in duration. Figures 12 through 24 depict a typical rash that was seen and its progression. Data for rash presentation are represented in Table 12.

The only factor examined that correlates with rash is presence of neutralizing antibodies above the limit of the assay, which is a dilution of 1:10, following primary infection with a correlation coefficient of 0.745 ($p=0.034$). One animal, 5612,which is a normally quite aggressive animal, showed symptoms of illness on days 184 through 186 in the form of lethargy.

Animal	Day 176	Day 177	Day 178	Day 179	Day 180	Day 181	Day 182	Day 183	Day 184	Day 185	Day 186
5595	0	0	0	0	3	4	3	2	0	0	0
5596	0	0	0	0	0	0	0	0	0	0	0
5597	0	0	0	0	0	0	0	0	0	0	0
5604	0	0	0	0	3	4	4	4	4	4	4
5598	0	0	0	0	2	2	3	3	3	4	4
5600	0	0	0	0	0	0	0	0	0	0	0
5609	0	0	0	0	3	4	4	4	3	3	2
5612	0	0	0	0	2	3	2	2	2	2	2
5594	0	0	0	0	0	0	0	0	0	0	0
5611	0	0	0	0	0	0	0	0	0	0	0

Table 1: Rash Level

Levels of rash are presented in this table. The numbers represent the number of areas of the swine that were affected by the rash on that day. The areas were dorsal side, ventral side, legs, and behind the ears. A value of 4 means that the animal was covered by a rash.

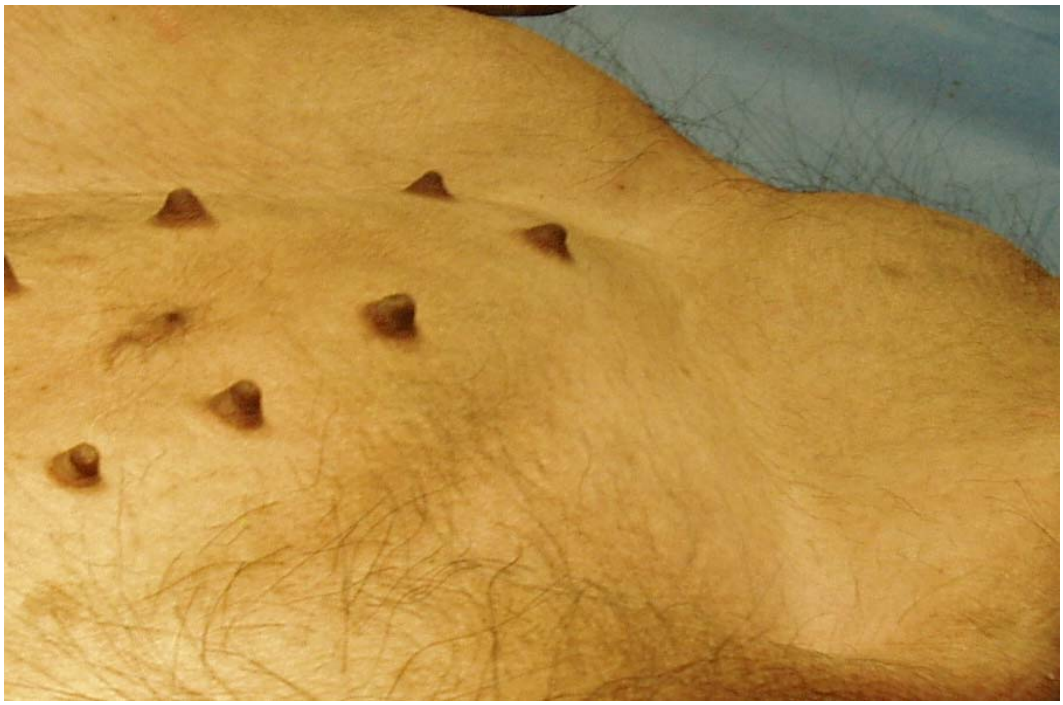

Figure 1: 5597 Day 181

This photo shows normal skin on the ventral side of an animal unaffected by the skin manifestation.

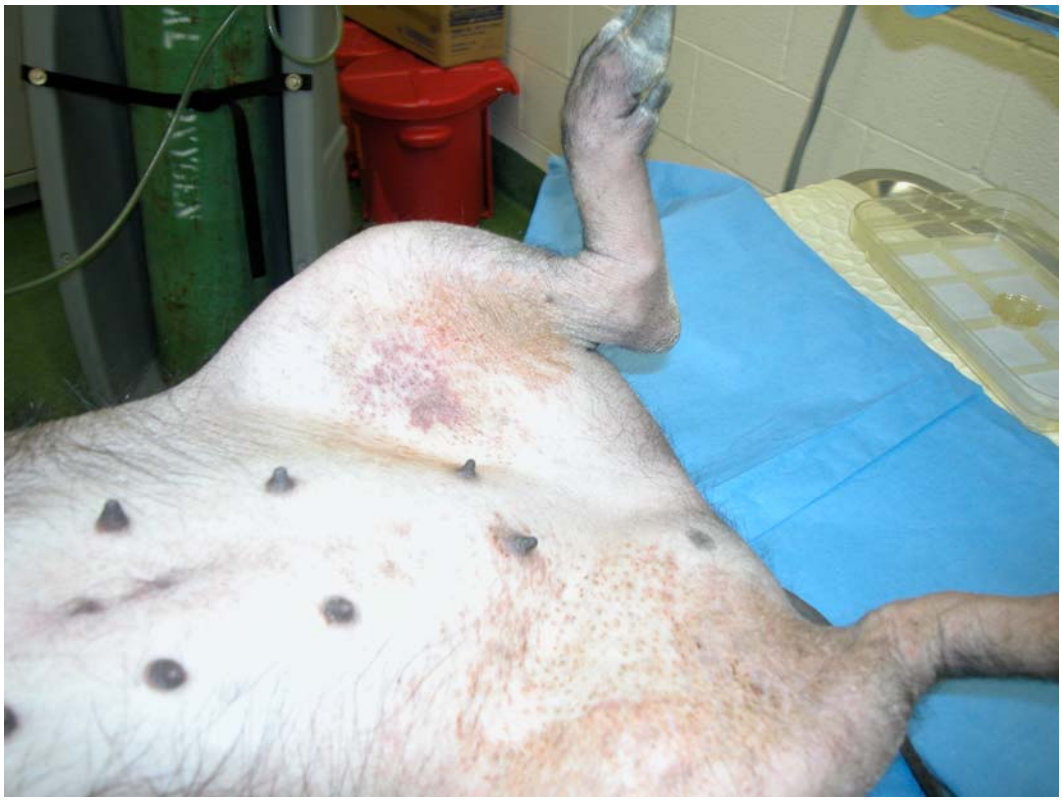

Figure 2: 5604 Day 181

On study day 180, a rash began to appear, and by day 181, the rash was severe and maculopapular in nature on the ventral side of this animal.

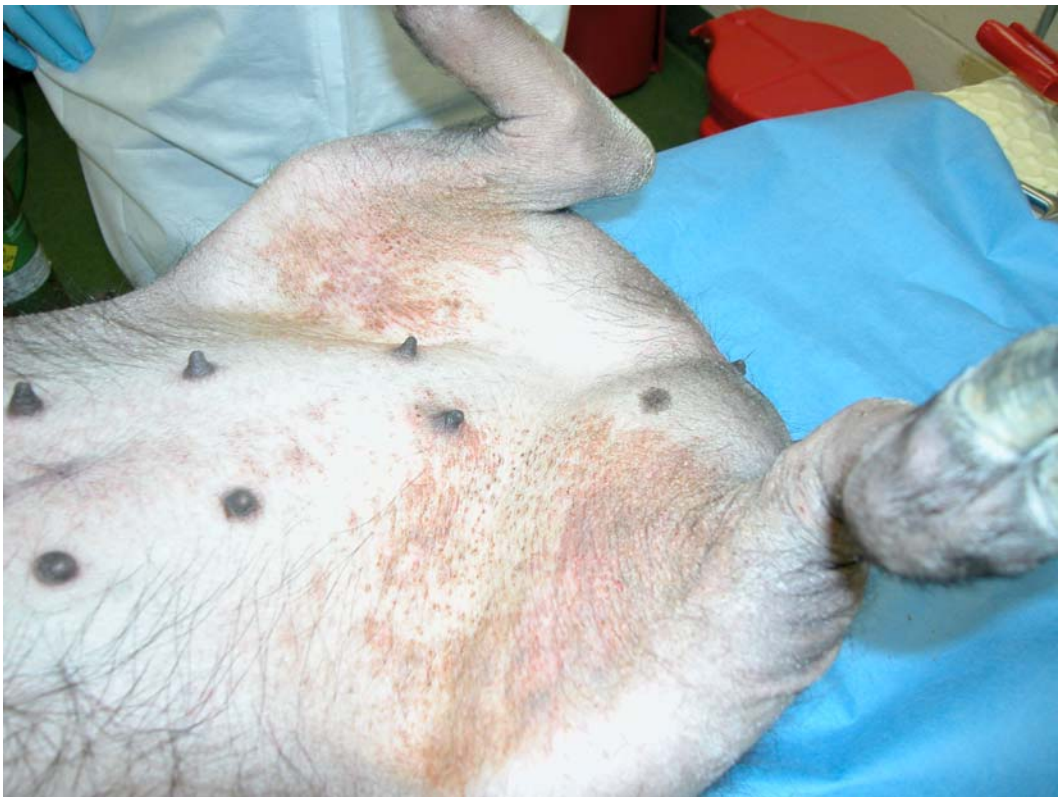

Figure 3: 5604 Day 183

By study day 183, the rash progressed and covered a greater area. Bruising developed in areas where the rash was dissipating.

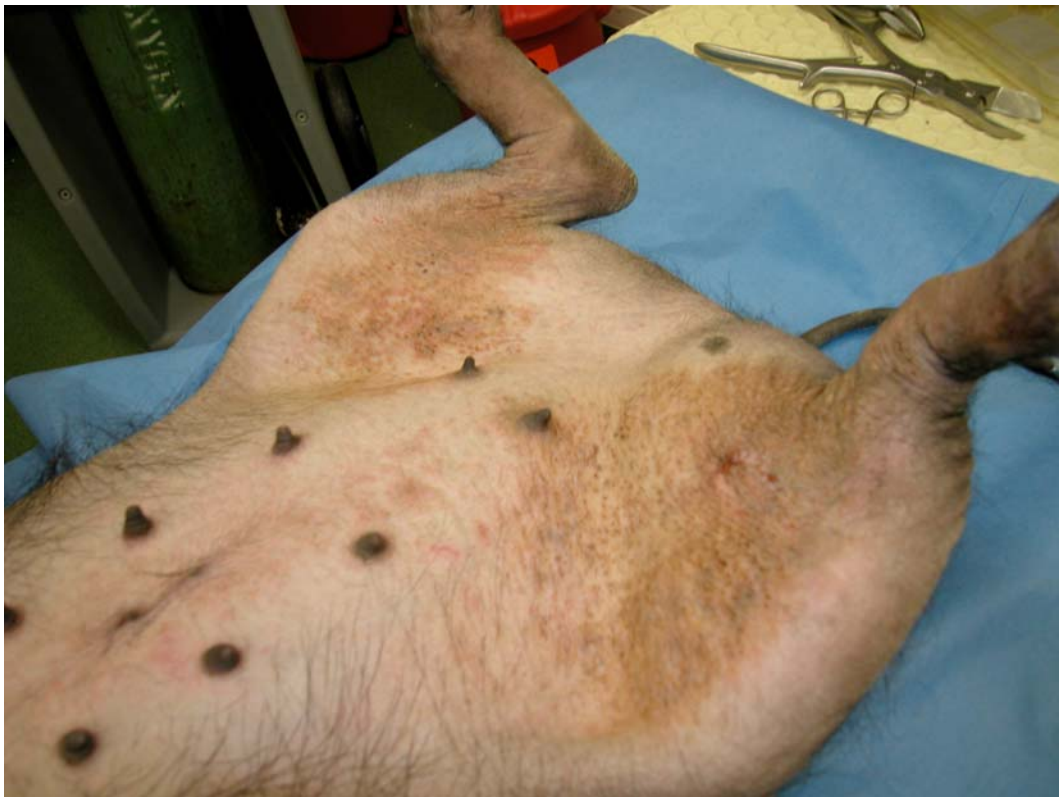

Figure 4: 5604 Day 184

By study day 184, the rash was still present and the bruising was becoming more extensive.

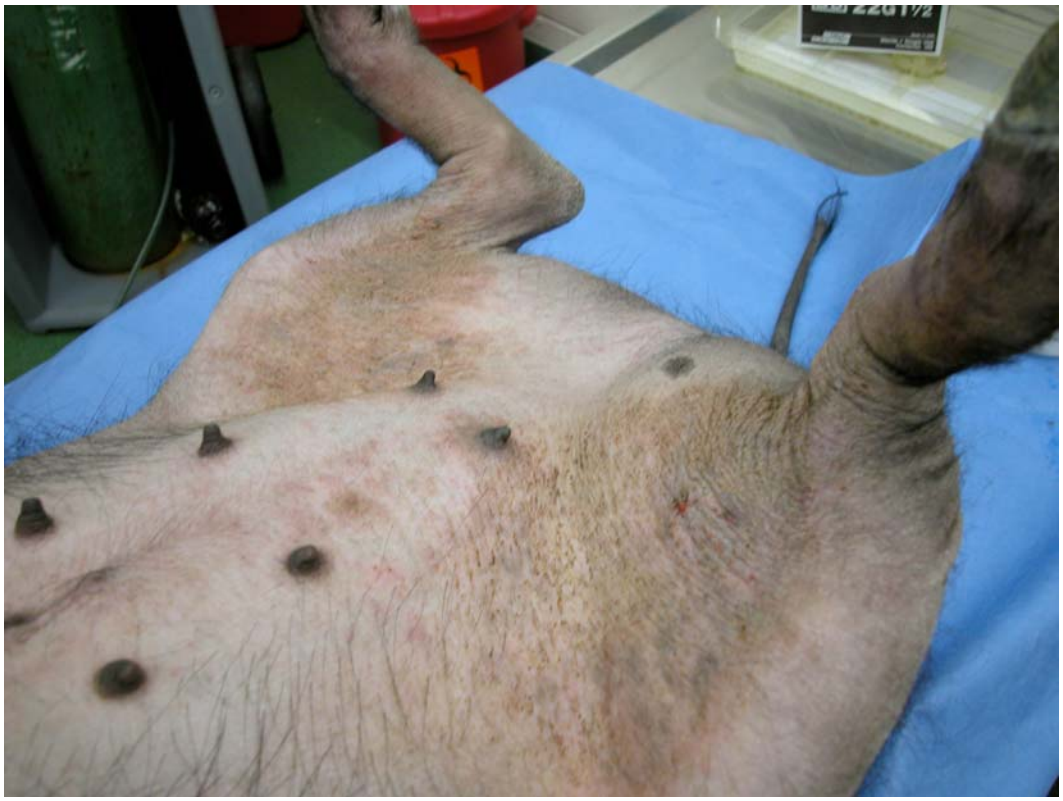

Figure 5: 5604 Day 186

By study day 186, the rash was barely present and the bruising was quite extensive. In addition the punch biopsy in the area of the rash was not healing well. The bruising was present in this animal until study day 197, and the punch biopsy was not completely healed until study day 204.

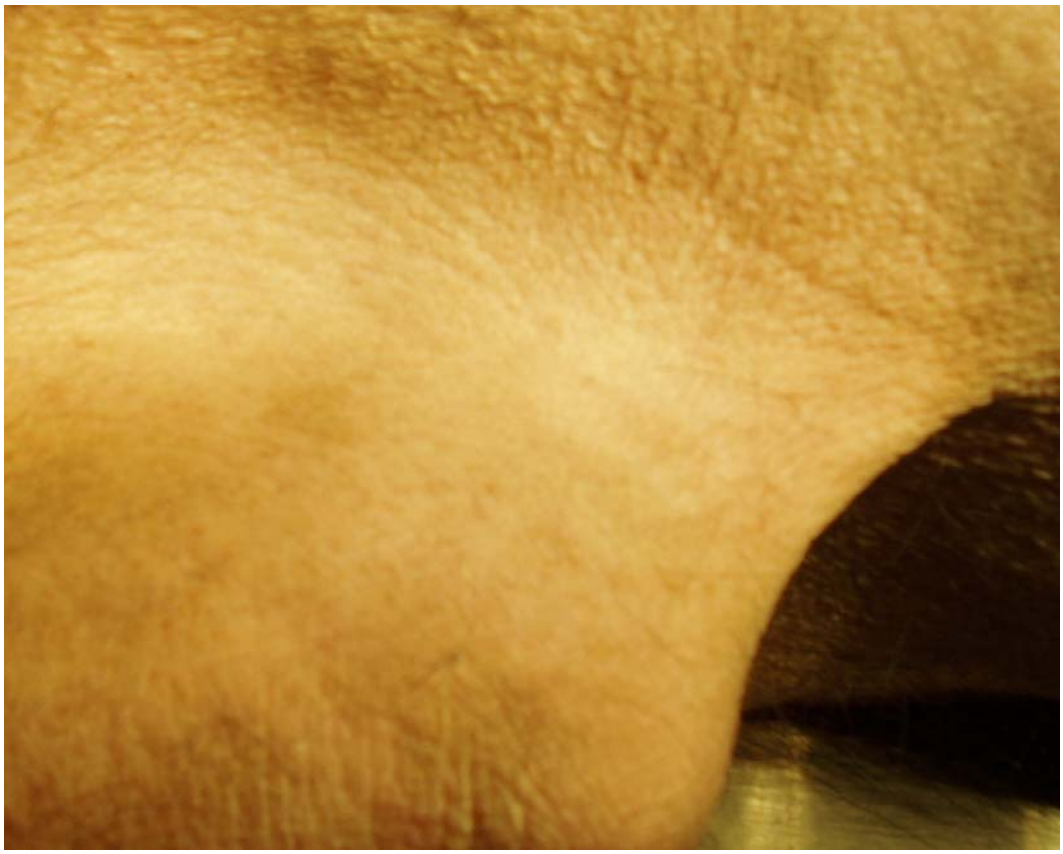

Figure 6: 5597 Day 181

This is a photo of normal swine skin on the interior of the right front leg.

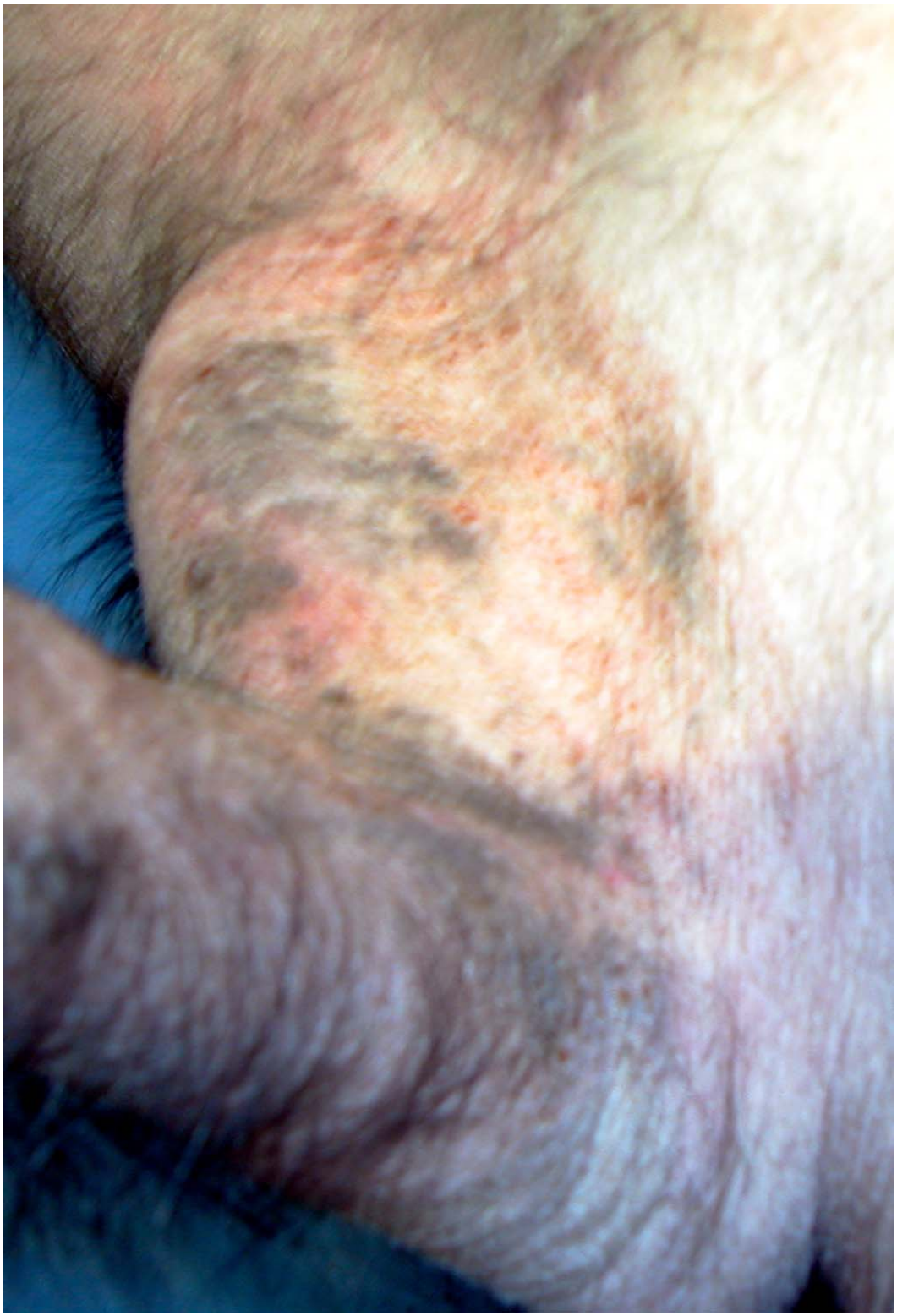

Figure 7: 5604 Day 181

On study day 180, this animal had a visible maculopapular rash accompanied by significant bruising on the interior of its front limbs.

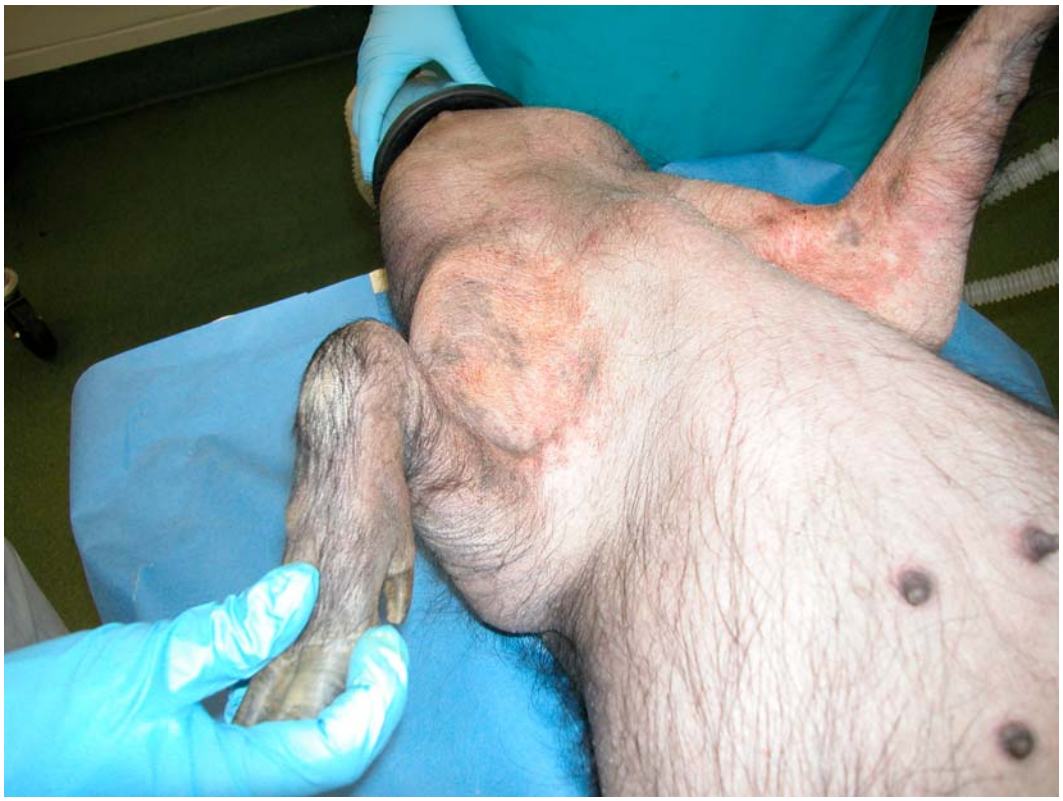

Figure 8: 5604 Day 183

By study day 183, the rash that was present only on the limbs spread across the sternum of this animal and onto its neck.

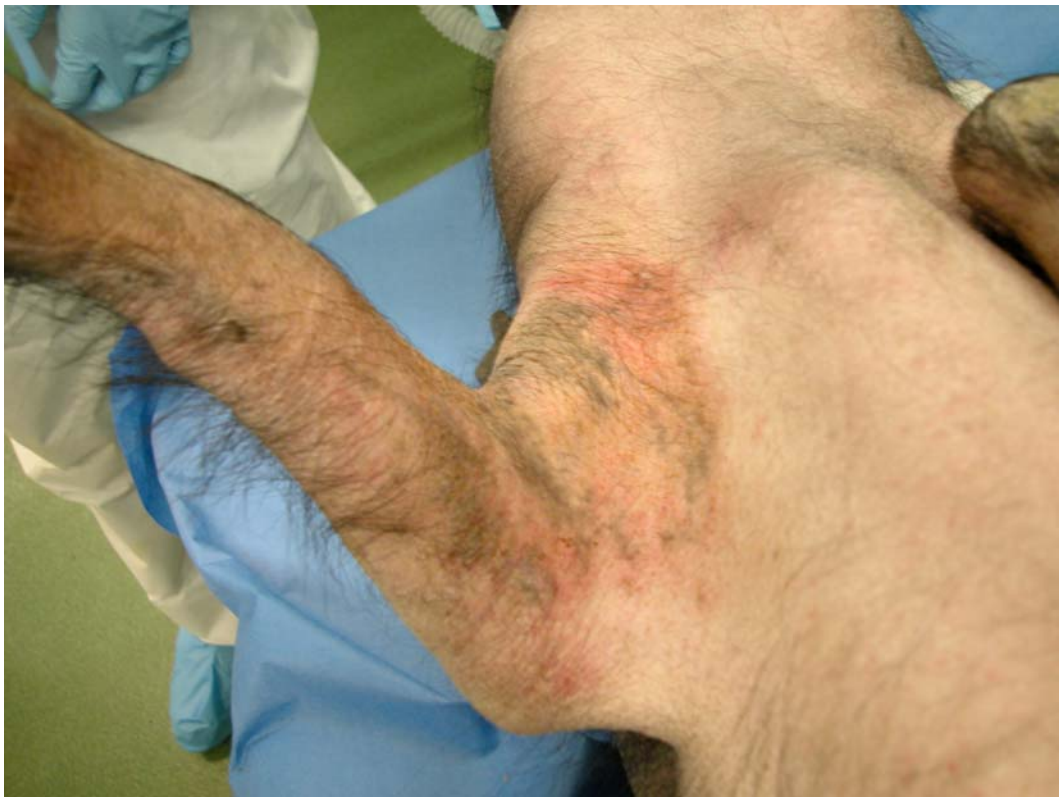

Figure 9: 5604 Day 184

On study day 184, the area was still marked by bruising, however the rash was replaced by an erythematous blush in this area.

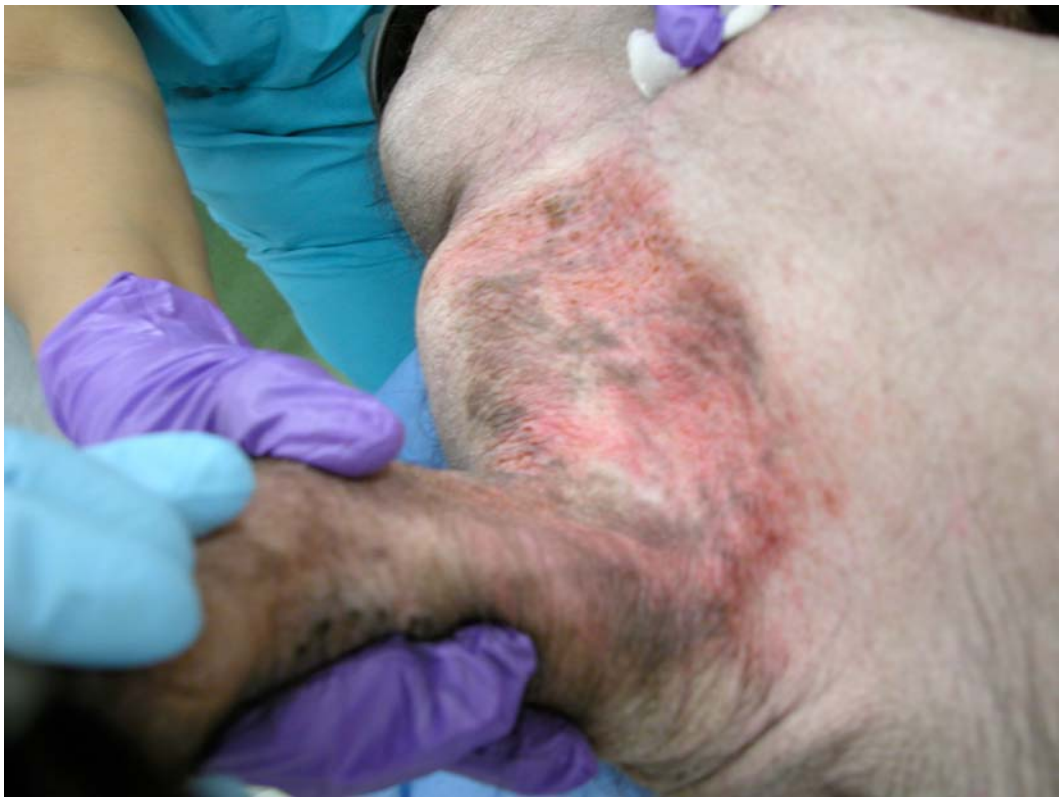

Figure 10: 5604 Day 186

The inner limb of this animal was very bruised, and the bruising was accompanied by extreme erythemia.

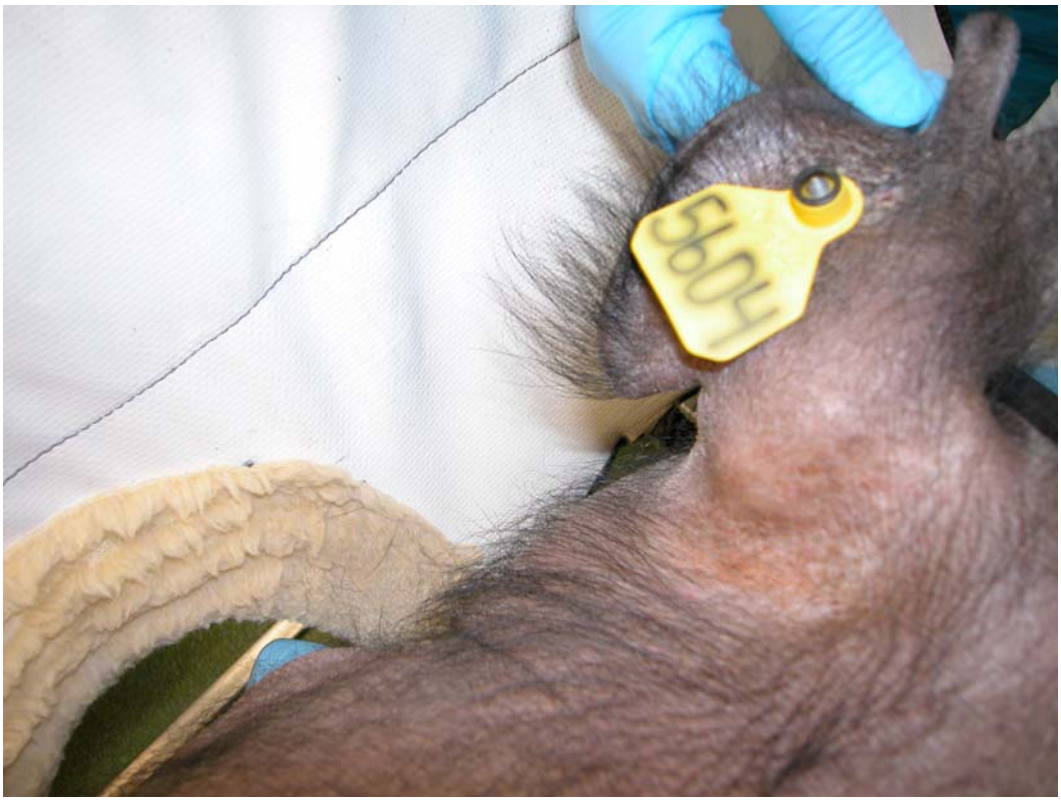

Figure 11: 5604 Day 184

Most of the animals also experienced a rash behind their ears.

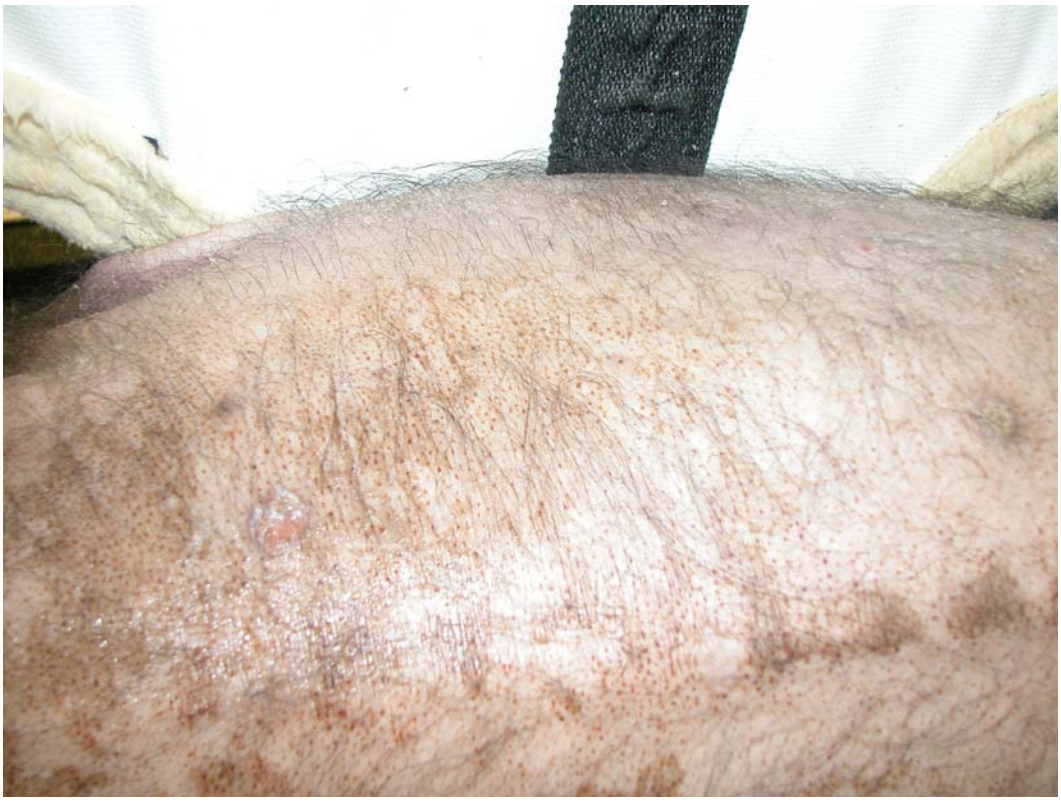

Figure 12: 5609 Day 182

The rash was often found on the dorsal side of these animals as well.

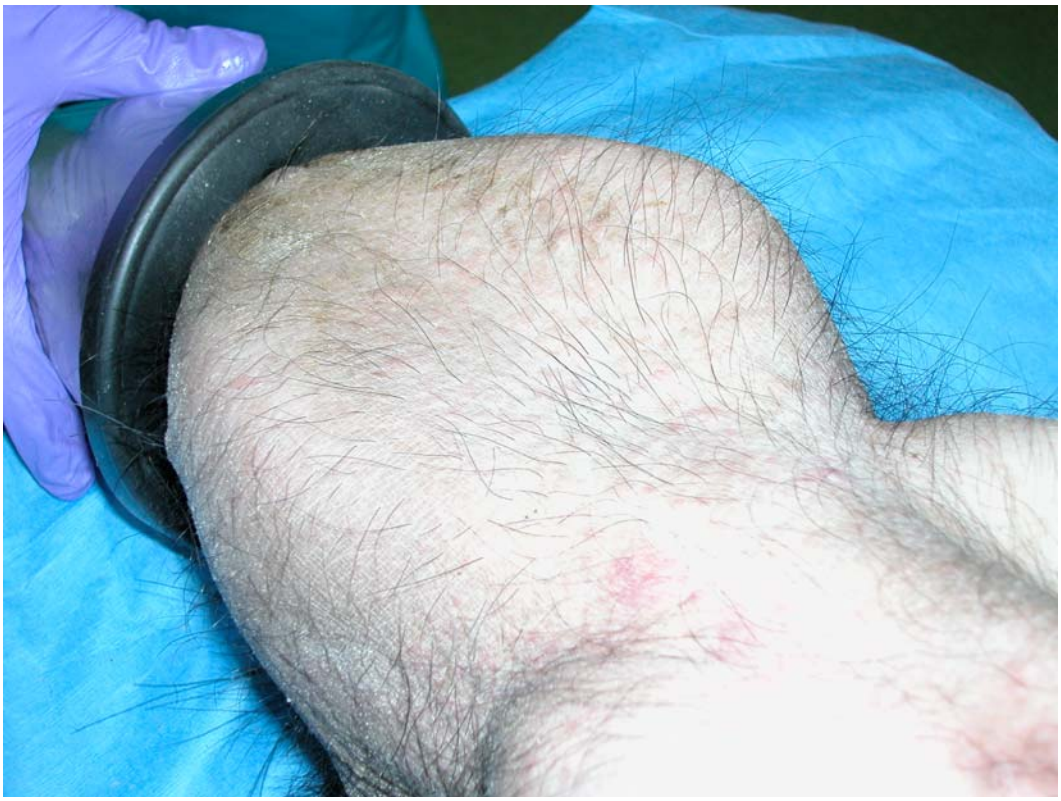

Figure 13: 5609 Day 182

In many animals the papules seen with the rash varied in size.

To determine if the rash was caused by an alteration in hemostasis, hematological samples were analyzed on study days 183, 185, and 186. From these samples, hemostasis appeared to be within normal limits for swine. A few of the animals did exhibit mild eosinophilia, which was the only abnormaility present from the complete blood counts on these animals.

Skin Pathology

A 4 or 8mm punch biopsy was taken from each animal on day 183 (Day 7 post infection) to help in the diagnosis of the cause of the rash. The punch biopsies from these animals were examined by the Pathology Department at NMRC, and

the findings are summarized in Table 13. Abnormal skin pathology consisted of dermal edema and perivascular infiltrates. The vessels in these animals had changes in the vessel walls marked by endothelial hypertrophy and exocytosis of neutrophils, and eosinophils were prominent in the epidermis. Only one animal that experienced a rash had normal skin pathology, which was most likely due to the biopsy coming from a site where no rash was present. It is also interesting to note that the two animals from Group 1 that did not have a visible rash had abnormal pathological findings.

Animal	Biopsy Site	Rash	Rash at Site	Findings
5595	Right Hindlimb	Yes	Yes	Abnormal
5596	Inner Right Hindlimb	No	No	Abnormal
5597	Right Ham	No	No	Abnormal
5604	Inner Left Hindlimb	Yes	Yes	Abnormal
	Inner Left Forelimb	Yes	Yes	Abnormal
5598	Non taken	Yes	NA	NA
5600	Inner Right Hindlimb	No	No	Normal
5609	Right Forelimb	Yes	Yes	Abnormal
5612	Inner Right Hindlimb	Yes	No	Normal
5594	Sternum	No	No	Normal
5611	Inner Left Hindlimb	No	No	Normal

Table 2: Skin Pathology, Secondary Homologous WP74 Challenge

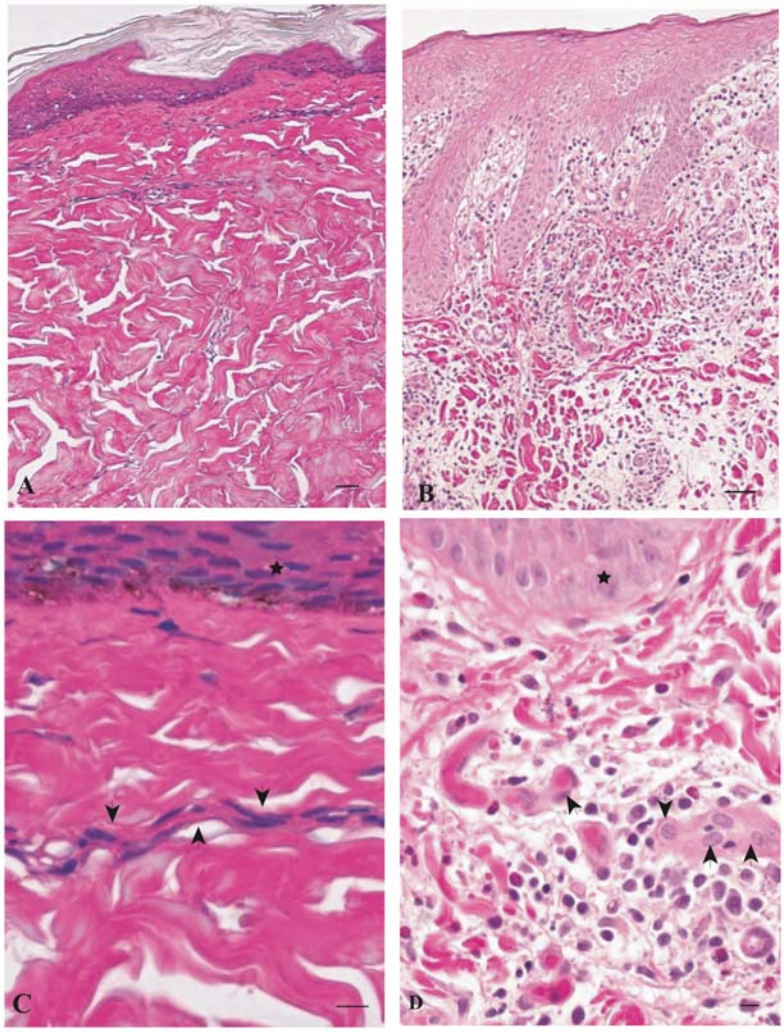

Figure 1: Skin Pathology

A. 5594(10X): The section is oriented with the epidermis at the top. The dermis typically contains abundant dense collagenous connective tissue.
B. 5604(10X): There is marked expansion of the dermis and separation of the dermal collagen bundles by edema fluid and infiltrates of mononuclear cells, neutrophils and eosinophils. The overlying epidermis is hyperplastic and edematous.
C. 5594(40X): This higher magnification demonstrates the normally inconspicuous vessels of the superficial dermis (arrowheads) and dense fibrous connective tissue, a normal feature of pig skin. Epidermis (*).
D. 5604(40X): There is endothelial hypertrophy and thickening of the vessel walls within the superficial dermis (arrowheads). The perivascular spaces are expanded by clear edema fluid and infiltrates of inflammatory cells. The basal cell layer of the epidermis (*) is hyperplastic.

Discussion

Our results support use of Yucatan Miniature swine as an animal model for dengue virus infection, vaccine, and pathogenesis studies. Following a primary infection with WP74, an asymptomatic viremia was observed. The primary infection induced dengue-specific IgM, IgG, and virus neutralizing responses. Upon secondary WP74 infection, the animals were significantly protected from viremia and their anti-dengue antibody responses were boosted. Additionally, the animals developed a skin manifestation similar to that observed in human disease.

An ideal animal model for dengue would have the following characteristics: 1) similar in anatomy and/or physiology to humans; 2) of sufficient size that multiple blood samplings can be taken in a small period of time; 3) widely available and relatively inexpensive; 4) susceptible to infection that can be measured; 5) show signs and or symptoms of disease; 6) susceptibility to viremia/disease symptoms is not age dependent; 7) develop immune responses to infection; and 8) vaccines for dengue are efficacious in these animals[44]. Yucatan Miniature swine were chosen as a potential animal model for dengue disease because they meet the first three criteria for an ideal dengue model for disease. Herein, we tested whether the animals would meet criteria four through seven. Viral replication was measured using cell culture isolation. Signs and symptoms that can be scored included temperature, hematocrit, outwardly signs such as lethargy, rash, bruising and tissue pathology. Immune responses were determined using ELISA and serum neutralization assays.

Cell culture virus isolation is a common method used for viremia determination in human or animal sera [65, 66]. For this study, Vero cell and SIGN cell virus isolation methods were used for viremia determinations. The two systems are similar in principle, such that if virus is present in test sera, the cells become infected and virus replicates to detectable levels. They do, however, differ in the receptors that are used in the infection process. Dengue viruses utilize heparin sulfate molecules[67, 68] as receptors on Vero cells and DC-SIGN receptors[69] on SIGN cells. The SIGN cell assay was recently developed in our laboratory, and this study described herein was an opportunity to validate the assay using non-human sera. The assay appeared to be more sensitive than the Vero cell assay after the primary WP74 infection. Animals in Group 2 had an average of 6.5 days of viremia by the Vero cell assay and 13.3 days by the SIGN cell assay. But, six months later, following the secondary infection, the sensitivity of the assay had diminished as compared to the Vero cell isolation assay. Animals in Group 3 had an average of 5 days of viremia by the Vero cell assay and only 3 days by the SIGN cell assay, and the viremia was not detected in both Group 3 animals by the SIGN cell assay. Possibly the loss of sensitivity of the SIGN cell assay in sera from the aging swine is a result of a serum component that inhibits infection of SIGN cells. A similar decreased sensitivity of the SIGN cell assay relative to the Vero cell assay has also been observed in specimens from viremic non-human primates that were similarly infected with dengue-1[70]. Because of the variable sensitivity of the SIGN cell assay, more confidence is given to the Vero cell assay for viremia results.

Williams has shown that swine infected with Japanese Encephalitis virus, Murray Valley Encephalitis virus, and Kunjin virus experienced 1 to 3 days of viremia[58]. Group 1 animals which received a lower dengue innoculum, had viremia of 1 to 7 days duration with 3.5 average days of viremia. All animals that were inoculated with the high dose of WP74, Group 2 and Group 3 animals, developed viremia with 3 to 9 days duration for a mean viremia of 6 days. The uniform and lengthy viremia that was observed in these swine is similar to that which is observed in non-human primates and humans. Raviprakash[48] and Kochel[47] have reported that WP74 produced viremia in duration of 6 and 4 days in *Rhesus macaques* and *Aotus nancymae*, respectively. Similar viremia patterns were also observed in *Cynomolgous macaques*[71]. Humans average 5 days of viremia following a primary dengue infection[16]. These results present an asymptomatic model of WP74 infection that is comparable to both the non-human primate model and asymptomatic experimental infections in humans[2].

Following primary WP74 infection, all animals that developed a detectable viremia also developed IgM, IgG, and low PRNT responses similar to those which are seen following a primary WP74 infection in non-human primates[47, 48]. The IgM responses observed in the two responding low dose animals trailed those of the high dose animals by four days. This delay was possibly due to a longer period needed for the lower innoculum of virus to replicate to levels sufficient to induce a measurable immune response.

One of the uses of a dengue infection/disease model is to test the protective efficacy of dengue virus vaccine candidates prior to human trials. To

determine if the observed immune responses post-primary WP74 infection are adequate to protect an animal from homologous infection, the animals were challenged with a high dose of WP74(10^7). Based on findings from infections of swine with other members of the Family Flaviviridae, it was hypothesized that these animals would be protected from a homologous dengue challenge. Previous findings show that swine can be protected from a secondary infection with a homologous flavivirus, and, in many cases, are protected from a secondary infection with heterologous viruses of the same family[58, 72]. All animals that had been previously exposed to WP74 demonstrated an 80% reduction in the days of viremia compared to the naive control animals. This finding suggests that protective immunological memory had been established in the two low dose animals that developed less than 2 days of viremia and barely detectable immune responses following primary WP74 infection. This hypothesis is also supported by the boosted PRNT responses following secondary infection in these animals. This response is comparable to that which is seen in humans with secondary homologous dengue challenges[2, 47].

The IgM responses observed in these swine experiencing a secondary homologous dengue-1 infection were varied. Following the secondary infection, the Group 1 and 2 animals had IgM responses that were lower in magnitude and duration than those observed following the primary infection.

When swine experience a secondary homologous or heterologous challenge with Japanese Encephalitis virus, Murray Valley Encephalitis virus, and Kunjin virus, boosting in immune responses as compared to a primary immune

response is observed[58]. The boosting is similar to that which is seen in humans following a secondary dengue virus infection. All secondarily WP74-infected swine demonstrated anamnestic PRNT responses, thus presenting an immunologic model for WP74 infection that is comparable to human and immunized non-human primate responses.

Dengue infections of humans are varied in their presentation, with primary infections typically manifesting either as dengue fever or having no measurable symptoms. However, secondary heterologous dengue infections are typically symptomatic and, in a small percentage of people, proceed to dengue hemorrhagic fever[1]. A similar manifestation is seen in the Yucatan Miniature pig model. During a primary WP74 infection, the animals showed no signs or symptoms of disease. Interestingly, during a secondary homologous WP74 infection, 63% of swine developed a rash. The pathology of the rash mirrored that which is seen in humans[73, 74]. This pathology includes apoptosis of capillary endothelia accompanied by vascular infiltration into the epidermis. The rash was accompanied by visible bruising and rash in the area from which the samples were taken. The presence of the rash after secondary homologous infection is unexpected since this is not what is observed with humans experiencing dengue infections. DHF is often associated with secondary heterologous infections and high viremia. Although disease symptomology was not obtained following a similar progression to that which is observed in humans, Yucatan Miniature swine may provide a model in which the elucidation of the reasons for development of severe disease is obtainable. As the same strain of virus was used for both primary

and secondary dengue virus challenges together with the fact that secondary viremia was not detected eliminates the viral factors thought to be involved in progression to severe disease. These factors are the association of higher viral titers with development of severe disease and that viruses which are though to be more virulent are thought to cause more severe disease.

These findings suggest that in the case of Yucatan Miniature swine that the progression to disease is due to some host factor, most likely due to immune response to dengue exposure, and possibly supports the idea that dengue diseases are caused by an autoimmune mechanism.

Our results demonstrate that Yucatan Miniature swine are susceptible to WP74 infection and most likely to other dengue viruses as well; nonetheless natural dengue infections or diseases have not been observed in any swine. As a mosquito only transmits 2-3 $\log_{10}$ PFU of virus per bite, it is unlikely that swine would be infected via mosquito bite since 5 $\log_{10}$ PFU of virus only measurably infected 50% of swine that were inoculated. As the SIGN isolation technique is semi-quantitative, we can estimate that the animals had 1-3 $\log_{10}$ PFU of virus per milliliter of serum. The amount of virus detected in the blood would not likely be sufficient to infect a susceptible mosquito, as they would need at least 10 PFU of virus per their 10 microliter blood meal, which is the highest estimated titer of blood that the swine experienced. In addition to the factors involved with mosquito transmission, it is not clear as to whether other species of swine are infectable with dengue viruses. As Yucatan Miniature swine are animals that are bred specifically for research purposes and are therefore housed in

environmentally closed areas, it is not probable that they would play any role in the natural infection cycle of dengue.

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