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Prevalence of Antibodies to West Nile Virus in Selected Farm Animals in Central Oklahoma

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Prevalence of Antibodies to West Nile Virus in Selected Farm Animals in Central Oklahoma

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ABSTRACT OF THESIS

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ABSTRACT:

This study examined the seroprevalence of West Nile Virus (WNV) specific Immunoglobulin G (IgG) antibodies among five different farm animal species including: *Bos taurus* (cattle), *Capra hircus* (goat), *Ovis aries* (sheep), *Sus scrofa* (pig), and *Gallus gallus domesticus* (chicken). Twenty samples were collected at three different time periods from each of the five species, totaling 300. March, April, May were grouped as the spring collection; June, July, August the summer collection; and September, October, November the fall collection. These grouping closely reflect the fluctuation in mosquito activity, specifically *Culex* species, which are the main vectors for WNV.

Seroprevalence was determined using an indirect antigen capture Enzyme-Linked Immunosorbent Assay (ELISA). A Kruskal-Wallis One Way Analysis of Variance (ANOVA) suggested a significant difference of WNV specific IgG titrations among species ($P = 0.009$). A Tukey multiple-comparison test could not determine which species were significantly different ($P > 0.05$). However, chickens had the largest amount of samples positive for WNV specific IgG. The ANOVA also suggested a significant difference among collection periods ($P = 0.033$) but the Tukey multiple-comparison test could not determine which collection period or periods were different ($P > 0.05$). However, the summer collection recorded the largest number of positive samples among collection periods.

Attempts to use diagnostic tests to detect the presence of antibodies specific to a particular flavivirus have been plagued by cross-reactivity. To determine if WNV was the antigen responsible for the IgG immune response detected by ELISA I also ran an ELISA with St. Louis Encephalitis Virus (SLEV) as the antigen. A Wilcoxon signed-rank test revealed that the antibody titrations against WNV antigen were significantly different than antibody titrations against SLEV antigen ($P < 0.001$). Eleven of sixteen samples testing positive

for WNV specific IgG showed cross-reactivity with SLEV. Of these eleven, all but one showed a four-fold difference or greater in endpoint titration to WNV antigen, in comparison to SLEV antigen. The exception showed a two-fold difference. This strongly suggests the antigen responsible for the IgG immune response was WNV. However, a co-infection with SLEV cannot be ruled out.

INTRODUCTION

West Nile Virus (WNV) was first discovered in 1937 when a woman from the West Nile region of Uganda came down with a case of febrile illness when participating in a malaria study (Smithburn et al., 1940). Since then WNV outbreaks have occurred in Africa and Europe, and WNV is present in some Asian countries (Dauphin et al., 2004). The first occurrence in the United States was documented in New York in 1999 (Lanciotti et al., 1999). After the outbreak in New York, WNV reached epidemic proportions quickly spreading throughout the United States eventually reaching California and the west coast by 2003 as reported by Riesen et al., (2004). The same authors showed that WNV has since spread north into Canada and south into Mexico, the Caribbean, and South America. The migratory patterns of birds, climate, and the transport of infected mosquitoes through commerce, all have apparently had a role in the quick spread of the virus throughout North America and into Central America (Riesen et al., 2004).

Solomon et al., (2003) state that WNV is a member of the family Flaviviridae and is one of 70 species belonging to the *Flavivirus* genus. It specifically is one of ten viruses belonging to the Japanese Encephalitis

Virus (JEV) serogroup (Solomon et al., 2003). Those species of the JEV serogroup that are most closely related to WNV based on envelope protein amino acid sequences include JEV, Saint Louis Encephalitis Virus (SLEV), and Murray Valley Encephalitis Virus (MVEV) (Stiasny et al., 2006). Stiasny et al., (2006) also reports that WNV is an icosahedral, positive sense (mRNA sense), single stranded, enveloped RNA virus.

Samuel and Diamond (2006) discuss the components of the WNV genome. The viral genome is translated into a single polyprotein which is then cleaved into three structural and seven non-structural proteins. The structural proteins include a capsid (C) protein which binds viral RNA, a premembrane (prM) protein which blocks premature viral fusion and an envelope (E) protein which is responsible for attachment of the virus, assembly and membrane fusion. Stiasny et al., (2006) describes the structure of the E protein as "herringbone-like." Oliphant et al., (2006) characterizes the three domains of the E protein which form head-to-tail homodimers on the virion surface. Domain I (DI) is the central structural domain made up of a 10-stranded β-barrel. Domain II (DII) is connected to two extended loops projecting from DI. At the end of DII is a conserved

amino acid sequence that is probably involved in fusion of the viral particle with the cell membrane. Domain III (DIII) is on the opposite side of DI forming a seven-stranded immunoglobulin-like fold. The non-structural proteins mediate viral transcription, replication and attenuate host antiviral responses (Samuel and Diamond, 2006).

Brinton (2002) describes the replication cycle of WNV. WNV virions, once bound to a currently unknown receptor, are endocytosed into the cell where a drop in pH causes the endosomal vesicle membrane to release the capsid into the cytoplasm. The genomic RNA is then released and translated into a single polyprotein. Various cellular and viral proteases produce mature viral proteins by cleaving the polyprotein at many sites. Viral RNA dependent RNA polymerase then copies the genomic RNA into complementary minus strands which serve as templates for new genomic RNAs. Virion assembly occurs in conjunction with rough endoplasmic reticulum membranes. These immature virions are then modified and transported to the plasma membrane in vesicles and released by exocytosis.

WNV is transmitted through a mosquito vector. Wright (2003) discusses the life cycle of mosquitoes in

Oklahoma that are involved with the transmission of WNV. The species which are most effective in transmission of WNV in Oklahoma include: *Culex pipiens quinquefasciatus, Culex tarsalis,* and *Culex restuans*. *Culex* species are reduced to very small numbers in the winter. Because of this it takes time for them to build up their populations. The peak number of *Culex* mosquitoes in Oklahoma occurs in August and early September. Lee and Rowley (2000) performed a study in Iowa that showed differences between *Culex* species in early and late summer. Their study suggested that *Culex restuans* is the dominant species in early summer with peak numbers occurring in June. After July 1, their numbers decrease and *Culex pipiens* becomes the dominant species in the genus. *Culex pipiens* populations peak in late August. USGS (2007) maps from 2003-2006 show the incidence of WNV in mosquitoes of Oklahoma peaking in June, August or early September depending on the year.

Turell et al., (2003) discuss the reasons the reservoir and amplifying host of WNV is a broad range of avian species. In avian species, the virus can quickly replicate and reach a concentration $>10^5$ plaque forming units per milliliter (PFUs/ml) in the blood. This is the minimum concentration needed to be transmitted by a

mosquito (Turell et al., 2003). Wright (2003) confers the replication of WNV in a mosquito. When a mosquito bites an animal with a sufficient viremia to transmit WNV, the bloodmeal and virus are ingested. The virus begins replicating and eventually spreads to the salivary glands. At this point the mosquito has the ability to transmit the virus with its next bite. Brinton (2002) discusses how WNV is capable of being transmitted to a wide range of vertebrate "dead end hosts." These "dead end hosts" are so named because the virus can not increase its concentration to $>10^5$ PFUs/ml of blood and thus cannot be transmitted by a mosquito, ceasing the transmission cycle. "Dead end hosts" represent a wide range of mammals, some being humans, horses, monkeys, deer, foxes, opossums, coyotes, and badgers (Brinton, 2002; Docherty et al., 2006). Also, the virus is capable of replicating in a wide range of cell lines from monkeys, humans, pigs, rodents, amphibians, and insects (Brinton, 2002). Because of this it has been hypothesized that the cell receptor the virus uses to gain entry to the cell, although currently unknown, is highly conserved (Brinton, 2002).

Table 1 shows the incidence of WNV in Oklahoma among horses, birds, and mosquitoes according to Oklahoma State

Health Department (2007) tabulations from 2002-2006. The table shows tabulations from the areas where samples in this study were obtained including Oklahoma, Canadian, Kingfisher, Logan, and Noble counties. A decrease in the number of animals positive for WNV infection is shown. This can be attributed to fewer numbers of tests performed in recent years due to a shift in funding and manpower to other areas of research. Thus, an accurate trend cannot be determined. It should be noted that chickens have never been tested for WNV infection by the Oklahoma State Health Department. Currently, the testing of mosquitoes for the presence of WNV is the sole method of WNV surveillance being performed in Oklahoma.

Brinton (2002) explains that most WNV infections go unreported because humans present with flu-like symptoms as well as a rash on the chest or back. Only in rare circumstances, usually weak immune systems or immunocompromised hosts, does WNV infection progress to flaccid paralysis, encephalitis, or meningoencephalitis (Brinton, 2002). Clinical signs of encephalomyelitis in horses and hamsters include muscle weakness, somnolence, tremors, and weight loss (Morrey et al., 2004).

A healthy immune system is needed to control WNV infection. Alpha, beta, and gamma interferon provide

immediate control to WNV in peripheral tissues, limiting viral load and halting its spread to the central nervous system (CNS) (Samuel and Diamond, 2004). Also, CD4+ T cells proliferate upon infection. Macrophages engulf antigens and present them to B cells which then bind to MHC-1 proteins and become activated. They then proliferate into plasma cells and begin secreting IgM antibodies at a high rate (Diamond et al., 2003). Once IgM is produced, the clearance of WNV from the bloodstream occurs (Sitati and Diamond, 2006). WNV specific IgM neutralizes viral particles and may trigger an adaptive IgG response that eliminates viral infection (Diamond et al., 2003). CD8+ T cells eliminate WNV from infected cells and thus remove its persistence. This is done by cytolytic mechanisms (Shrestha and Diamond, 2004). Fas, perforin, and granzymes A and B pathways of cellular cytotoxicity induce apoptosis and are important in controlling WNV infection (Wang et al., 2004). Notably, homozygous deficiencies of a synthetase involved in the RNase L pathway results in high mortality in mice (Mashimo et al., 2002). WNV is a neurotropic virus that gains entry to the blood brain barrier and central nervous system when TNF-α increases its permeability (Samuel and Diamond, 2006). When WNV gains entry into

the central nervous system, flaccid-paralysis and meningoencephalitis occurs (Guarner et al., 2004).

Purdy et al., (2004) compared two serological tests developed to detect WNV infection. Enzyme-Linked Immunosorbent Assay (ELISA) technique has been used largely in clinical diagnosis for the detection of antibodies to WNV in animal serum. ELISA has been developed to compete with the Plaque Reduction Neutralization Test (PRNT). PRNT uses a live virus to infect an animal cell culture producing plaques and the subsequent neutralization of the virus by antibodies present in serum. This technique is labor-intensive and dangerous since the lab is working with a live viral particle. However, it is still considered the gold standard because it reflects the actual neutralization of the virus similarly to what occurs in the host's blood. ELISA technique is a safe alternative because it uses a non-infectious recombinant viral protein secreted from genetically engineered COS-1 cells (African green monkey fibroblasts transformed by Simian Virus-40). It is also more cost efficient and less time consuming because many samples can be accommodated on a single plate.

One drawback to any test that detects the presence of antibodies specific to a particular flavivirus is the

cross reactivity of the antibodies to other flaviviruses. Cross-reactivity of antibodies makes it difficult to determine which flavivirus was the cause of infection. Oliphant et al., (2006) states that a major reason for this is the majority of antibodies produced in a WNV infection are specific for Domain II of the envelope protein. Domain II is highly conserved among flaviviruses and antibodies specific for this region of the envelope will cross-react with other flaviviruses (Oliphant et al., 2006).

According to Dr. Chang at the Centers for Disease Control and Prevention (2007) SLEV is the only other flavivirus currently known to be present in Oklahoma and would require a test of cross-reactivity. Purdy et al., (2004) state that SLEV was the leading cause of epidemic flaviviral encephalitis in the United States before the introduction of WNV into the western hemisphere in 1999. From 1964 to 2002, about 4,500 human cases of SLEV infection were reported (Purdy et al., 2004). Most cases of SLEV go unreported because infection presents clinically with flu-like symptoms and only in cases of a weakened immune system or an immunocompromised host does it progress to flaccid-paralysis and meningoencephalitis (Purdy et al., 2004). Similarly to WNV, its vectors are

also members of the *Culex* genus of mosquitoes. However, the species differ depending on the location in the U.S (Tsai and Mitchell, 1988). In Oklahoma and the south central United States, the primary mosquito vector for SLEV is *Culex pipiens quinquefasciatus*. Also like WNV, amplifying hosts of SLEV are a wide range of avian species including cardinals, robins, chickens, bobwhites, and quail (McLean et al., 1985). Dead end hosts for SLEV are a broad range of mammals but studies of infectivity among mammals are lacking in comparison to WNV. Mammals previously reported to be infected by SLEV include mice, humans, hamsters, monkeys, rabbits, chipmunks, and sloths (McLean et al., 1985; Monath et al., 1980; Seymour et al., 1983).

MATERIALS AND METHODS

Serum Samples

Blood samples from *Bos taurus* (cattle), *Capra hircus* (goat), *Ovis aries* (sheep), *Sus scrofa* (pig), and *Gallus gallus domesticus* (chicken) were obtained from three slaughterhouses located in Edmond, Oklahoma. Blood samples were collected in different ways for each animal. Blood samples from cattle and pigs were collected in sterile beakers when the throat of the animal was cut. Blood samples from goats and sheep were drawn from the external jugular vein with a syringe. Blood samples from chickens were drawn from the subclavian vein by syringe. All blood samples were injected into gel clotting tubes and inverted five times to thoroughly mix the blood with the clotting agent coated on the inside of the tube. After clotting, tubes were centrifuged to remove any cells and serum samples were stored in a freezer at -20°C with 0.1% sodium azide as a preservative (Focus Diagnostics, 2005). Twenty samples from each of five species were collected during each of three collection periods. This resulted in a total of 300 samples. The main periods of mosquito activity occur between March and November in Oklahoma. This time was divided into three collection periods. The first collection period included

March, April, and May (spring), the second June, July, and August (summer), and the third September, October, and November (fall).

ELISA Protocols

An indirect antigen capture ELISA technique (Fig. 1) was developed by Johnson et al., (2000) of the CDC. This protocol was slightly modified to accommodate the species in this study. Indirect antigen capture ELISA was used to detect the presence or absence of WNV-specific IgG in each serum sample. Ninety-six well Maxisorp Nunc microtiter plates (Bethyl Laboratories, Inc.) were coated for 18-24 h at 4°C in a humidified chamber with 100 µl/well of mouse anti-WNV IgG 4G2 antibody (Hennessey Research, Inc.) diluted 1:2,000 in coating buffer (0.015 M sodium carbonate, 0.035 M sodium bicarbonate, pH 9.6). The plate was washed by adding 200 µl/well of phosphate buffered saline with 0.05% Tween 20 (PBST) pH 7.6 $\pm$ 0.2, two times. Wells were blocked with 300 µl/well for 1 h at room temperature with freshly prepared 5% non-fat dry milk (Difco, Sparks, MD) diluted with PBST. The plate was subsequently washed five times with PBST, 200 µl/well. Fifty µl/well of non-infectious recombinant WNV antigen made from COS-1 cells (Hennessy Research, Inc.) and diluted 1:50 in PBST was added and the plate

incubated at 4°C for 18-24 h in a humidified chamber. The plate was then washed five times with PBST, 200 µl/well. Fifty µl/well of test sera diluted 1:400 in PBST was added and incubated at 37°C for 1.5 h in a humidified chamber. Positive and negative control sera were diluted at 1:800 dilution with PBST in triplicates. The plate was again washed five times with PBST, 200 µl/well. Fifty µl/well of 1:2,000 horseradish peroxidase (HRP) conjugated anti-IgG Fc portion, specific for each animal species including rabbit anti-goat, goat anti-pig, goat anti-chicken (Bethyl Laboratories, Inc., Montgomery, TX), rabbit anti-sheep, and goat anti-bovine (Rockland Immunochemicals, Inc., Gilbertsville, PA) diluted in PBST with 5% non-fat dry milk was added and incubated at 37°C for 1 h. The plate was washed ten times with PBST, 200 µl/well. Bound conjugate was detected by adding 50 µl/well of 3,3',5,5'-Tetramethylbenzidine (TMB) substrate (Bethyl Laboratories, Inc., Montgomery, TX) for 10 min. at room temperature. The substrate reaction was stopped with 50 µl/well of 1 N H_2SO_4 and the reactions' optical densities were then measured at an A_{450} with a Multiskan EX ELISA plate reader.

For detection of cross-reactivity of IgG to SLEV (Fig. 2) the procedure was essentially the same except

the plates were initially coated with mouse anti-flavivirus IgG antibody 4G2 (CDC, Boulder, CO) and a non-infectious recombinant SLEV made from genetically modified Chinese hamster ovary cells was used as the antigen (CDC, Boulder, CO) (Davis et al., 2001).

Test Validation and Calculation of Positive/Negative absorbance ratio values

Before the samples could be considered each plate had to be validated by comparing the average triplicate values of known positive serum with viral antigen with the average triplicate values of know positive serum with normal antigen (Johnson et al., 2000). Positive and negative control sera containing WNV specific IgG for goat, sheep, and chicken were obtained from Amy Glaser of Cornell University. Positive and negative control sera for pigs were obtained from serum samples in this study. Positive and negative control sera for cattle could not be obtained therefore goat reference serum was substituted with everything else being equal except the necessary HRP conjugated IgG Fc portion anti-goat antibody. A ratio of $\geq$ 2.0 validated the plate. If the ratio was < 2.0 the samples on the plate were not considered. Each average sample with viral antigen was compared with average normal antigen. A Positive

antigen/ Normal antigen (P/N) ratio of $\geq$ 2.0 was considered positive and < 2.0 was considered negative (Johnson et al., 2000).

WNV Statistical Analyses

The data were statistically analyzed using SigmaStat 3.5. Because the data were not distributed in a normal fashion, a non-parametric Kruskal-Wallis One Way Analysis of Variance (ANOVA) on ranks was performed. This procedure was done separately five times to compare WNV specific IgG antibody titer levels with each factor including: collection period, species, location, sex and age. The testing of each factor gave a separate P value. Zar (1999) denotes that this determined if the differences in the median values among the treatments groups within each factor were greater than would be expected by chance alone. Tukey pairwise multiple comparison tests were then performed to determine which treatment groups within each factor differed from the others. All tests were performed at a 95% level of significance shown by a $P < 0.05$.

Cross Reactivity Statistical Analysis

A Wilcoxon Signed Rank Test was performed on the titration levels of IgG antibodies specific for WNV and SLEV. Zar (1999) describes this analysis, saying that

the Wilcoxon Signed-Rank test involves the ranked calculation of differences between paired data. The sum of positive ranks is then compared with the sum of negative ranks. This comparison determines if the change that occurred within the treatments is greater than would be expected by chance alone. This test was performed at a 95% level of significance shown by a $P < 0.05$.

RESULTS

Detection of WNV specific IgG

Of the five species tested for presence of WNV specific IgG (Table 2), chicken samples totaled 50% of all positives, with two positive samples in the spring collection, five in summer collection, and one in fall collection. Pig samples accounted for 25% of all positive samples with one positive in the spring collection, two positives occurring in summer collection and one in the fall collection. Sheep had 18.75% of all positive samples with three positives occurring in the summer collection. Goat samples were responsible for 6.25% of all positive samples with only one positive in the spring collection. None of the cattle samples were positive in any of the three collection periods spring, summer or fall. Statistical analyses were significant ($P = 0.009$) in showing a difference in titration values among species but the multiple comparison test was not strong enough to determine the species that were different ($P > 0.05$). Chickens had the highest incidence of positives among all species. The peak mosquito season represented by the summer collection had 62.5% of all positives, the spring had 25%, and the fall 12.5%. The statistical analysis showed a difference ($P = 0.033$)

between collection periods but the multiple comparison test was not strong enough to detect which collection period or periods were different ($P > 0.05$). However, the summer collection period had the largest number of positives. WNV antibody titration values were also statistically compared with regards to age ($P = 0.190$), sex ($P = 0.053$), and location ($P = 0.630$). All of these were not significant although it should be noted that females had very close to a significant P value of 0.053.

Detection of Cross Reactive IgG

The sixteen samples that tested positive for IgG antibodies to WNV antigen were then tested for cross-reactivity with SLEV antigen (Table 3). Differences in ELISA titrations between the two antigens were strongly significant ($P < 0.001$). Five of the samples positive for WNV specific antibodies had no significant cross-reactivity and eleven were cross-reactive with SLEV antigen. Ten of the eleven cross-reactive samples exhibited $\geq$ 4-fold difference in titration values to WNV antigen compared to SLEV antigen. The exception, sample P12a, showed a 2-fold difference in titration levels.

DISCUSSION

Out of 300 animal samples tested for the presence of WNV specific IgG antibodies, sixteen tested positive. These sixteen positives included eight chickens, four pigs, three sheep, and one goat. Differences among the titrations ranging from 1/800 in sheep to 1/25,600 in chickens were seen among species and collection periods although a multiple comparison test was not strong enough to determine which species and which collection periods were significantly different. An increase in sample size would increase the power of the test, possibly alleviating this problem (Zar, 1999). Among species, chickens were observed to have the highest prevalence of antibodies to WNV. This agrees with Brinton (2002) that has shown avian species to be amplifying hosts of WNV due to the ability of the virus to reach high enough concentrations to be transmitted by a mosquito in these species.

Chickens in other states, including Colorado and Missouri, have been tested as a method of WNV surveillance. In 2002, Colorado had three chickens test positive out of a total of 1,342 samples (0.2%). In 2003, 213 chickens tested positive out of a total of 1,426 samples (14.9%)(Comstock, 2007). In 2004, Missouri

had 28 chickens out of 50 total samples (56.0%) test positive for WNV (Diehl, 2007). This study had 8 out of 60 samples test positive (13.3%). This value is similar to Colorado in 2003 but dissimilar to Colorado in 2002 and Missouri in 2004. Recently, many states have stopped sentinel chicken testing as a method of WNV surveillance. Chickens were shown to be a poor predictor of WNV transmission in humans (Comstock, 2007; Diehl, 2007; Scott, 2007). Using mosquitoes for WNV surveillance has been a good predictor of WNV transmission in humans, is less time consuming and requires fewer resources. Currently, the sole method of WNV surveillance for most states including Oklahoma involves randomized testing of mosquitoes for viral RNA using reverse transcriptase-polymerase chain reaction (RT-PCR) technique (Comstock, 2007; Diehl, 2007; Oklahoma State Health Department, 2007; Scott, 2007).

Among collection periods, the summer collection period had the largest number of positives recorded. This also is in accordance with Wright (2003) who has reported that greater numbers of *Culex restuans, Culex pipiens quiniquefasciatus,* and *Culex tarsalis* are seen in these months in Oklahoma. In addition, the peak incidence of WNV in mosquitoes in Oklahoma occurs

primarily in late summer or early fall (Oklahoma State Health Department, 2007). Higher numbers of mosquitoes would increase the number of vectors allowing the virus to spread more rapidly during these months (Gu et al., 2004).

No differences in WNV antibody titration levels were seen among the sexes of the animals in this study. This is supported by a lack of reported instances of either sex being more susceptible to infection or producing more antibodies upon infection than the other. No differences were seen among age groups when the animals were divided into young and mature, with one year being the age where the animals were considered mature. All of the species tested were four years or less. An age related decrease in the immune response would not occur until later in these animal's lives and thus would have no impact on this study. Docherty et al., (2006) also showed no significant difference in sex or age among wild mammals from Wisconsin that had antibodies to WNV. Also, no differences in WNV antibody titration levels were seen among locations of the animals. All of the animals were in a limited collecting area of five counties and no large differences in climate or physiogeography are present in central Oklahoma.

Previous testing of livestock in Oklahoma was limited to horses (Oklahoma State Health Department, 2007). Table 1 shows a decreasing trend of horses positive for WNV from 2002-2006. However, this is due to a decrease in the number of tests that were performed on these animals (Cooper, 2007). This decline in testing is due to a commercial WNV vaccine that is currently available for horses. Data from this study shows that WNV is infecting livestock in central Oklahoma, although at a low rate. Interestingly, the cattle in this study were not shown to be infected with WNV while horses are commonly infected as indicated by Table 1. Both are large mammals and found in similar settings on a farm yet horses seem to be more susceptible to WNV infection than cattle. This difference in infectivity could be due to some physiologic difference that makes horses more susceptible to infection or some ecologic difference that prevents *Culex* mosquitoes from passing the virus to cattle. Chickens, however, have not been tested in Oklahoma as a possible method of WNV surveillance. These data suggest sentinel chicken flocks would be a good source for surveillance of WNV activity in the future.

Sixteen samples were positive for WNV antibodies including eight chickens, four pigs, three sheep, and one

goat. Of these sixteen eleven showed cross-reactivity with SLEV. These eleven included seven chickens and four pigs. However, five of the sixteen samples positive for WNV antibodies did not show cross-reactivity to SLEV. These included one goat, one chicken, and three sheep. These five were definitively infected with WNV.

The eleven samples including seven chickens and four pigs that did show a cross-reaction with SLEV antigen can be considered from two view points. The first is that these samples were infected with WNV and the antibodies were simply cross-reacting with SLEV. WNV and SLEV share epitopes on their envelope proteins allowing antibodies produced to WNV infection to cross react with SLEV. In all eleven of these samples there was a greater concentration of antibodies to WNV than SLEV. A greater concentration of antibodies would mostly likely be produced to the virus that was responsible for the infection.

A second perspective that cannot be ruled out is that co-infection of both WNV and SLEV took place. Both viruses are present in the area (USGS, 2007) and both are probably capable of replicating in all of the species upon exposure to the virus (Monath et al., 1980; Seymour et al., 1983; Brinton, 2002; Docherty, 2006). A reverse

transcription-polymerase chain reaction (RT-PCR) test would be needed to unequivocally determine which virus or viruses were responsible for the antibody response or responses (Pupo et al., 2006). In this case, however, IgG seroconversion does not occur until the virus has been neutralized in the serum by IgM and cellular clearance occurs shortly thereafter through cytolytic mechanisms. Thus, an RT-PCR would be without merit.

Furthermore, the limitations of the ELISA must be mentioned. A different method of detecting WNV specific IgG antibodies called antibody capture IgG (GAC) ELISA has recently been shown by Purdy et al., (2004) to be significantly more sensitive and specific. This method captures all IgG antibodies for the specific animal and then allows WNV specific IgG antibodies to react with viral antigen. The viral antigen is then detected with a conjugated antibody. This method in theory should be less specific and less sensitive because it captures all IgG antibodies, whereas the antigen capture IgG ELISA (ACG) only allows WNV specific IgG to be bound. The capture of all IgG antibodies with a broad range of specificity may competitively inhibit the small number of WNV specific IgG antibodies. However, the ACG ELISA has previously had a 64% positive and 78% negative predictive

value in the same study comparing ELISA formats for human serum samples. The GAC ELISA has an 86% positive and 100% negative predictive value in the same study, respectively (Purdy et al., 2004). IgG has previously been shown to be more cross reactive in comparison to IgM (Johnson et al., 2000), with cross-reactivity occurring in 78% and 44% of human serum samples in the ACG format, respectively (Purdy et al., 2004). Another study that has shown IgG to be more cross reactive than IgM, had a cross-reactivity occurring in human samples with a range of 15.7-100% for IgG and 0-18.2% for IgM (Niedrig, 2007).

Testing for either IgM or IgG has its own benefits due to the kinetics of the antibody response. Ratterree et al., (2004) shows that in Rhesus monkeys, median antibody titers of IgM were significant in 10 to 45 days after WNV mosquito challenge. IgM is a signal of recent WNV infection. In comparison IgG median antibody titers were significant 21 to >63 days post inoculation in the same study. WNV specific IgG is evidence of a somewhat recent primary infection or it can be an indication of a secondary infection because it is produced quickly in high concentrations upon re-infection (Goldsby et al., 2003). The WNV specific IgG detected in this study can not give a timetable as to the infection or re-infection

of WNV. It does, however, show that WNV is active in central Oklahoma among farm animals, especially chickens. It is probably also present in many nearby wild animals in similar proportions.

Future studies could include comparisons of the ACG and GAC format as well as comparisons of IgM and IgG antibodies titers. A correlation between Plaque Reduction Neutralization Test titers and ELISA titers could also be examined. In addition, the use of a broader range of animal species including humans, a larger and more even distribution of locations, an equal representation of sexes, and a larger range of ages could be employed in future studies.

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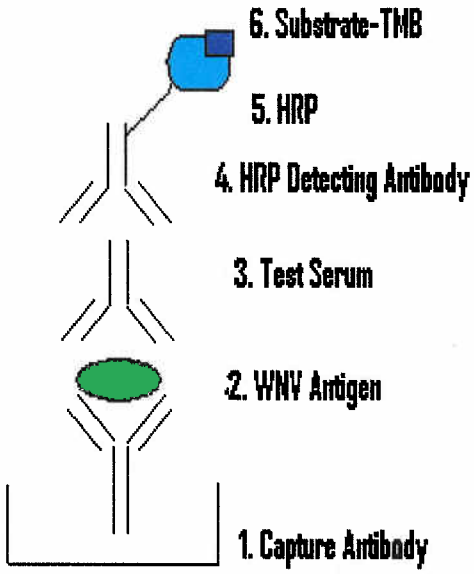

Figure 1. Illustration of the Enzyme-Linked Immunosorbent Assay with WNV as the antigen.

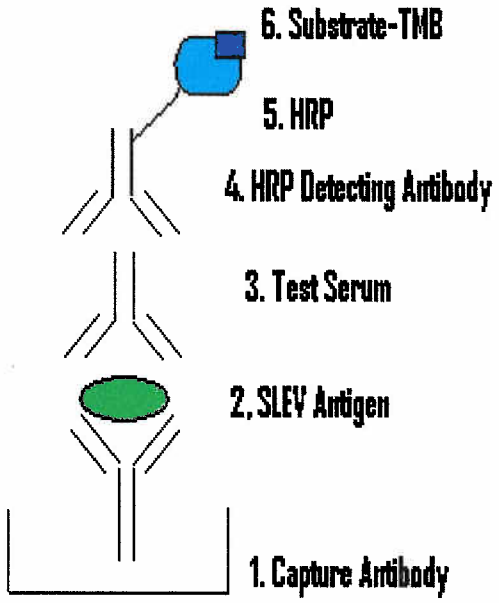

Figure 2. Illustration of the Enzyme-Linked Immunosorbent Assay with SLEV as the antigen.

Table 1. An Oklahoma State Health Department survey of West Nile Virus incidence in horses, birds, and mosquitoes in five counties. The numerator indicates the number of positive cases and the denominator indicates the total number of tests for that category (Oklahoma State Health Department, 2007).

	Oklahoma County			Canadian County			Kingfisher County			Logan County			Noble County		
	H*	B*	M*	H	B	M	H	B	M	H	B	M	H	B	M
2002	72/NA**	51/329	0/5	18/NA	1/51	0/0	7/NA	0/11	0/0	20/NA	5/50	0/0	5/NA	0/2	0/0
2003	7/NA	63/123	2/50	1/NA	0/0	0/0	0/NA	0/0	0/0	1/NA	0/0	0/0	1/NA	0/0	0/0
2004	2/NA	20/68	0/0	1/NA	0/0	0/34	0/NA	1/2	0/0	0/NA	0/9	0/0	0/NA	0/0	0/0
2005	6/NA	na***	0/0	1/NA	na	0/0	1/NA	na	0/0	1/NA	na	0/0	1/NA	na	0/0
2006	0/NA	na	0/1	0/NA	na	0/0	0/NA	na	0/0	0/NA	na	0/0	0/NA	na	0/0

*H=Horse; B=Wild Bird; M=Mosquito.

**NA=Horse denominator data not available.

***na=Wild Birds were not tested after 2004.

Table 2. Results of testing five species for West Nile Virus antibodies in three collection periods. The numerator indicates the number of samples testing positive for West Nile Virus specific IgG antibodies and the denominator shows the total number of samples tested for that category.

	March, April, May (spring)	June, July, August (summer)	September, October, November (fall)	Totals
Sheep	0/20	3/20	0/20	3/60(5%)
Pig	1/20	2/20	1/20	4/60(6.7%)
Bovine	0/20	0/20	0/20	0/60(0%)
Goat	1/20	0/20	0/20	1/60(1.7%)
Chicken	2/20	5/20	1/20	8/60(13.3%)
Totals	4/100(4%)	10/100(10%)	2/100(2%)	16/300(5.3%)

Table 3. Cross-reactivity of samples positive for West Nile Virus antibodies are shown by Enzyme-Linked Immunosorbent Assay endpoint antibody titer levels for West Nile Virus and St. Louis Encephalitis Virus antigens.

Species	Sample	Collection period*	WNV-ELISA titration	SLEV-ELISA titration
Goat	G7a	Spring	1/800	0
Pig	P12a	Spring	1/12,800	1/6,400
Pig	P12b	Summer	1/12,800	1/800
Pig	P20b	Summer	1/12,800	1/1,600
Pig	P20c	Fall	1/6,400	1/1,600
Chicken	C9a	Spring	1/25,600	1/1,600
Chicken	C11a	Spring	1/25,600	1/6,400
Chicken	C3b	Summer	1/25,600	1/800
Chicken	C7b	Summer	1/25,600	1/6,400
Chicken	C8b	Summer	1/25,600	0
Chicken	C10b	Summer	1/25,600	1/3,200
Chicken	C16b	Summer	1/25,600	1/3,200
Chicken	C3c	Fall	1/25,600	1/3,200
Sheep	S5b	Summer	1/800	0
Sheep	S8b	Summer	1/800	0
Sheep	S18b	Summer	1/800	0

*Spring is denoted by the months March, April, May; Summer by June, July, August; and Fall by September, October, November.