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Protocols

The optimisation and application of a novel BaseScope™ RNA-ISH assay for the detection of foot-and-mouth disease virus in carrier African buffalo (*Syncerus caffer*) from South Africa

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SUMMARY

The detection of foot-and-mouth disease is limited to BSL-3 laboratories and its detection in carrier animals require increased test sensitivity. *In-situ*-hybridisation utilises the propensity of a labelled single-stranded sequence of DNA or RNA to anneal to a complementary target. It can be performed on formalin-fixed tissues and with some of the most recent advances, show an increased test sensitivity. BaseScope™ incorporates an additional signal amplification step, which makes it possible to detect RNA splicing variants, point mutations, small insertions or deletions, and short RNA targets (50–300 nucleotides). This study aimed to adjust and optimise the BaseScope™ assay to detect foot-and-mouth disease virus in a novel, carrier wildlife species, i.e., buffalo. Specific steps were adjusted to attempt to address some of the rigidity involved in the workflow. However, none of the in-house reagents or equipment attempted as an alternative to the original and prescribed workflow was successful. This demonstrates the fastidious nature of this diagnostic modality and the synergistic characteristics of a commercial assay. However, keeping tissues in formalin for up to 7 days and storing cut sections for up to 3 months did not have a negative impact on the results. This further demonstrated the reliability of BaseScope™.

1. Introduction

The first description of using *in-situ* hybridisation (ISH) to detect nucleic acids dates back to 1969 (Gall and Pardue, 1969). Hybridisation utilises the propensity of a labelled single-stranded sequence of DNA or RNA (the probe) to anneal to a complementary target (Jin and Lloyd, 1997). Variations on the traditional method, in conjunction with rapid, methodical advances, have made ISH a feasible molecular tool to use in diagnostics and research (Egger et al., 1994, Panoskaltsis-Mortari and Bucy, 1995, Wilcox, 1993, Wilkinson, 1998).

Radioisotope and non-isotope labelling are the primary methods for labelling and detecting the specific hybridisation reaction. Radioisotope labelling is detected by radiographic film or emulsion autoradiography, whereas non-isotope labelling is detected by immunohistochemistry or histochemistry. Non-isotope labelling has become the preferred method due to advantages that include increased labelled probe stability, rapid results, and increased resolution. Although ISH can be applied to either cell specimens or tissue sections, intact cells are preferred as there will be decreased nucleotide sequence damage (Jin and Lloyd, 1997). This allows for anatomically meaningful interpretation of test results by preserving cell or tissue integrity whilst localising gene expression. Fresh-frozen and formalin-fixed tissue can be used as specimens for this assay (Wilcox, 1993). It has numerous applications in research and as a valuable diagnostic tool, i.e., detecting infectious diseases, cytogenetics, and gene expression. Some of the most recent advances include detecting multiple target nucleic acids and amplifying the detection systems or the target DNA/RNA by polymerase chain reaction (PCR), which increases the test sensitivity (Kerstens et al., 1995).

The causative agent of foot-and-mouth disease (FMD) is foot-and-mouth disease virus (FMDV) species *Aphthovirus vesiculae*. This single-stranded, positive-sense RNA virus belongs to the genus *Aphthovirus* within the *Picornaviridae* family. Foot-and-mouth disease, first described in Venice in 1514 is a highly contagious, transboundary disease of mainly cattle, pigs, sheep and goats (Fracastorius, 1946). It causes decreased livestock production with a low mortality rate but a moderate to high morbidity rate (Belsham, 1993, Pereira, 1981, Sobrino et al.,

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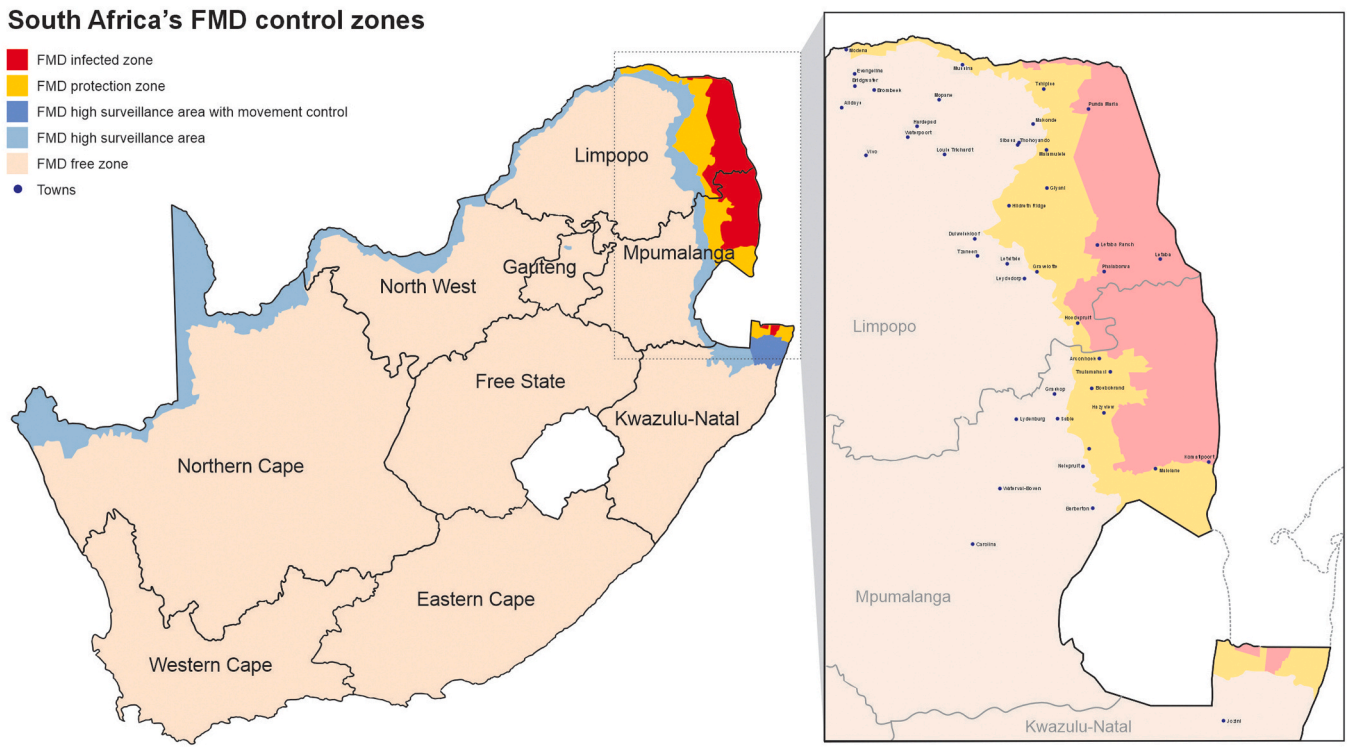

Fig. 1. Schematic representation of the FMD control zones in South Africa.

2001). The virus can spread rapidly and cause outbreaks over vast geographic areas, leading to significant economic consequences and trade restrictions (Belsham, 1993, Sobrino et al., 2001). The disease can also affect many species of wild ungulates, although the majority will be subclinical infections and/or carriers (Belsham, 1993, Sobrino et al., 2001, Pereira, 1981). Humans, equipment and/or animal transport can facilitate viral transmission (Brooksby, 1982, Donaldson and Kitching, 1989). In addition, airborne transmission, often over large distances, has been documented (Brooksby, 1982, Donaldson et al., 1987). Animals, including cattle, can become persistently infected with FMDV (Salt, 1993). The carrier status is species and virus strain-dependent (Salt, 1993). There are seven strains/serotypes of the virus, termed A, O, C, Asia 1 and Southern African Territories (SAT)-1, -2 and -3. The SAT serotypes are the predominant strains in South Africa, with African buffalo being an epidemiologically important carrier species. Carrier animals play an important role in the epidemiology of FMD as they are primarily responsible for disease maintenance and serve as a significant stumbling block to controlling and eradicating the disease (Salt, 1993, Vosloo et al., 2002).

The Kruger National Park (KNP) is South Africa's largest game reserve and is home to many bird and animal species, including more than 40,000 African buffalo. An FMD control zone (Fig. 1) was established around the park due to the important role of buffalo as carrier animals in which FMDV persists in the oropharynx of exposed animals or tissue samples for months. Before 2019, South Africa maintained an FMD-free zone without vaccination. Since then, multiple FMD outbreaks in the FMD-free zone have resulted in the suspension of the country's FMD free status. The movement of fresh tissue samples for research from the FMD control zone is strictly controlled, and the diagnosis or research of this disease is restricted to biosafety level 3 (BSL-3) laboratories. Fixing tissues in formalin inactivates this highly contagious virus, which makes it possible to move these samples to laboratories that are in areas where FMD does not occur, and to use techniques such as PCR, immunohistochemistry and ISH in BSL-2 laboratories.

Compared to traditional ISH, the BaseScope™ RNA assay is a novel and proprietary method to observe single RNA molecules in slide-mounted samples. This assay does not require the RNA-free environment used for traditional ISH. It is based on Advanced Cell Diagnostics' patented signal amplification and background suppression technology to improve test sensitivity further. Compared with the RNAscope™, BaseScope™ incorporates an additional signal amplification step, which makes it possible to detect RNA splicing variants, point mutations, small insertions or deletions, and short RNA targets (50 – 300 nucleotides). The major drawbacks of ISH, whether traditional or modern, include its fastidious nature and the extended turnaround time. Its application in veterinary science is limited to research due to the need for specialised and expensive equipment and high running costs.

This study aimed to adjust and optimise the BaseScope™ assay to detect FMDV in a novel, carrier wildlife species, i.e., buffalo.

2. Materials and methods

2.1. Diagnostic samples

Tissues used in the current study were obtained from African buffalo (n = 30) culled and slaughtered, respectively, in the KNP. The test buffalo (n = 15) were culled as part of the park's population control/harvest management strategies. Their respective FMD statuses were determined by PCR performed at the Transboundary Animal Disease Laboratory of the Onderstepoort Veterinary Research-Agricultural Research Council (TAD) as previously described (Innis et al., 2012, Callahan et al., 2002). The assay can detect viral RNA in less than two hours and is a reliable, rapid and sensitive diagnostic test for the detection of FMDV (Callahan et al., 2002). Negative diagnostic control tissues were sourced from an unrelated, approved project (REC 036–18) performed on African buffalo (n = 15) that repeatedly tested negative for FMDV by PCR. These buffaloes were PCR-negative for FMDV at 30 – 60 days intervals for three rounds, with the final round coinciding with the slaughter date. Positive diagnostic control tissues from adult cattle (n = 6) experimentally infected with FMDV were also tested. All animals included were of varying age, gender and body weight.

The following tissue samples were collected from all the animals

Table 1
Custom probe design request information.

Target type	RNA
GenBank accession number	KJ144904.1
Target nucleotide sequence in FASTA format	>KJ144904.1 Foot-and-mouth disease virus – type SAT 2 isolate SAT2/KNP/51/93 P2/P3 polyprotein gene, partial cds Callahan 3D P TCCTTTGCACGCCGTGGGAC (4015–4034)
Species of target gene	Virus
Species of sample	Bovine (Bos taurus)
Sequence number	127532
Order number	NPR−0045649

included in this study: tip of the ear (left or right), eyelid (left or right), lip (top or bottom), oropharyngeal tissue, retropharyngeal lymph nodes (medial and lateral), palatine tonsil, lung, coronary band (all four limbs) and interdigital skin (all four limbs). Post collection, the samples were placed in 10 % buffered formalin and transported to the histopathology laboratory, Section of Pathology, Department of Paraclinical Sciences, Faculty of Veterinary Science (FVS), University of Pretoria, Pretoria, South Africa. Formalin fixed tissues were processed to formalin-fixed, paraffin-embedded (FFPE) blocks within 7 – 14 days post collection. The formalin-fixed, paraffin-embedded (FFPE) tissue samples were processed, embedded and sectioned according to the Department of Agriculture (DA) -accredited standard operating procedures. To avoid repeated trimming of blocks and, minimizing the loss of tissues for follow-up studies, all the blocks were cut into 4 µm thick sections until the blocks were too thin to cut any more sections. Cut sections were floated in warm water, picked up onto microscope slides, dried, and stored for 7 days to three months.

2.2. Analytical controls

The conditions for the assay were first optimised using FMDV positive and negative formalin-fixed paraffin-embedded control blocks prepared with the assistance of staff from TAD. A monolayer of baby hamster kidney (BHK)-21 cells (ATCC, Manassas, VA, USA) were grown in Glasgow's cell minimum essential medium (MEM) (Gibco™, ThermoFisher Scientific, Waltham, MA, USA) containing 5 % foetal bovine serum and 1× penicillin-streptomycin-glutamine antibiotic supplements in 75 cm^2 cell culture flasks (Corning™, Corning, New York, USA). Three positive control flasks were inoculated at the multiplicity of infection (MOI) ratios of 0.1, 1.0, and 2.5 with SAT 3 FMDV (KNP 10/90/3) (ARC-OVR, Pretoria, South Africa), whereas the negative control flask remained uninoculated. Cells were incubated at 37 °C and 5 % CO_2 for approximately 13 h. More prolonged incubation resulted in advanced cytopathic effect (CPE) and detachment of a large proportion of cells. At 13 h, CPEs corresponding with MOI were observed and recorded for all flasks. Cells were then trypsinised with trypsin-EDTA and harvested in RPMI medium containing 10 % foetal bovine serum (ThermoScientific™, ThermoFisher Scientific, Waltham, MA, USA). Harvested cells were suspended in 4 % paraformaldehyde and fixed by 15 min (min) incubation at room temperature (RT) before they were rewashed twice with phosphate-buffered saline (PBS) and suspended in prewarmed HistoGel™ (ThermoScientific™, Richard-Allan Scientific). The samples were kept at 4 °C overnight to allow the HistoGel to solidify. The fitness for use of the prepared FFPE cells as positive and negative controls was confirmed by triplicate RT-qPCR of cell block sections (Heid et al., 1996). Samples were then transferred to the FVS histopathology laboratory and paraffin embedded.

2.3. Probe design

The custom BaseScope™ design request (a 1ZZ probe named BA-V-FMDV-SAT2-pp-1zz-st targeting 3969–4014 of KJ144904.1) was based

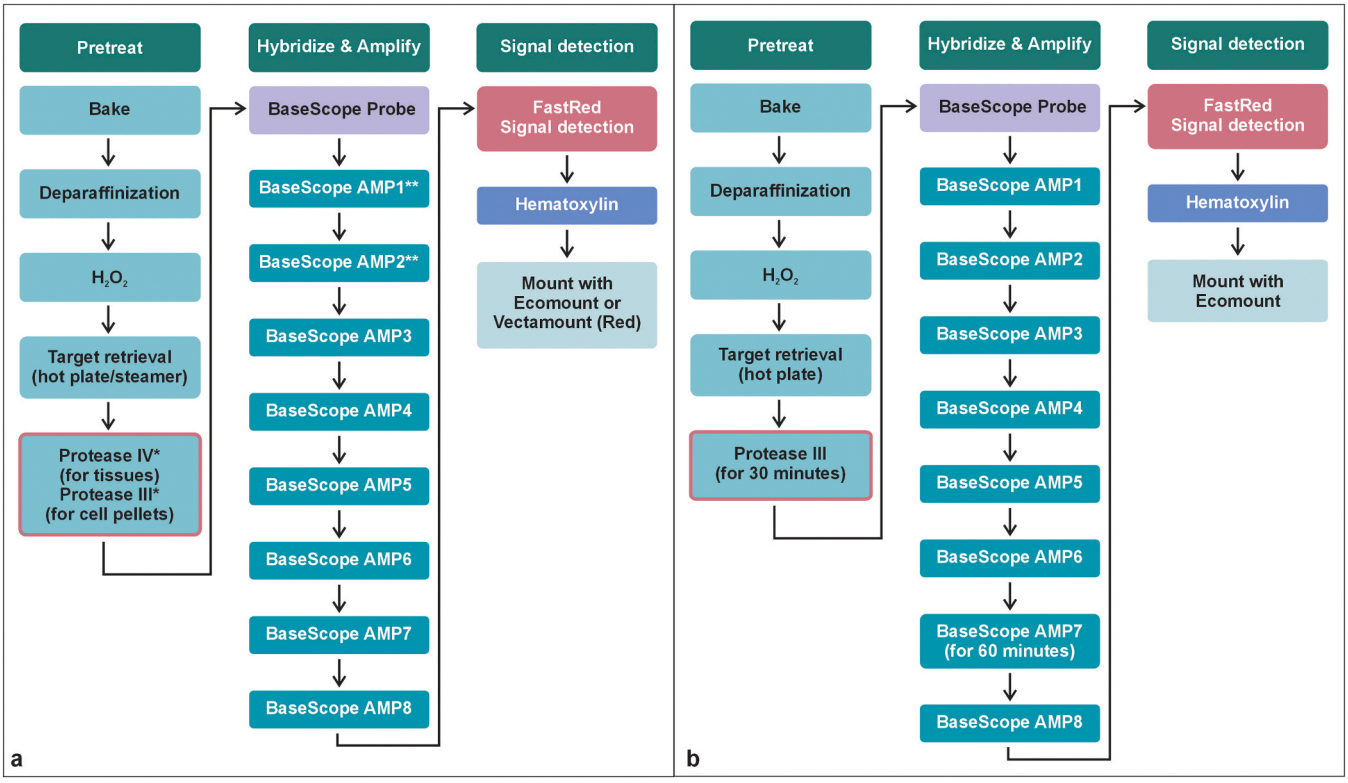

Fig. 2. The steps involved in the pretreatment, hybridisation, amplification and signal detection pathways of the BaseScope™ workflow where a) represents the original BaseScope™ workflow and b) the optimised workflow.

Table 2
The steps included in the prescribed BaseScope™ assay compared to the adjustment performed.

Specific step	Prescribed method	Alternate method
Duration of formalin fixation	Unknown	7–14 days
Cut and store cell and tissue sections	Unknown	7 days to 3 months
Pre-treatment	Incubate in provided hydrogen peroxide solution	In-house 25 % hydrogen peroxide
Pre-treatment	Incubate in provided hydrogen peroxide solution	In-house 3 % methanol-hydrogen peroxide
Target retrieval	Use a glass beaker and hot plate	Microwave
Target retrieval	Protease III for 15 min	Protease III for 30 min Protease Plus Protease IV In-house proteinase K
Hybridisation	HybEZ™ oven	In-house humidified oven
	AMP7 for 30 min	AMP7 for 60 min
Signal detection	FastRed A and B	NovaRED

Table 3
Outcome of the PCR and ISH performed on buffaloes and cattle.

Buffalo number	PCR result	ISH result
Experimental cases		
1	+	+
2	-	-
3	+	+
4	Inconclusive	+
5	+	+
6	-	-
7	+	+
8	+	+
9	+	+
10	+	+
11	+	+
12	+	+
13	+	+
14	+	+
15	+	+
Total positive cases	**12/15**	**13/15**
Negative cases		
1	-	-
2	-	-
3	-	-
4	-	-
5	-	-
6	-	-
7	-	-
8	-	-
9	-	-
10	-	-
11	-	-
12	-	-
13	-	-
14	-	-
15	-	-
Total positive cases	**0/15**	**0/15**
Positive cattle		
1	+	+
2	+	+
3	+	+
4	+	+
5	+	+
6	+	+
Total positive cases	**6/6**	**6/6**

on the parameters listed in Table 1.

2.4. In-situ hybridisation

A novel ISH assay (BaseScope™ Technology, Advanced Cell Diagnostics, Hayward, California, USA) was used . The Advanced Cell Diagnostics HybEZ II Hybridization System (supplied by Whitehead Scientific) was used with the BaseScope™ kit and custom probe. This system included an oven, humidity control tray, slide holder, wash tray and humidifying paper. The probe was applied to the tissue samples, hybridised overnight, and the signal was detected using light microscopy. Fig. 2 indicates the steps included in the BaseScope™ workflow. The BaseScope™ was performed in four rounds over eight to ten months.

2.5. ISH optimisation

The current study required stringent attention to optimisation due to the intricacies of the method itself, its application to both cell cultures (positive control) and tissue sections (test cases), as well as its application on a novel animal species, i.e., buffalo. Considering the prescribed steps, the complete ideal workflow summates to a minimum of 8 working hours, without any provision for unavoidable delays. Due to the stringent nature of the workflow, specific steps were adjusted to enable the processing of an increased number of slides per batch rather than to decrease the workflow duration. To limit the cost, specific reagents and solutions were substituted with readily available in-house solutions or local products (Table 2). These adjustments were performed on the positive cell culture and the negative control rather than the experimental tissue to minimise the consumption of consumables. During optimisation, a single variable was adjusted at any given time, lending validity to the outcome.

2.6. Pre-treatment and target retrieval

Slides were deparaffinised by incubating in xylene for 5 min at room temperature (RT) followed by 2 min in 100 % alcohol at RT and air dried for 5 min at 60 °C in an oven. Slides were incubated in the provided hydrogen peroxide solution *(*Alternative method)* for 10 min at RT.

**Alternative method: The provided hydrogen peroxide solution was replaced with an in-house 25 % hydrogen peroxide solution (diluted in distilled water). In addition, it was also replaced by in-house 3 % methanol-hydrogen peroxide solution.*

It was then washed in distilled water three to five times and then placed in the provided target retrieval reagent to boil for 30 min *(*Alternative method)* at 98 – 102 °C in a beaker on a hot plate *(**Alternative method)*. Using the beaker and hot plate instead of a commercial steamer was described as an alternative, acceptable variation in the original method. The slides were fully immerged in the solution and the beaker was covered with aluminium foil whilst boiling. The target retrieval reagent was prepared by diluting 70 ml of provided target retrieval solution with 630 ml distilled water.

**Alternative method: Slides were boiled in the target retrieval reagent for 15 min.*

***Alternative method: Target retrieval was attempted using a laboratory microwave oven (Whirlpool-Dual Crisp Technology) for standard immunohistochemical antigen retrieval. It was set to mimic the temperature and duration in the prescribed method, 15 min. at 100 °C.*

The slides were rinsed in distilled water, washed in 100 % alcohol for 3 min. and dried in oven at 40 °C for 5 min. The Immedge™ hydrophobic barrier pen created an area of interest and was allowed to dry at RT. This was followed by incubating the slides in the provided protease III *(*Alternative method)* at 40 °C in the HybEz™ oven and washed in distilled water three to five times.

**Alternative method: Slides were incubated in the provided protease IV solution, provided protease plus solution and an in-house proteinase K solution, respectively, as alternatives.*

2.7. Hybridisation

Slides were placed on the HybEZ™ slide rack, and each section was entirely covered with approximately four drops of the appropriate

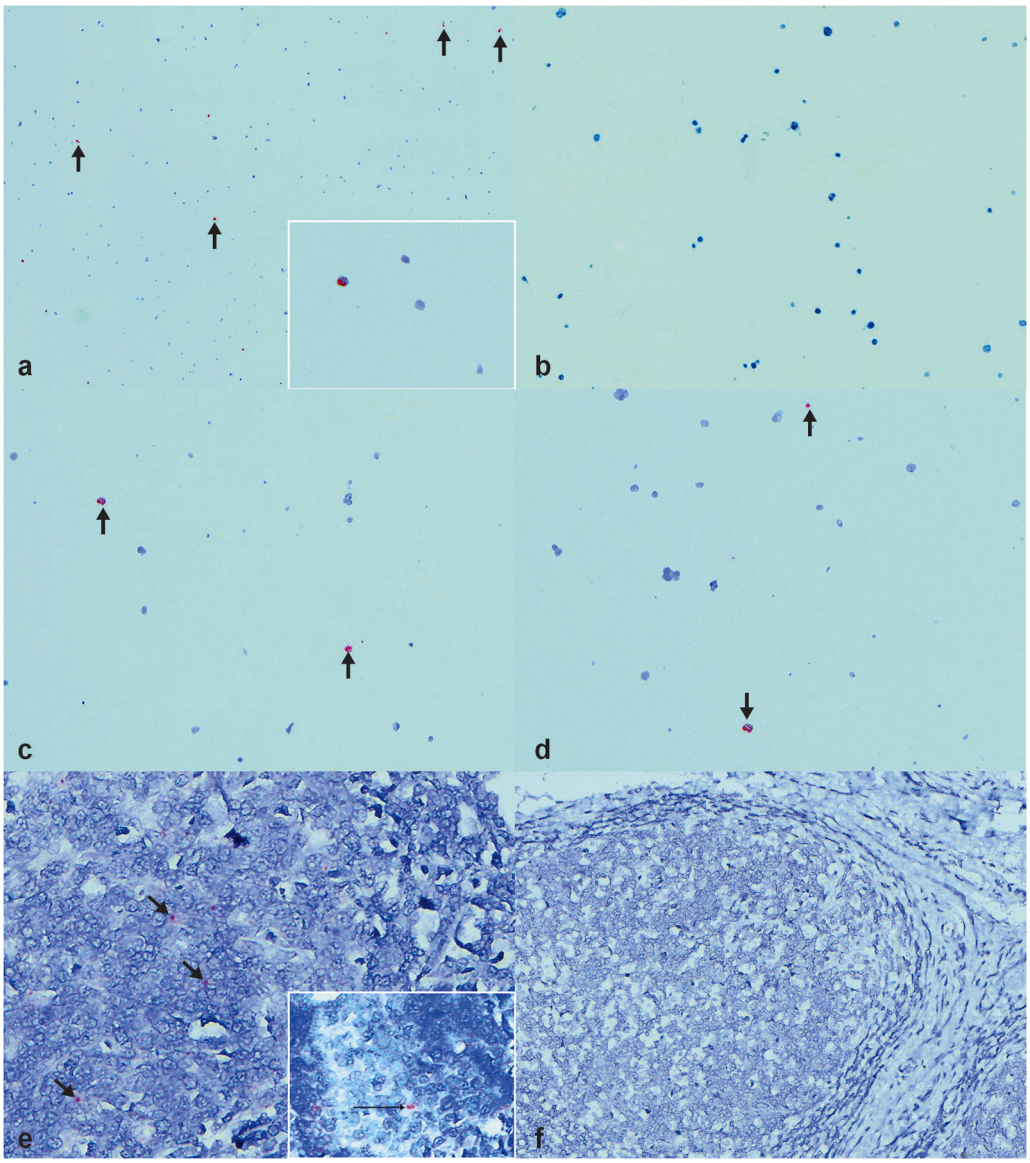

Fig. 3. Findings using RNA BaseScope™ in FMDV positive and negative cell culture controls as well as FMDV positive and negative carrier buffalo. a) Fine red granules (line arrows) in a positive cell culture section at various magnifications b) No staining in a negative cell culture section c) ISH-positive cell culture control stored for 7 days d) ISH-positive cell culture control stored for >7 days but <3 months e) Fine red granules in the cytoplasm of cells in PCR-positive carrier buffalo f) No staining in a PCR-negative buffalo.

probe. The slides were then incubated for 3 h at 40 °C by inserting the sealed tray containing the HybEZ™ slide rack into the HybEZ™ oven *(*Alternative method).*

**Alternative method: The hybridisation step in the HybEZ™ oven was replaced by placing the sample in a laboratory oven at the prescribed temperature with a tray of distilled water to mimic the required humidity.*

Following incubation, excess liquid was removed, and the slides were washed twice in wash buffer for 2 min. at RT by submerging the slides in a dish filled with wash buffer and occasionally agitating the dish. The wash buffer was included in the kit and was prepared by diluting it with distilled water to reach a 2 % solution. Reagents, AMP1 to 6, in the kit were sequentially hybridised to the sections in the oven at 40 °C, AMP1 for 30 min, AMP2 for 30 min, AMP3 for 15 min, AMP4 for 30 min, AMP5 for 30 min and AMP6 for 15 min. AMP7 and AMP 8 were hybridised to the sections at RT. AMP7 for 60 min. *(*Alternative method)* and AMP8 for 15 min. At every step, four drops from the ampoule were applied to the slides and the slides were incubated by inserting the sealed tray containing HybEZ™ slide rack into the HybEZ™ oven.

**Alternative method: AMP7 was hybdridised to the sections at RT for 30 min.*

Following each of the eight incubation steps, excess liquid was removed from the slides, and the slides were washed twice in wash buffer for 2 min. at RT.

2.8. Detection

Hybridising was followed by the detection step in which the sections were covered in RED solution *(*Alternative method),* incubated on the sealed tray containing the HybEZ™ Slide Rack for 10 min at 40 °C and washed three to five times in distilled water.

**Alternative method: The detection step was adapted by using in-house NovaRed as an alternative to the RED solution.*

The RED solution was prepared beforehand by mixing 1 vol of RED-B with 60 vol of RED-A. Sections were then counterstained with 50 % diluted Mayer's haematoxylin for 10 s then blued by rinsing with tap water for 30 s. Slides were dried in a 60 °C dry oven for 15 min, cooled

Table 4
The steps included in the prescribed workflow, its adjustment and their respective outcomes with the BaseScope™ assay.

Specific step	Prescribed method	Outcome	Alternate method	Outcome
Durantion of formalin fixation	-	-	7–14 days	Positive
Cut and store cell and tissue section	-	-	Between 7 days and 3 months	Positive
Pre-treatment	Incubate in provided hydrogen peroxide solution	Positive	In-house hydrogen peroxide	Negative
Pre-treatment	Incubate in provided hydrogen peroxide solution	Positive	In-house 3 % methanol-hydrogen peroxide	Negative
Target retrieval	Use a glass beaker and hot plate	Positive	Microwave	Negative
Target retrieval	Protease III for 15 min	Negative	Protease III for 30 min	Positive
			Protease Plus	Negative
			Protease IV	Negative
			In-house proteinase K	Negative
Hybridisation	HybEZ™ oven	Positive	In-house humidified oven	Negative
	AMP7 for 30 min	Negative	AMP7 for 60 min	Positive
Signal detection	FastRed A and B	Positive	NovaRED	Negative

for 5 min. at RT, briefly dipped in xylene, mounted using EcoMount before the xylene dried and covered with a coverslip. The slides were not dehydrated in alcohol since the RED substrate is alcohol sensitive.

2.9. Interpretation

Results were dichotomised as positive or negative for the presence of FMDV. A light microscope (Olympus BH-2) was used to determine the presence of labelling compared to the PCR results per case. Positive labelling consisted of the presence of bright pink punctate dots. For each tissue section at least 10 high power fields were evaluated for the presence of specific labelling. In addition, the stained sections were scanned with a Motic Easyscan Pro 6 Digital Slide Scanner. The digital slides were viewed using the Motic DSAssistant digital software to obtain digital images that represented specific labelling.

3. Results

Out of the 15 test buffalo, 12 were PCR positive for FMDV, two were negative, and one had an inconclusive PCR result (Table 3). The ISH results mirrored the PCR results except for buffalo number 4, which was also ISH positive despite the inconclusive PCR result. See Fig. 2 for a basic schematic of the optimised workflow applied.

All the cell and tissue samples fixed in formalin for 7–14 days were ISH-positive in PCR-positive animals. The sections (control and test) that were stored for >7 days but <3 months were ISH-positive in buffaloes that tested PCR-positive. In addition, there was no noticeable difference in the labelling intensity in sections that were stored for a week compared to those stored for months (Fig. 3).

The pre-treatment steps adjusted by replacing the provided hydrogen peroxide solution with an in-house hydrogen peroxide and 3 % methanol-hydrogen peroxide solution were negative. The use of the in-house laboratory microwave oven for target retrieval was unsuccessful. Target retrieval performed for 15 min. did not have a positive result, whereas 30 min. incubation did. The following variables were included instead of those prescribed for the target retrieval: protease IV (provided), protease plus (provided) and proteinase K (in-house), but none of these revealed positive results.

Replacing the hybridisation step with the in-house laboratory oven was unsuccessful. The extended AMP7 incubation time revealed superior positive results compared to the initial time of 60 min. Lastly, the signal detection step was unsuccessfully replaced by NovaRED solution (Table 4).

4. Discussion

Traditional ISH has been widely applied in the diagnostic and research field in humans and, to a lesser extent, in the veterinary field. However, it has a low sensitivity and specificity due to detecting highly expressed genes. Novel ISH advances include RNAscope™ and BaseScope™ technology, offering target-specific probe designs that result in highly specific staining due to their ability to minimise nonspecific off-target signals. (Neau et al., 2019) Although RNAscope™ and, to a lesser degree, BaseScope™ technology have been applied to various genes, tissues, and species, they have rarely been performed in wildlife and have not been used on African buffalo tissue. In addition, there is a single report where RNAscope ™ has been applied to detect FMD in carrier cattle. (Litz et al., 2024) We optimised a complete protocol for BaseScope™ staining for FMDV in African buffalo.

Formalin creates cross-links between proteins and nucleic acid sequences responsible for degrading the RNA and, in turn, influencing test sensitivity and specificity. (Bingham et al., 2017, Howat and Wilson, 2014, Grabinski et al., 2015) Research indicated this is often only experienced when tissues are stored in formalin for longer than 180 days. (Wu et al., 2022, Colburn et al., 2024) The nature of the current study design precluded formalin fixation of less than 32 h as prescribed. This was an unavoidable adjustment and constant across all the samples subject to testing. Sample collection was extended over a few days, then only transported from the KNP in the Limpopo province to the FVS in the Gauteng province. On arrival at the laboratory, the samples were electronically captured, cut and processed into FFPE blocks, further contributing to the delay. Despite the current study's extended formalin fixation period, it was revealed that a formalin fixation period of up to 14 days did not significantly influence the outcome of the test results.

According to the DA-accredited standard operating procedures of the histopathology laboratory of the FVS, control FFPE blocks may be sectioned in advance and stored ideally for no longer than 7 days. The BaseScope™ was performed in four rounds over eight to ten months. The rounds were based on the initial optimisation of the actual workflow (before placing a repeat order) as well as the liquidity of the project itself. Thus, the reason why it was possible to perform the BaseScope™ on control sections >7 days but <3 months. Based on our findings, it did not influence the positive outcome of the ISH results.

We determined that the epitope retrieval followed by protease incubation for 30 min. instead of 15 min. at 40 °C allowed optimum RNA detection. An AMP7 incubation time of 60 min. at RT revealed an increased signal intensity. In addition, none of the in-house reagents or equipment attempted as an alternative to the workflow revealed any significant positive results, deeming them incapable of acting as replacements. It also supports the fastidious nature of this specific diagnostic modality and the synergistic characteristics of a commercial assay.

Despite being cost prohibitive, time consuming and requiring specialised equipment, ISH has noteworthy advantages compared to other diagnostic modalities including PCR. It has the potential for increased test sensitivity especially considering recent advances like the RNAscope ™ and BaseScope™ technology. An important factor in the face of low viral loads for example in subclinical infections and/or carrier animals. It can be applied to formalin-fixed tissue, a valuable aspect where the

transport of fresh tissue is prohibited, regulated or limited.

The aim was to apply and optimise this novel assay in the veterinary field, particularly to wildlife tissues infected with FMDV. Although the successful outcome of this diagnostic modality revealed selected insights into the pathogenesis of this disease, specifically related to carrier animals, it was not within the scope of this study. It could serve as valuable information for future studies.

CRediT authorship contribution statement

Melvyn Quan: Writing – review & editing, Supervision, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Angelika Loots:** Writing – review & editing, Supervision, Methodology. **Lieza Odendaal:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization. **Alischa Henning:** Writing – original draft, Visualization, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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