STABILITY OF SENTINEL CHICKEN SERUM AT DIFFERING TEMPERATURES FOR WEST NILE VIRUS DETECTION

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ABSTRACT

The control of arboviruses is aided by surveillance programs. The use of sentinel chickens is a commonly used surveillance tool with operational benefits for mosquito control. However, sentinel chicken programs have associated costs related to animal husbandry, sample collection, and for out-sourced testing such as shipping costs. This study investigated the impact of eliminating cold shipping conditions often required for shipping samples to outside laboratories. Storage of sentinel chicken samples at room temperature (22°C) and 50°C for up to 6 days post blood draw and serum separation showed no difference in the reportable results with a commercially available competitive ELISA assay despite there being significant differences among some of the temperatures/days post blood draw. Eliminating the need for cold shipping conditions and the need for overnight shipping may reduce costs for mosquito control program.

Key Words: Costs, ELISA, chicken serum, stability, temperature, WNV

Mosquito-borne viruses continue to pose a threat to veterinary, wildlife, and public health on a global scale (Langevin et al 2001, Ramirez et al 2018). With the lack of preventative measures, such as vaccinations, and lack of treatment options, the control of arboviral activity is most effectively accomplished through the management of vector species, which can be enhanced through the use of surveillance measures (Ramirez et al 2018, Danforth et al 2021). Arboviral surveillance is thus a critical function to any effective mosquito control program (van den Hurk et al 2012). One commonly used surveillance tool is the use of sentinel chickens (Crans 1986, Olson et al 1991, Khan et al. 2017). Although the use of sentinel chickens is not always effective (Crans 1986, Day and Lewis 1992, Ramirez et al 2018), studies have shown how sentinel chickens can be one of the most sensitive indicators of virus activity in an area (Day and Lewis 1992, Reisen et al 1994, Ramirez et al 2018) and can lead to more precise data collection (Reisen et al 1994, Langevin et al 2001).

In Florida, sentinel chicken programs have been used since 1978 (Day and Lewis 1992) and are still used by mosquito control programs throughout the state today. Many programs rely on the data generated by these surveillance efforts to make informed decisions regarding their treatment initiatives (van den Hurk et al 2012). Sentinel chicken programs are also able to provide early warnings for human disease risk within communities (Day and Lewis 1992).

An established sentinel program does have inherent associated costs. In-house testing requires the cost of sentinel chicken husbandry, sample collection, and testing assays. Out-source testing requires the cost of sentinel chicken husbandry, sample collection, shipping of samples to outside laboratories, and if required, testing fees. Shipping costs regularly include the need for cold storage throughout sample collection and shipping (often accomplished through the use of dry ice or chilling packs; Reisen et al 1994) which also requires larger shipping containers – which increases costs as well. This study aimed to investigate the stability of sentinel chicken serum stored without the use of cooling agents as well as at higher temperatures that might be observed during the collection and shipping process.

Previously confirmed by the Florida Department of Health Laboratory, WNV-positive and WNV-negative sentinel chickens were bled using a 3-mL syringe and a 25-gauge needle with a brachial venipuncture (Johnson et al 2003, Florida Department of Health 2021). Blood was transferred into 3.5-mL serum separator tubes (SST) and allowed to clot for > 30 minutes. SSTs were then centrifuged at >2,000 revolutions per minute for at least 10 minutes (Grasedieck et al 2012). Serum was then removed from the SST and transferred into a 2-mL microcentrifuge tube (stock tube) that were pre-labeled with the individual bird IDs for storage. Stock tubes were stored in a temperature controlled incubator (22°C or 50°C) for the duration of sample collection. Subsamples (160 µL) were taken from the stock tubes and placed in a new 2-mL microcentrifuge tubes on days 0 (initial blood draw and serum separation), 1, 2, 3, 4, 5, and 6 post blood draw. After subsamples were collected these subsamples were immediately stored in a -80°C freezer until testing.

Sentinel chicken serum was tested for WNV antibodies using a commercially available competitive enzyme-linked

immunosorbent assay (ELISA) (Innovative Diagnostics, Grabels, France). In brief, controls and samples were mixed with dilution buffer and transferred into the pre-coated microwells of the ELISA plate. Plates were allowed to incubate for 90 minutes at room temperature and then washed three times (Model 1575 Immunowash Microplate Washer, Bio Rad, Hercules, CA) with approximately 300 µL of wash solution. Diluted conjugate was then added to each microwell and the plates were allowed to incubate for another 30 minutes at room temperature. Plates were washed again and substrate solution was added to all wells and allowed to incubate in the dark for 15 minutes at room temperature. Stop solution was then added to all wells and the plates were read at 450 nm (iMark Microplate Absorbance Reader, Bio Rad, Hercules, CA). Optical density (OD) values were then converted into a S/N% using the following equation: $((OD_{sample} / OD_{average\ negative\ control}) * 100)$. S/N% of >50 are considered negative, >40 but ≤50 are considered doubtful, and ≤40 are considered positive (ID.Vet 2020).

Subsamples from each day of collection were tested in triplicate. Comparison of sample averages between collection days was conducted using an ANOVA in Microsoft Excel (version 2016).

Sixteen blood draws were taken from 10 birds for analysis in this study (Table 1). OD values for specific samples and days were averaged and plotted on bar graphs. Samples P 1-4 and N 1-4 were held at 22°C for up to 6 days (Figure 1) and samples P 5-9 and N 5-7 were held at 50°C for up to 6 days (Figure 2). All results from each sample and their replicates were positive or negative as expected based on their pre-study status. Subsamples from day 6 of samples P 2 and N 1-2 were not collected as the stock serum had run out. Collection of subsamples from day 5 were missed for samples P 4 and N 4.

Significant differences were observed among the length of storage for samples P 1, P 4-8, N1-3, and N 5-7 (P 1: $F(6, 14)=5.18$, $p=0.005$; P 4: $F(5, 12)=3.46$, $p=0.036$; P 5: $F(6, 14)=3.26$, $p=0.032$; P 6: $F(6, 14)=10.22$, $p=0.0002$; P 7: $F(6, 14)=3.50$, $p=0.025$; P 8: $F(6, 14)=8.29$, $p=0.0006$; N 1: $F(5, 12)=9.23$, $p=0.0009$; N 2: $F(5, 12)=9.04$, $p=0.005$; N 3: $F(6, 14)=5.47$, $p=0.004$; N 5: $F(6, 14)=62.80$, $p<0.0005$; N 6: $F(6, 14)=125.61$, $p<0.0005$; N 7: $F(6, 14)=4.80$, $p=007$). No significant differences were observed among the length of storage for samples P 2-3, P 9, and N 4 (P 2: $F(5,12)=0.79$, $p=0.579$; P 3: $F(6, 14)=1.96$, $p=0.140$; P 9: $F(6, 14)=1.75$, $p=0.181$; N 4: $F(5, 12)=3.06$, $p=0.052$).

Other studies have investigated the integrity of serum and the detectability of a variety of components at different storage conditions and lengths of time. Grasedieck and colleagues (2012) demonstrated that there was no significant difference in the total RNA concentrations that were detected from serum after a year of storage at -80°C compared to storage at -20°C. Timms and colleagues (2007) determined that differing collection and handling methods influenced the protein profiles obtained from serum samples. Cruickshank-Quinn and colleagues (2018) observed differences in the metabolite abundance of serum based on time it takes to process samples.

This study demonstrated the stability of sentinel chicken serum kept above the chilled conditions which are typically used for storage and shipping. Despite there being significant differences observed between the S/N% among the collection days for a majority of the samples in this study, these differences are negligible as none of the differences effected the positivity or negativity of the respective samples (Figures 1 and 2). However, all negative samples had the highest S/N% (most negative value) on day 0 and then became more positive (lower S/N%) in the following days of storage. There was no real trend in which day experienced the "least negative" value. With this observation, there is the potential of a sample initially being negative, or in the doubtful range, on day 0 to then test positive after subsequent storage at higher temperatures. Unfortunately, it is difficult to experimentally show this potential as finding a sample that falls in or near the doubtful range is rare, and if found, often falls back into the negative range on a later blood draw (unpublished data). Conversely, some samples started trending back more negative by day 6. These results support the idea that storage of sentinel chicken serum at room temperatures or up to at least 50°C does not influence the detectability of WNV antibodies in competitive ELISA assays for up to at least 6 days post blood draw and serum separation.

Knowledge of the stability of sentinel chicken serum could be a considerable cost saving measure for mosquito control programs that ship their samples to outside laboratories with dry ice or other chilling measures. Also, the frequently required overnight shipping, which often has high associated cost, may no longer be require based on these results. As an example, if samples are collected on a Monday but the weekly receiving cutoff from the outside laboratory isn't until Wednesday, then there is no need to spend the extra funds to get your samples there on Tuesday. This is especially important for programs with limited budgets for arboviral surveillance (Peper et al. under review).

Knowledge of serum stability for sentinel chickens at higher temperatures is also beneficial to the operational side of mosquito control. If samples get delayed in shipment or left in the back of a field truck for a time, the results from this study help us understand the potential for these samples to still be of use for analysis. However,

Table 1. Bird identification number and associated Sample ID and West Nile virus status prior to sample collection and testing.

Corresponding Sample	Bird ID	Predetermined WNV Status
P 1–4	583	Positive
N 1–4	565	Negative
P 5	62	Positive
P 6	66	Positive
P 7	61	Positive
P 8	82	Positive
P 9	80	Positive
N 5	1080	Negative
N 6	1085	Negative
N 7	1086	Negative

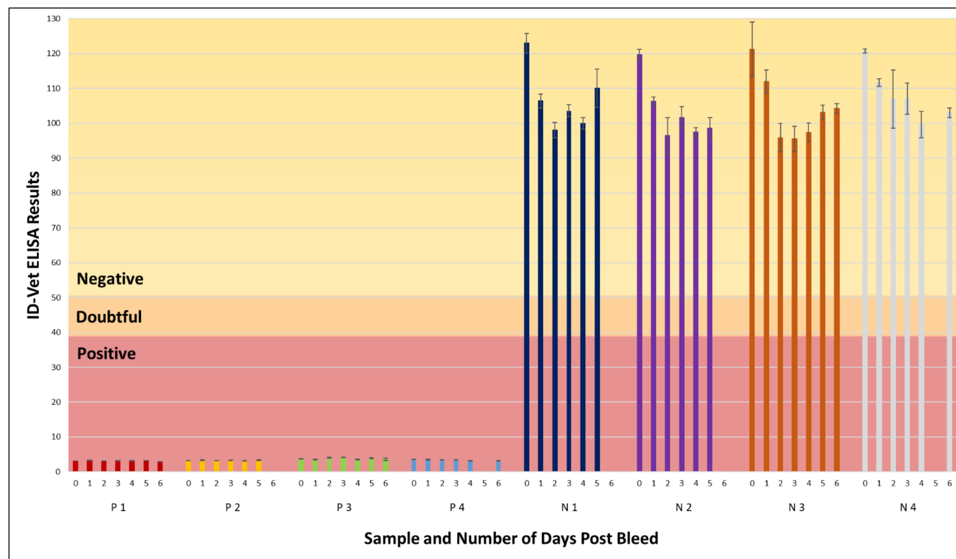

Figure 1. West Nile virus Competitive ELISA results for samples stored at 22°C.

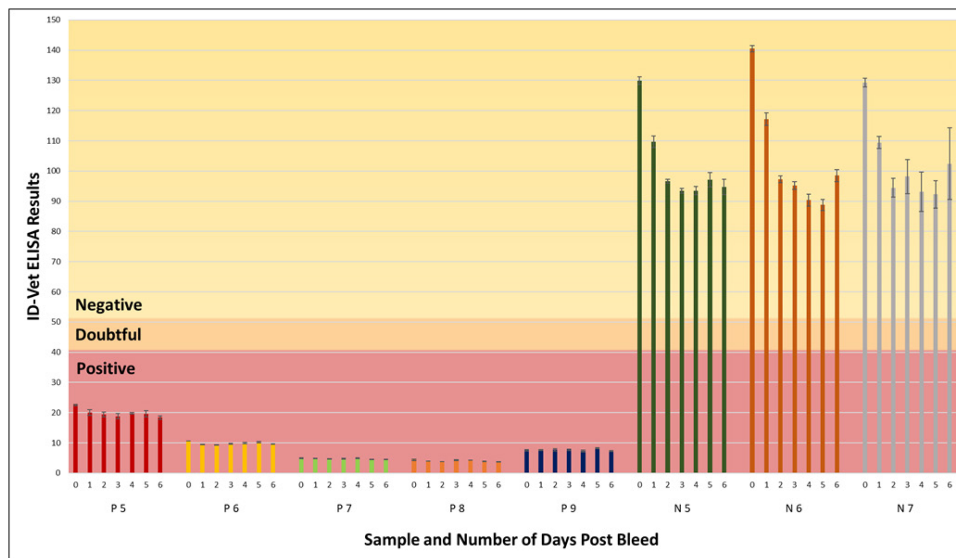

Figure 2. West Nile virus Competitive ELISA results for samples stored at 50°C.

some circumstances may expose samples to higher temperatures than used in this study. The elimination of dry ice in shipping would also reduce potential shipping hazards associated with the sublimation of carbon dioxide (Caldwell et al 2006) and packaging size of shipping container needed for transport.

The use of sentinel chickens for arboviral surveillance is a tried and true practice where the benefits often outweigh the negatives. One limitation, of course, for some mosquito control programs is the cost associated with shipping samples to outside laboratories for testing and this study helps lay the groundwork for updated sample handling protocols that may help reduce some of those associated costs.

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