SRAS Final Report: Understanding the mechanisms of West Nile virus (WNV) infection and induced lesions in *Crocodylus porosus*

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ABSTRACT

West Nile virus (WNV) is a significant cause of economic loss in the saltwater crocodile (*Crocodylus porosus*) farming industry, primarily due to skin lesions known as "pix" caused by the infection. The mechanisms of development of the lesions are yet to be elucidated. This study aimed to investigate the dynamics of WNV infection and the immune responses in experimentally infected saltwater crocodile hatchlings to better understand the pathogenesis of the lesions. Using virus isolation techniques and quantifying viral genome loads in tissues through RT-qPCR, we showed that the virus initially replicates in the spleen and liver, and later in the pancreas. Overall, the findings of this study suggest that the kidney and gastrointestinal tract serve as the main sites of viral replication, which leads to cloacal shedding. However, the connection between viral replication and the development of skin lesions remains to be fully clarified.

INTRODUCTION

West Nile virus (WNV) is an orthoflavivirus and zoonotic arbovirus that now occurs almost globally. Infections and diseases caused by WNV have been reported in several animal species, including humans, horses, sheep, birds, and reptiles (Brault, 2009; Byas and Ebel, 2020; Byas et al., 2022; Habarugira et al., 2020b). Infected hosts can exhibit a wide range of clinical presentations, from subclinical infections to severe neurological forms (Habarugira et al., 2020b). Susceptible hosts may experience severe neurological, digestive, renal, and skin-related signs (Barzon et al., 2013a; Barzon et al., 2014; Barzon et al., 2013b; Habarugira et al., 2020b). In our previous research, we demonstrated the role of WNV in the development of skin lesions in farmed saltwater crocodiles (*Crocodylus porosus*) (Habarugira et al., 2020a).

Under farming conditions, 10% to 30% of saltwater crocodiles may develop skin lesions, which can lead to depreciation and rejection by the tanning industry (Isberg et al., 2019). Histologically, these lesions are characterized by lymphohistiocytic dermatitis. Interestingly, similar lymphoid aggregates were observed in the gastrointestinal tract of experimentally infected hatchling crocodiles; however, no viral antigen was detected in any of the lesions through immunohistochemistry. Despite this, viral RNA was found in cloacal swabs and water samples, indicating that the virus is shed into the water. In reptiles, the waste products from the gastrointestinal and urinary systems combine in a single chamber called the cloaca, where they are excreted via the vent (Holz, 2020; Kuchel and Franklin, 2000). Moreover, even though the experimentally infected crocodiles were never exposed to natural infection through mosquito bites, nor were they experientially re-infected, levels of neutralizing antibodies continued to rise over several months post-infection. This suggests a possible persistence of the virus in some tissues or ongoing maturation of the immune response. Understanding disease diagnosis and control in any host requires a thorough understanding of the pathogenesis, including pathogen tissue tropism, infection kinetics, and innate immune responses - essentially the host-pathogen interactions (Rouse and Sehrawat, 2010; Thakur et al., 2019; Varela et al., 2013).

Our previous study did not definitively resolve the tissue tropism of virus replication in infected saltwater crocodiles (Habarugira et al., 2020a). Nevertheless, based on our findings, we hypothesized that skin lesions are immunopathological in nature, resulting from a strong innate immune response during acute viral infection, followed by an adaptive immune response that clears the virus (Habarugira et al., 2020a; Habarugira et al., 2022).

In this study, we aimed to elucidate the WNV infection kinetics and tissue tropism in saltwater crocodiles.

METHODS

Four month old crocodile hatchlings were infected with 10^5 IU of a Kunjin strain of WNV (WNV_{KUN}), in 100 µL of sterile PBS as previously described (Habarugira et al., 2020a). The control group was sham infected with 100µL of sterile PBS (mock-infected). Infected animals were co-housed with uninfected animals (in–contact control) (Figure 1). Infected, uninfected control and in-contact control animals were sampled daily for the first 7 days post infection (dpi) and every second day from 9 dpi. Collected samples included blood, tissue, cloacal swabs, and water.

Aseptically collected blood was processed, and the obtained serum was tested in blocking ELISA and virus neutralization test as described previously (Vet et al., 2020). Cloacal and oral swabs were collected and processed and tested by RT-qPCR as previously described (Habarugira et al., 2020a).

A set of collected tissues including liver, spleen, kidney, oral cavity mucosa, the glosso-epiglottic fold mucosa, skin and the third eyelid were tested for viral load by $TCID_{50}$ assay on selected cell lines and indirect quantitation by detection of viral RNA by RT-qPCR. Other tissues, including brain, eye, tongue, lung, heart, esophagus, stomach, small and large intestines, cloaca, liver, pancreas, spleen, kidney, and skin were assessed for pathology by microscopy and immunohistochemistry.

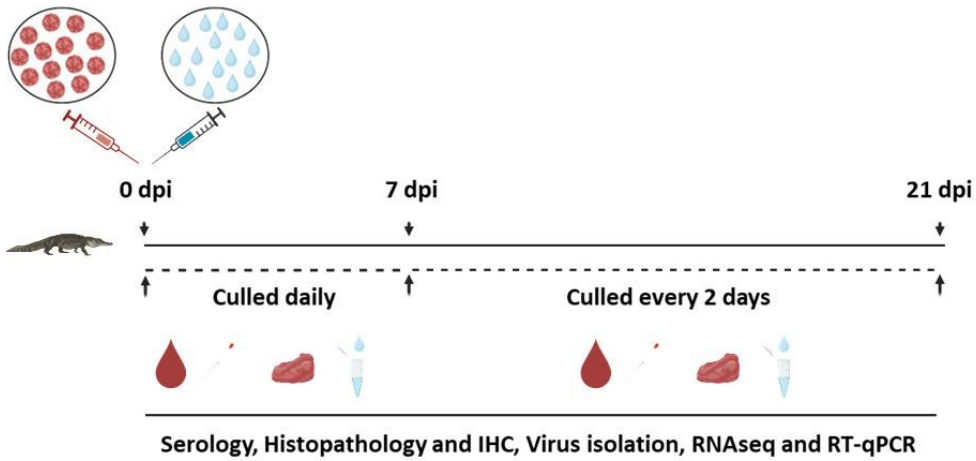


Figure 1. An illustrative summary of WNV infection kinetics experiment in saltwater crocodiles. Samples, including blood, tissues, cloacal swabs and water were collected daily for the first 7 dpi and every second day from day 9 dpi.

RESULTS

Virus titration from serum

Due to severe cytotoxicity exerted by crocodile serum on Vero and BSR cells, direct virus isolation was unsuccessful. In contrast, viral RNA was detected in most serum samples collected from infected animals, as well as in one of the contact control animals, starting from day one post-infection and continuing until the end of the experiment at 21 days post-infection (dpi) (Figure 2 A and B). At least 60% of the infected animals were viremic during the first 7 to 9 dpi, as shown in Figure 3A. None of the mock-infected animals showed any signs of viremia (Habarugira et al., 2025).

Seroconversion

Virus-neutralizing antibodies were first detected in the saltwater crocodiles challenged with WNV on day 9 dpi, with antibody titers ranging from 20 to 160 (Figure 2C). By 17 dpi, three in-contact animals had developed virus neutralizing antibodies, with titers between 20 and 40 (Figure 2C). No mock-infected animals showed detectable WNV neutralizing antibodies (Habarugira et al., 2025).

WNV detection by RT-qPCR in cloacal swabs

Cloacal swab samples were collected from each saltwater crocodile at the time of termination. Viral RNA was first detected in virus-challenged animals at 3 dpi. Viral RNA was found in 40% of the infected animals that were culled between 3 and 6 dpi. All infected animals culled at 9 dpi tested positive for WNV RNA in their swabs. The first instances of RNA-positive cloacal swabs from in-contact control animals were also detected at 9 dpi, with half of these animals showing positive results for viral RNA (see Figure 3A and B). From 11 dpi onward, the proportion of RNA-positive cloacal swabs in both the infected and in-contact control groups decreased until the end of the experimental period at 21 dpi (Figure 3B).

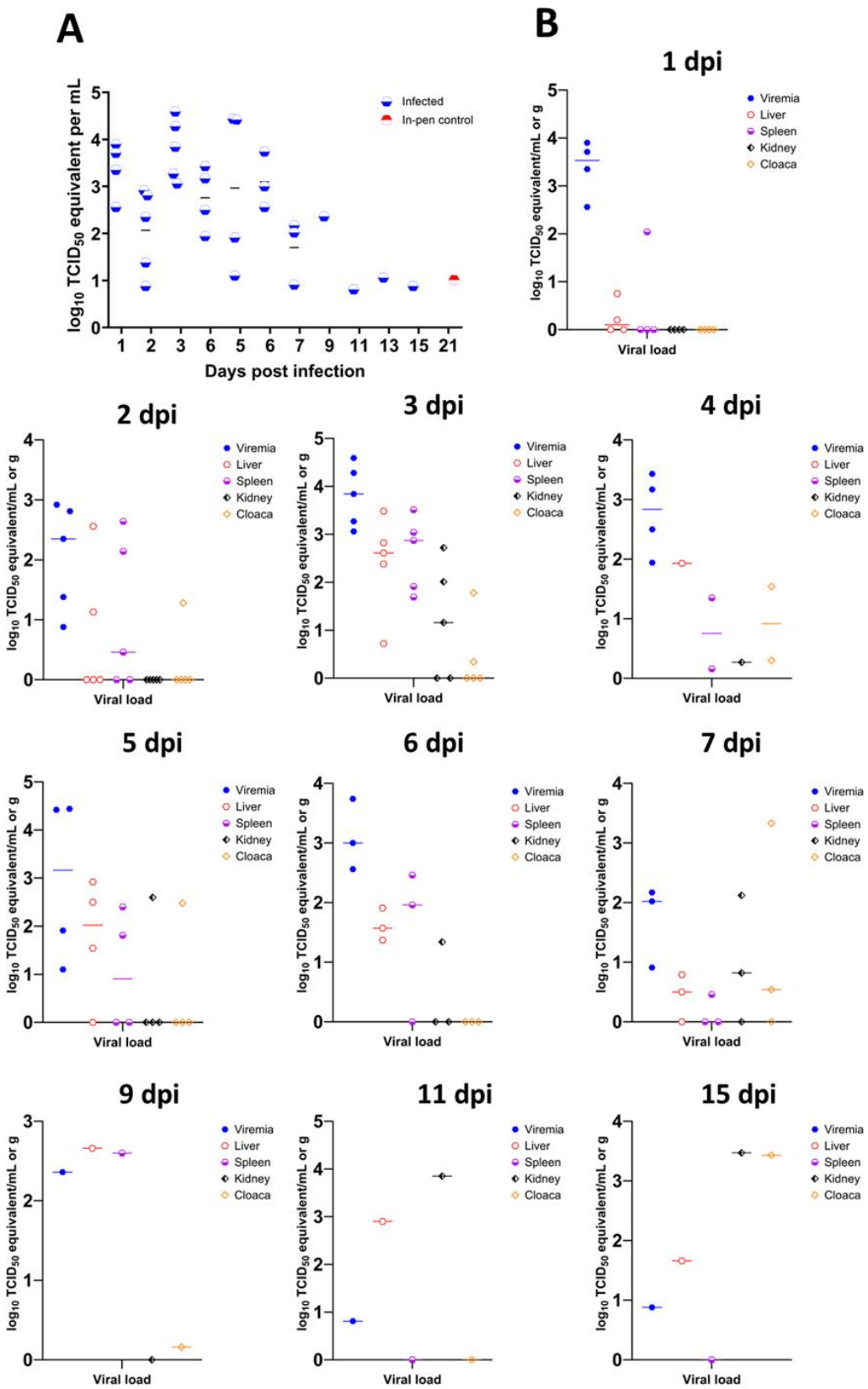


Figure 2. Viremia and viral load in selected tissues of infected saltwater crocodiles in relation to viremia measured by RT-qPCR at various timepoints. A. viremia per timepoints expressed in log10 $TCID_{50}$ per mL of blood. B. Viral load in liver, spleen, kidney and cloacal mucosa in relation to viremia in each timepoint. Only tissues from animals with viremia were examined.

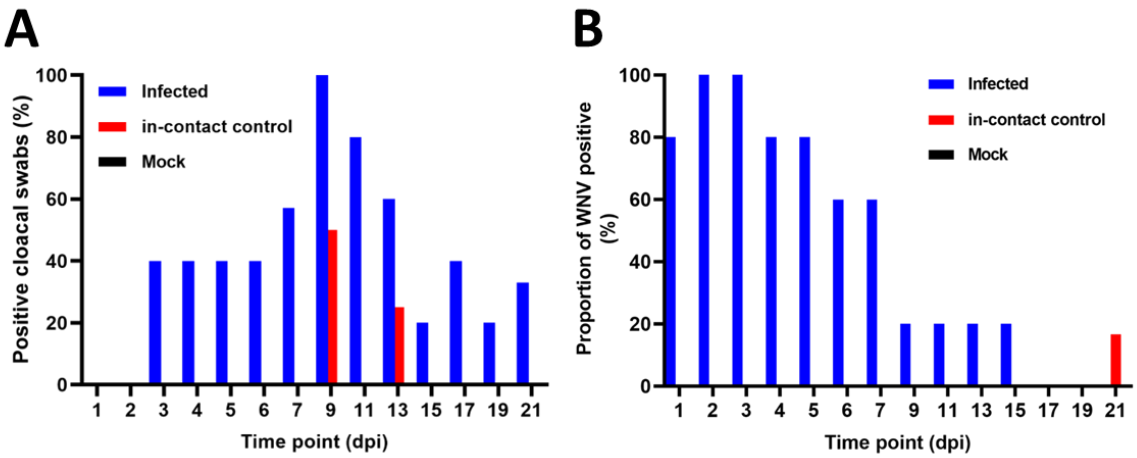


Figure 3. Proportion of WNV positive among infected, in-contact control and mock-infected crocodiles. A. Proportion of cloacal swabs positive for WNV by RT-qPCR at different time points post experimental WNV challenge. B. The proportion (%) of crocodile hatchlings positive for WNV by RT-qPCR in blood at various time points post-infection.

Gross and microscopic pathology

Macroscopic skin lesions were observed during necropsy in four animals at 5-, 6-, and 17-dpi, respectively Figure 4). Notably, only one animal, examined at 17 dpi, exhibited a typical pix lesion, which was confirmed through histological analysis (Figure 4A, B). Histologically, focal to multifocal infiltrates of lymphocytes and histiocytes, along with lymphoid aggregates, were detected in various tissues, including the conjunctiva, liver, spleen, kidneys, stomach, both small and large intestines, cloacal mucosa, and pancreas at multiple time points (Figure 4C – F): two animals terminated at 3 dpi, one at 5 dpi, two at 6 dpi, and one at 7 dpi. Mild intraepithelial and epidermal infiltration of lymphocytes and monocytes was noted, as well as mild perivascular lymphocyte infiltration in the dermis (Figure 4B and 5A). However, none of these skin samples tested positive for viral nucleic acid, nor was any viral antigen detected by immunohistochemistry (IHC) (Habarugira et al., 2025).

Viral antigen was identified via IHC using the anti-flavivirus NS1 monoclonal antibody 4G4 in the spleen of two animals terminated at 3 and 5 dpi, respectively. Additionally, viral antigen was found in three pancreas samples from animals that were terminated at 13 dpi (n = 1) and 15 dpi (n = 2) (Figure 5C, D). The latter three animals had detectable WNV-neutralizing antibodies at the time of their termination (Habarugira et al., 2025).

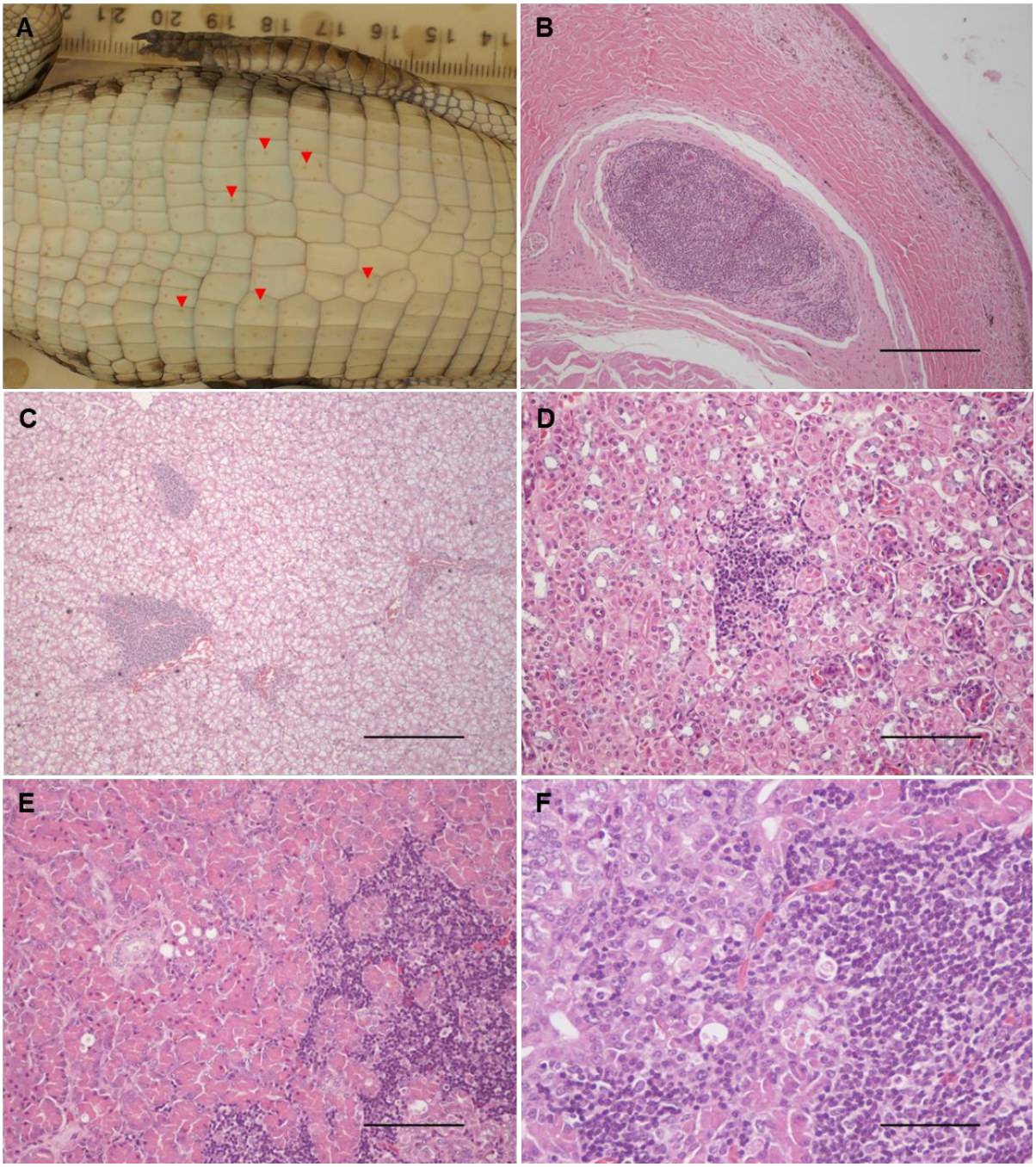


Figure 4. Lymphoid aggregates in different tissue sections. A. Macroscopic "pix" lesions (red arrow heads) in the skin of an infected crocodile. B. Lymphoid aggregates in a skin from an infected crocodile. C. Periportal lymphoid aggregates in the liver with mononuclear infiltration in periportal zones. D. Interstitial lymphoid aggregates the in kidney. E and F. Exocrine pancreatic cell degeneration with interstitial mononuclear infiltration (E 20x; F, 40x). Scale bars: A and C 625 µm; D and E 125 µm; F 65 µm.

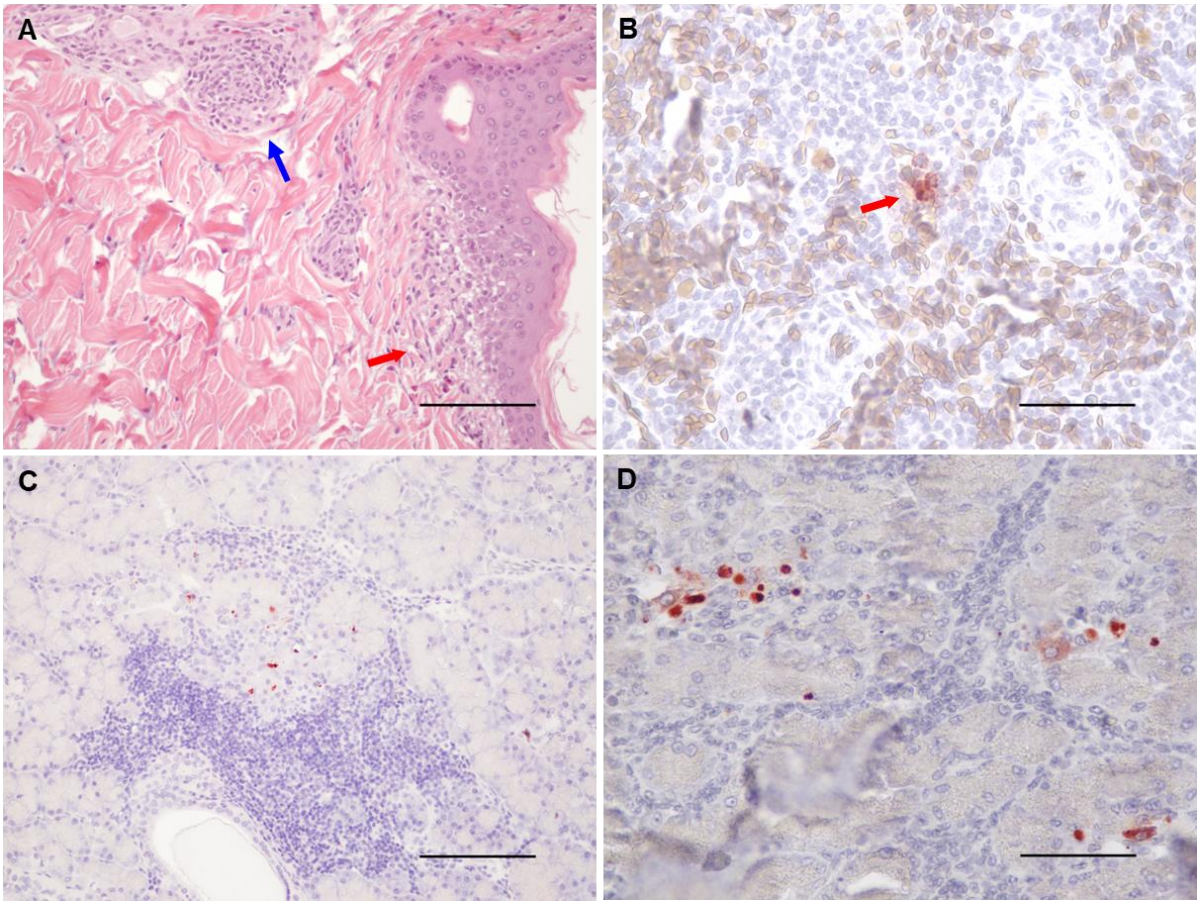


Figure 5. Histopathology lesions in infected saltwater crocodiles. A. Mild intraepithelial and epidermal infiltration of mononuclear cells (lymphocytes and monocytes) (red arrow), mild perivascular lymphocyte infiltration in the dermis (blue arrow), scale bar 1250 µm. B. Immunohistochemical detection of WNV NS1, using monoclonal antibody 4G4, in spleen from a crocodile infected with WNV_{KUN}. There are viral protein-positive monocytes (red arrow), scale bar 125 µm. Note that the brown round oval cells in panel B are nucleated erythrocytes. C. and D. Immunohistochemical detection of WNV NS1 (red signals) in exocrine pancreas from animals terminated 13 and 15 dpi, scale bars 250 and 65 µm, respectively.

DISCUSSION

In this study, we further investigated the pathobiology of WNV infection in hatchling saltwater crocodiles expanding from our previous study (Habarugira et al., 2020a). We previously demonstrated the role of West Nile Virus (WNV) in the development of skin lesions, referred to as "pix," in saltwater crocodiles. Additionally, we reported the fecal-oral transmission of the virus under simulated farming conditions and established that the levels of viremia in the crocodiles could support mosquito transmission (Habarugira et al., 2023; Habarugira et al., 2020a). Several unanswered questions such as virus tropism, replication and source of virus shedding in infected animals led to this short-term kinetics study.

As we previously reported (Habarugira et al., 2020a), none of the infected animals became clinically sick. Only 6% of the animals developed grossly detectable skin lesions during the 21 days' study period. Under farm conditions in Australia, 10 – 30% of animals may develop pix skin lesions in any given season (Habarugira et al., 2020a; Isberg et al., 2019), and our previous studies, as well as experience from studies of alligators, suggest lesions may develop over a period of up to three months post-infection (Habarugira et al., 2023; Habarugira et al., 2020a; Klenk et al., 2004). The low incidence of lesions in this study is expected since animals were terminated sequentially. By 21 dpi, only four WNV-challenged animals and five in-contact controls were left. This likely prevented lesion development in others terminated earlier. Notably, 13 animals terminated at earlier time points displayed

lymphohistiocytic dermatitis microscopically in skin samples from the abdominal region, suggesting these cellular infiltrates may be precursors to lesion development.

Despite the lack of a clinical disease, we detected infectious WNV, viral RNA and virus protein were detected in crocodile plasma and tissues, including the liver, spleen, kidney, pancreas, cloaca and third eyelid (Figure 2, 3 and 5). Isolating infectious viruses from serum and whole blood samples was hindered by the toxic nature of crocodile blood on commonly used cell lines for virus isolation, necessitating further studies.

The presence of viral RNA at a later stage of infection, even after the detection of neutralizing antibodies, indicates that the virus may continue to replicate and persist in certain tissues. Further studies are needed to explore the mechanisms behind this persistence and its duration.

In this study, we observed a direct association between small aggregates of lymphohistiocytic cell infiltrates and viral protein in the pancreas, as determined by immunohistochemistry (IHC). In our previous investigations, we detected West Nile Virus (WNV) RNA in the lymphoid aggregates of skin lesions; however, we did not find replicating virus or viral antigens present (Habarugira et al., 2023; Habarugira et al., 2020a; Isberg et al., 2019). The phenotype of the immune cells in these infiltrates has not yet been established due to the lack of suitable reagents for reptiles. Therefore, it remains unclear whether these lymphoid cells are actively exerting antiviral activity or if they are merely present as bystanders; however, the former seems more plausible.

This study provides insights into the pathophysiological responses of saltwater crocodiles to experimental WNV infection. However, additional research is needed to better understand the interaction between WNV and the host. Our findings could serve as a foundation for further investigations into how crocodilians interact with WNV and other pathogens.

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