Rift Valley Fever Viral RNA Detection by *In Situ* Hybridization in Formalin-Fixed, Paraffin-Embedded Tissues

Running head: Pan-RVFV FFPE ISH

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Abstract

Sporadic outbreaks of Rift Valley fever virus (RVFV), a zoonotic, mosquito-borne *Phlebovirus,* cause abortion storms and death in sheep and cattle resulting in catastrophic economic impacts in endemic regions of Africa. More recently, with changes in competent vector distribution, growing international trade, and potential use for bioterrorism, RVFV has become a transboundary animal disease of significant concern. New and sensitive techniques that determine RVFV presence, while lessening the potential for environmental contamination and human risk, through the use of inactivated, non-infectious samples such as formalin-fixed, paraffin-embedded (FFPE) tissues are needed. FFPE tissue *in situ* hybridization (ISH) enables the detection of nucleic acid sequences within the visual context of cellular and tissue morphology. Here we present a chromogenic pan-RVFV ISH assay based on RNAscope® technology that is able to detect multiple RVFV strains in FFPE tissues, enabling visual correlation of RVFV RNA presence with histopathologic lesions.

Keywords: Rift Valley fever virus, diagnostics, *in situ* hybridization, ruminants, virology, ISH

Introduction

Rift Valley Fever (RVF) virus (Family: *Phenuiviridae*, genus: *Phlebovirus*) is a zoonotic arbovirus endemic to Africa and the Arabian Peninsula. It is an enveloped, negative-sense RNA virus with a tripartite genome, consisting of the ~6.4 kb large (L), ~3.8 kb medium (M) and ~1.7 kb small (S) segments (Faburay et al. 2017).

RVF is considered to be a high consequence transboundary animal disease and a potential public health concern. Work with it requires BSL-3+ containment and in the US, also Select Agent clearance. RVF outbreaks in ruminants are characterized by abortion storms and neonatal deaths, and to a lesser degree morbidity and mortality in adult ruminants. Infected tissues must be handled with care due to their zoonotic risk causing human infections, which can vary from mild febrile illness to severe liver disease, encephalitis or even death, often occurring following exposure to infected animal fluids or tissues (Faburay et al. 2017).

RVF virus (RVFV) has a broad tissue tropism but is typically found in the liver where it causes a necrotizing hepatitis and in lymphoid tissues and the kidney (Odendaal, et al. In Press). In experimental animal studies with virulent strains, viral nucleoprotein is readily detectable by immunohistochemistry (IHC) in tissues until 5 days post-infection (Faburay et al. 2016, Wilson et al. 2016). While, histopathologic lesions consistent with RVF are seen at later time-points post-infection, current virus detection techniques typically yield negative results.

Formalin-fixed, paraffin-embedded (FFPE) tissues offer a safe format for handling and testing viral pathogens (Haddock et al. 2016). IHC and to a lesser extent PCR and *in situ* hybridization (ISH) have been used for pathogen detection in these

tissues. However, to date, only IHC, the detection of viral antigen, has been used for RVFV and serves as a confirmatory diagnostic test in South Africa (Odendaal et al. In Press). While both ISH and PCR detect viral RNA (vRNA) in tissues, ISH like IHC additionally provides histomorphologic context, thereby enabling the correlation of microscopic lesions with the presence of virus (Cassidy and Jones 2014).

Recent technological advances have improved the accuracy and accessibility of RNA ISH. One such technology, RNAscope® (Advanced Cell Diagnostics [ACD], Hayward, CA), uses short branched DNA probes to enhance the detection of vRNA in FFPE tissues and may be as sensitive at existing qPCR technology (Wang et al. 2012, Vasquez et al. 2018). To our knowledge, so far RVFV ISH has only been used to detect RVFV RNA in fixed cells (Wichgers Schreur and Kortekaas 2016). Therefore, the objective of this study was to develop a highly sensitive ISH assay for the detection of RVFV RNA in FFPE tissues.

Materials and Methods

The RVFV infected FFPE ruminant tissues used in this study originated from prior experimental studies (Faburay et al. 2016, Wilson et al. 2016) and the 2010 South African outbreak (Odendaal et al. In Press). RVFV infection was previously confirmed using either RVFV-specific triplex or M segment RT-qPCR, for the experimentally and naturally infected animals, respectively (Odendaal et al. In Press, Faburay et al. 2016, Wilson et al. 2016). In the experimental studies, 4-5 month-old lambs and calves were inoculated subcutaneously with 2 mL of 1×10^6 plaque-forming units of RVFV Kenya 128-15B 2006 (Ken06) or Saudi Arabia 2001-1322 (SA01) (Faburay et al. 2016, Wilson et al. 2016). We tested pan-RVFV FFPE tissue ISH on liver from a Ken06 4 days post-

inoculation (dpi) sheep and a liver and lymph node from a 5 dpi Ken06 sheep, lambs #55 and #44 respectively (Faburay et al. 2016). We also tested one liver sample from a 3 dpi Ken06-infected calf, #41, and two liver samples from a 4 dpi SA01 infected calf, #37 (Wilson et al. 2016). Finally, we tested a mixed tissue block, from SA2010 outbreak adult sheep 10-3268, containing lung, liver, spleen and kidney (Odendaal et al. In Press). Uninfected control tissues for all tissue types and species tested came from either mock-inoculated animals in the aforementioned experimental studies or were species matched necropsy tissues from the Kansas State Veterinary Diagnostic Lab (KS VDL).

ACD synthesized the pan-RVFV ISH probe according to the investigator provided target sequence and a list of excluded sequences including host species and nearest neighbor viruses. Ten sequences of RVFV representative of phylogenetic variance of up to 5% in the target region of the L segment, were aligned, including MP-12, Ken06, Clone 13, ZH548, ZH501, Smithburn, Lunyo, SA-57, SA-51 and SA01. The resulting probe (516121-V-RVFV-L) targets an L segment consensus sequence located between nucleotides 20-997 of the Rift Valley fever virus segment L, complete genome (NCBI Reference Sequence: NC_014397.1). ACD recommended negative and positive control probes targeting dihydrodipicolinate reducatase B of *Bacillus subtilis* and sheep housekeeping gene peptidylprolyl isomerase B (PPIB) respectively, were also used.

The chromogenic ISH assays were conducted according to the manufacturer's protocol. Briefly, 4 µm tissue sections were prepared using standard procedures of the KS VDL. These were rehydrated, pretreated, hybridized and detected using the RNAscope® 2.5 HD Brown Kit then counterstained and mounted. For RVFV RT-qPCR on the FFPE tissues on the previously untested SA2010 tissue block, we used a single tube

deparaffinization and tissue digestion protocol followed by automated magnetic bead extraction or silica based spin column protocol for recovery of RNA and the RVFV triplex RT-qPCR assay (Wilson et al. 2013). Additional modifications made were reduction of the final concentration of primers and probes, halving of the volume of nucleic acids used, and use of a 20 min reverse transcriptase reaction with the PCR master mix (Quanta BioSciences). Ct values reported are the means of three replicates for RVFV L, M and S gene detection. IHC results were previously published (Odendaal et al. In Press, Faburay et al. 2016, Wilson et al. 2016).

Results

The pan-RVFV ISH assay readily identified vRNA in lesions in both species' livers (**Fig. 1A-B**) and did not label uninfected liver (**Fig. 1C**). The assay also worked well on the other infected tissue types (**Fig. 1D**). The negative and positive control probes performed as expected including the sheep PPIB positive control probe on cattle tissue, as expected due to close gene homology (data not shown).

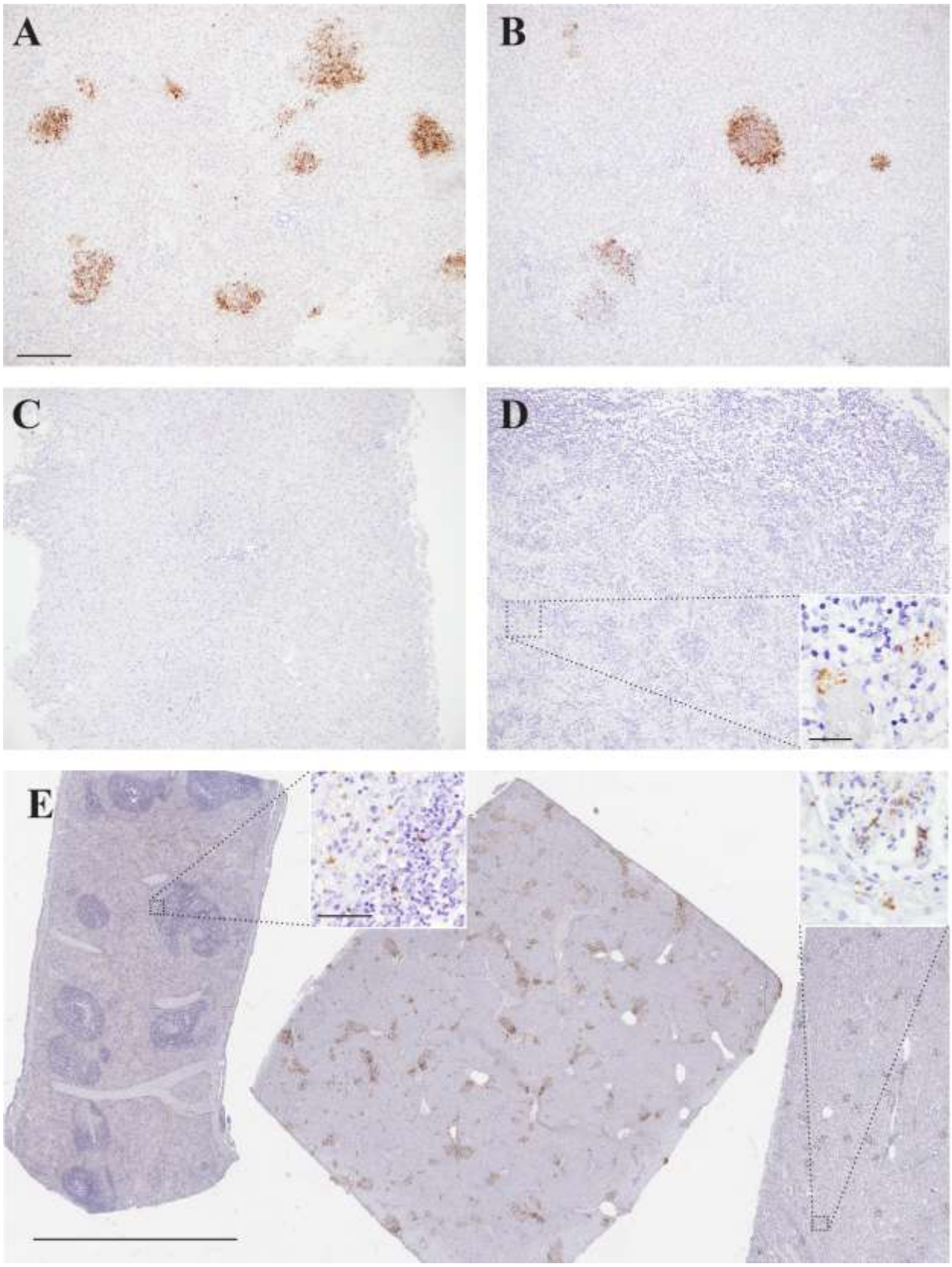
A
B
C
D
E

Figure 1. Pan-RVFV ISH detects multiple strains of RVFV in ruminant tissues

The pan-RVFV ISH probe labeled RVFV L segment vRNA (brown) in (A) Ken06 infection induced hepatic necrosis lesions in a sheep (A) and cattle (B). No signal was present in uninfected control tissues, (C), sheep liver. (D) shows that macrophages circulating in the medullary sinuses of an infected mesenteric lymph node contain RVFV RNA, inset is a magnification of the region outlined by the dashed box. The signal was punctate and cytoplasmic consistent with RVFV intracytoplasmic replication. The bar for the main images (A-D) is 200 μm and for the insets is 25 μm. (E) is a portion of a digitally scanned whole slide of pan-RVFV ISH labeling of the mixed tissue block for SA2010 sheep 10-3268. Spleen, liver, lung (not shown) and kidney were positive for vRNA. Insets show magnifications of the regions outlined by the dashed boxes in the spleen and kidney respectively. In liver as in (A) and (B), signal correlated with RVFV induced hepatic necrosis. ISH signal in the spleen correlated with previously observed splenic necrosis and both glomeruli and degenerating and necrotic tubular epithelium in the kidney were positive. Bar for main image is 3 mm and for insets is 50 μm.

Table 1 summarizes IHC, PCR and ISH results for all tissues. The ISH results correlated 100% with the triplex RT-qPCR and prior IHC (Faburay et al. 2016, Wilson et al. 2016) with the exception of one liver sample from calf #37 SA01 and sheep 10-3268's lung, kidney and spleen. The Ken06 tissue blocks were all heavily infected as indicated by low mean RT-qPCR Ct values, ranging from 21.3-23.6. In the liver, ISH labeling correlated with histopathology lesions and in the lymph node vRNA was found in the cytoplasm of circulating macrophages as reported prior by IHC (Faburay et al. 2016). Subjectively, ISH produced a stronger, more widespread and more readily interpreted signal than seen prior with IHC (Faburay et al. 2016, Wilson et al. 2016, Odendaal et al. In Press).

Table 1: ISH correlates with IHC and RT-qPCR

Animal #	Species	Tissue	RVFV virus strain	DPI	IHC	RT-qPCR (Ct) [a] L / M / S			ISH
55	sheep	liver	Ken06	4	+	21.7	21.5	23.6	+
44	sheep	liver	Ken06	5	+	23.5	23.7	26.2	+
41	cattle	liver	Ken06	3	+	21.3	22.2	22.7	+
37	cattle	liver	SA01	4	+	32.6	31.7	34.4	+
37[a]	cattle	liver	SA01	4	-	ND	ND	ND	+
10-3268[b]	sheep	liver	SA2010	N/A	+	20.9	20.4	23.7	+
70	sheep	liver	Uninfected	N/A	-	ND	ND	ND	-
44	sheep	lymph node	Ken06	5	+	35.5	28.4	ND	+
10-3268[b]	sheep	lung	SA2010	N/A	-	20.9	20.4	23.7	+
10-3268[b]	sheep	spleen	SA2010	N/A	-	20.9	20.4	23.7	+
10-3268[b]	sheep	kidney	SA2010	N/A	-	20.9	20.4	23.7	+

DPI = days post inoculation, Ct = cycle threshold, ND = not detected, N/A = non-applicable, [a] mean Ct of triplicates, bead extraction, [b] separately collected sample from same organ that was processed into a separate FFPE block, [c] PCR was run on all tissues as a mixed tissue block specimen, consequently all PCR results listed are the same for each organ

The SA01 liver samples from calf #37, were selected for their low vRNA level (Ct 30), determined using RT-qPCR on fresh unfixed tissue, and the observation of viral antigen in only one tissue block (Wilson et al. 2016). Moreover, the IHC negative block was also initially negative when tested by FFPE tissue triplex RT-qPCR using both column and bead extraction techniques with 100 µl lysate. However, increasing the lysate volume to 200 µl for bead extraction yielded a suspect result, specifically a mean Ct of 35.1 for the M segment. By pan-RVFV ISH both liver blocks were positive, albeit the vRNA labeling was less pronounced for the block that was previously negative by the other tests.

The SA2010 outbreak sheep mixed tissue block 10-3268, from a sheep with a positive liver by M segment RT-PCR on fresh unfixed tissue (Odendaal et al. In Press), was positive by triplex RT-qPCR (mean Ct values of 20.9/20.4/23.7 for L/M/S segments respectively). By IHC, the liver was strongly positive while the lung, kidney and spleen were negative (Odendaal et al. In Press). However, on histopathologic review there was a moderate amount of necrosis in the spleen and the kidney had a moderate amount of acute tubular injury. In the liver, stronger labeling with similar intralesional distribution was seen by ISH when compared to IHC. Interestingly, all four organs were ISH positive, albeit with lung, kidney and spleen more sparsely labeled than the liver (**Fig. 1E**).

Discussion

This is the first report of a pan-RVFV ISH for detection of RVF vRNA in FFPE tissues. RNAscope® ISH technology is a highly sensitive RNA ISH platform previously shown to accurately detect viral nucleic acids in FFPE tissues (Vasquez et al. 2018). We found that pan-RVFV ISH readily identified vRNA regardless of infecting strain in all species' tissues tested (**Fig. 1**). Additionally, in this study, the ISH results correlated well with results from IHC and RT-qPCR. Finally, the SA01 low vRNA experimental block and the SA2010 outbreak mixed tissue block results suggest that pan-RVFV ISH is more sensitive than IHC and possibly RT-qPCR on FFPE tissues.

Further evaluation of pan-RVFV ISH including establishment of the assay's diagnostic accuracy and precision is underway.

Conclusions

The RNAscope® pan-RVFV ISH assay has great potential as a confirmatory diagnostic test or research tool, including for use with archival FFPE tissues. Importantly,

ISH may offer superior sensitivity to IHC while still enabling testing to be conducted outside high containment on verified inactivated RVFV infected FFPE tissues. Unfortunately, high costs currently limit the application of the RNAscope® pan-RVFV ISH assay as a routine diagnostic test but the authors are hopeful that these costs will drop as the technology matures.

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Author Disclosure Statement

No competing financial interests exist.

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