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# Chemical inactivation of foot-and-mouth disease virus in bovine tongue epithelium for safe transport and downstream processing

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ABSTRACT

Epithelial tissue or vesicular fluid from an unruptured or recently ruptured vesicle is the sample of choice for confirmatory laboratory diagnosis of foot-and-mouth disease (FMD). However, in '*FMD-free*' countries the transport and downstream processing of such samples from potentially infected animals present a biosafety risk, particularly during heightened surveillance, potentially involving decentralised testing in laboratories without adequate biocontainment facilities. In such circumstances, rapid inactivation of virus, if present, prior to transport becomes a necessity, while still maintaining the integrity of diagnostic analytes. Tongue epithelium collected from cattle infected with FMD virus (FMDV) of serotype O (O/ALG/3/2014 – Lineage O/ME-SA/Ind-2001d) or A (A/IRN/22/2015 – Lineage A/ASIA/G-VII) was incubated in the PAXGene Tissue System Fixative (pH 4) and Stabiliser (pH 6.5) components respectively, in McIlvaine's citrate-phosphate buffer (pH 2.6) or in phosphate-buffered saline (PBS, pH 7.4) at room temperature for 2, 6, 24 or 48 h. Following incubation, tissues were homogenised and tested by virus isolation and titration using LFBK$_{\alpha V\beta 6}$ cells. The integrity of FMD viral RNA was assessed by RT-qPCR (3D$^{pol}$ coding region), Sanger sequencing of the VP1 region and transfection of LFBK$_{\alpha V\beta 6}$ cells to recover infectious virus. Viable virus could be recovered from samples incubated in PBS for at least 48 h. The PAXgene Tissue System Stabiliser component yielded variable results dependent on virus serotype, requiring at least 6 h of incubation to inactivate A/IRN/22/2015 in most samples, whereas the Fixative component required up to 2 h in some samples. McIlvaine's citrate-phosphate buffer rapidly inactivated both viruses within 2 h of incubation. There was no demonstrable degradation of FMD viral RNA resulting from incubation in any of the buffers for up to 48 h, as assessed by RT-qPCR, and 24 h by sequencing and transfection to recover infectious virus. McIlvaine's citrate-phosphate buffer (pH 2.6) is easy to prepare, inexpensive and inactivates serotype A and O FMDV in epithelial tissue within 2 h, while maintaining RNA integrity for downstream diagnostic processes and virus characterisation.

## 1. Introduction

Outbreaks of foot-and-mouth disease (FMD), a major livestock disease affecting cloven-hoofed animals such as sheep, cattle, goats and pigs, in FMD-free countries have significant socio-economic implications due to direct (reduced production, mortality in young stock) and indirect losses (control costs and restricted trade in meat and other livestock products). The annual impact of FMD in terms of production losses and control effort costs in endemic regions has been estimated to be between US$6.5 and 21 billion, while an outbreak in an FMD-free region is estimated to result in losses exceeding US$1.5 billion (Knight-Jones and Rushton, 2013). Modelling of a large multi-state FMD outbreak in Australia estimated revenue losses between AU$49.3 and 51.8 billion over 10 years, in addition to control costs between AU$60 and 373 million. Smaller, state-contained outbreaks are estimated to result in revenue losses between AU$5.6 and 6.2 billion over 10 years (Buetre et al., 2013).

FMD is caused by FMD virus (FMDV), a member of the *Aphthovirus* genus in the *Picornaviridae* family and consists of six currently circulating serotypes with distinct immunological characteristics, and many

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strains within each serotype (OIE, 2021). FMDV is quickly inactivated at acidic or basic pH (Bachrach et al., 1957), however mutations in the viral genome can lead to increased capsid stability to pH or other environmental parameters (Mateu, 2010).

The initial diagnosis of FMD cases in '*FMD-free*' countries will be handled by centralised high-containment national/reference laboratories, where available. However, once an outbreak is declared, in order to avoid overwhelming the central laboratories, heightened diagnostic surveillance in potentially affected areas would result in state or regional laboratories within the country performing the FMD virus exclusion on samples. Testing in these secondary laboratories would include only methods that do not include virus culture, i.e. detection of nucleic acid by real-time reverse transcription-polymerase chain reaction (RT-qPCR). Epithelial tissue or vesicular fluid from an unruptured or recently ruptured vesicle is the sample of choice for FMD laboratory diagnosis (OIE, 2021). For samples to be submitted to reference laboratories with adequate biological containment (BSL3-Ag), epithelial samples should be submitted in transport medium, tissue culture medium or PBS (OIE, 2021) to maintain virus viability. However, samples submitted in these buffers would be unsuitable for submission to state/regional laboratories without the required biocontainment, since any positive sample might result in that laboratory being deemed contaminated, adding more pressure to the control effort. This necessitates the implementation of additional biosafety precautions to keep these laboratories operational (FAO, 2013). Chemical treatment of samples at the site of collection, to enable rapid virus inactivation while maintaining sample integrity, presents a feasible measure to prevent contamination of state/regional laboratories during FMD outbreaks in FMD-free countries and can be considered in endemic countries that lack high containment level laboratories.

We previously evaluated the ability of commercially available nucleic acid preservation buffers, DNA/RNA Shield (Zymo Research, CA, USA) and RNAlater (Thermo Fisher Scientific, MA, USA), to inactivate FMDV in epithelial tissue (Horsington et al., 2020). Although promising, these buffers required at least 24 h of incubation for inactivation which would not render potentially infectious samples safe during transportation and upon first arrival at state/regional laboratories. This timeframe also does not suit an emergency response where exclusion testing needs to be performed as rapidly as possible. The objectives of the present study were to test the ability of additional buffers, either commercially available or prepared in-house, to inactivate FMDV in freshly collected epithelial samples and assess the ability of these to preserve RNA integrity to allow downstream molecular testing and virus characterisation.

The animal work and the laboratory assays were performed at the BSL4vet containment facility of the Friedrich-Loeffler-Institut in Riems (FLI), Germany. All the protocols for experimentation with live cattle were approved by the Australian Centre for Disease Preparedness Animal Ethics Committee (AEC 1979) and the State Office of Agriculture, Food Safety and Fisheries Mecklenburg-Vorpommern, Germany (file no. LALLF M-V 7221.3-2-82 026/17).

Eight cattle of between 5 and 9 months of age were obtained from a commercial livestock producer in Germany and were housed in the BSL4vet animal facility at FLI to acclimatise for 7 days before the commencement of the experiment. Clarified homogenates of vesicular material from cattle previously experimentally infected with FMDV A/IRN/22/2015 (Lineage A/ASIA/G-VII) and FMDV O/ALG/3/2014 (Lineage O/ME-SA/Ind-2001d) were provided by Wageningen Bioveterinary Research, Lelystad, The Netherlands. Titres were adjusted to $10^7$ PFU/ml by dilution in Dulbecco's modified Eagle medium (DMEM) containing 5% foetal bovine serum (FBS) and antibiotics (penicillin, streptomycin, gentamicin and amphotericin B). Each animal was deeply sedated using xylazine (0.3 mg/kg body mass administered intramuscularly) and then inoculated into the epithelium of the tongue with 0.1 ml of cattle-derived virus (either FMDV O/ALG/3/2014 or FMDV A/IRN/22/2015; $1 \times 10^6$ PFU/ml – four cattle for each strain) each at six sites. Pre-emptive pain relief before infection was provided as per the protocol (meloxicam, 0.5 mg/kg, intravenously). Cattle were examined daily for development of vesicles at the site of inoculation and at two days post inoculation, vesicular lesions were considered sufficient for collection and in line with ethics requirements. The cattle were humanely slaughtered as per approved protocols and epithelial flaps from the tongues collected into tubes containing Dulbecco's modified Eagle medium (DMEM) containing 5% FBS and antibiotics (penicillin, streptomycin, gentamicin and amphotericin B).

The epithelial samples were sterilely cut into pieces approximately 20–25 $mm^2$ using a scalpel and placed in pre-marked tubes kept on ice. Inactivation experiments were initiated on the day of sample collection. A total of 32 epithelial pieces were prepared per animal, resulting in 128 samples each for A/IRN/22/2015 and O/ALG/3/2014. This was sufficient for 8 samples per time point (n = 4) per buffer (n = 4), per virus. Of the 8 biological replicate samples per time point, 4 were immersed in each buffer immediately (1 ml per tube), whereas the other 4 were first homogenised manually for 30 s using a plastic micro pestle (Eppendorf, Hamburg, Germany), and then fully immersed in each buffer (1 ml). The buffers used were the PAXgene Tissue System (PreAnalytiX, Qiagen/BD, Switzerland) Fixative (pH 4) and Stabiliser (pH 6.5) components respectively, McIlvaine's citrate-phosphate buffer (pH 2.6) and phosphate-buffered saline (PBS, pH 7.4). The proprietary PAXgene Fixative is composed of methanol (50 – 70% w/w), acetic acid (10 – 20% w/w) and polyethylene glycol (1–10% w/w) according to the MSDS. The PAXgene Stabiliser composition is not given by the manufacturer but it has a stated pH of 6.5. McIlvaine's buffer was prepared as follows: 21.8 ml of disodium phosphate ($Na_2HPO_4$) stock solution (0.2 M) and 178.2 ml of citric acid ($C_6H_8O_7$) stock solution (0.1 M) were mixed to prepare a 200 ml volume (pH 2.6) and filtered through a 0.2 μm filter to sterilise. The samples were incubated at room temperature (22 ± 2 °C) for 2, 6, 24 or 48 h. Following incubation, tubes were centrifuged briefly (2000*g*, 2 min) to collect the tissue at the bottom, and the inactivation buffer was removed by pipette. Fresh PBS (1 ml) was added to each tube to wash out excess inactivation buffer, the tubes were centrifuged briefly again, and the PBS was removed by pipette and discarded. Another 1 ml of fresh PBS was added to each tube, along with a 5 mm sterile stainless-steel ball and the samples were homogenised at 30 Hz for 3 min using a Qiagen Tissuelyser II (Qiagen GmbH, Germany). After homogenisation, the tubes were centrifuged for 3 min at 16,000 × *g* and the supernatant was collected for further processing and testing, or storage at − 80°C.

Homogenates were tested for the presence of infectious FMDV by inoculating monolayers of αVβ6-expressing porcine kidney cells ($LFBK_{\alpha V\beta 6}$ cells, LaRocco et al., 2013; LaRocco et al., 2015), as described previously (Dill and Eschbaumer, 2019; Horsington et al., 2020). Briefly, monolayers of $LFBK_{\alpha V\beta 6}$ cells grown in 24-well cell culture trays were washed with PBS, inoculated with 100 μl sample homogenate in 200 μl serum-free DMEM, and incubated for 1 h at 37 °C. The cells were then washed with PBS and overlaid with 1 ml DMEM containing 5% FBS and antibiotics and incubated at 37 °C with 5% $CO_2$. The monolayers were examined daily for cytopathic effect (CPE) up to 72 h after inoculation. If no CPE was observed, cells and supernatant were frozen/thawed, clarified and inoculated onto fresh $LFBK_{\alpha V\beta 6}$ monolayers for a second passage. The presence or absence of FMDV was confirmed using a standard double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) (Roeder and Le Blanc Smith, 1987) with polyclonal antibodies specific for serotypes O or A. Virus titrations were performed on all positive samples using the homogenates that had been stored at − 80 °C (and thawed once). Homogenates were 10-fold serially diluted in serum-free DMEM and virus titres were determined by adding serial dilutions to 96-well microtitre plates and adding $LFBK_{\alpha V\beta 6}$ cells in suspension to all the wells. Virus titres were calculated as $TCID_{50}$/ml (Kärber, 1931).

Manual homogenisation of tissue before immersion in buffer did not have any detectable effect on sample inactivation compared to immersion without manual homogenisation (results not shown). Therefore the

**Table 1**
Inactivation of A/IRN/22/2015 and O/ALG/3/2014 viruses as assessed by virus isolation in 24-well culture plates, showing the number of wells positive. All virus isolation results were confirmed by antigen capture ELISA on culture supernatants. Values in brackets represent the median ± standard deviation of $log_{10}$ $TCID_{50}$/ml titres where VI positive samples were titrated.

| Buffer | Inactivation period | A/IRN/22/2015 | O ALG/3/2104 |
|---|---|---|---|
| **PAXgene Fixative** | **2** | 1/8 (*LDL) | 0/8 |
| | **6** | 0/8 | 0/8 |
| | **24** | 0/8 | 0/8 |
| | **48** | 0/8 | 0/8 |
| **PAXgene Stabiliser** | **2** | 8/8 (2.5 ± 0.6) | 2/8 (*LDL) |
| | **6** | 8/8 (3.1 ± 0.9) | 0/8 |
| | **24** | 4/8 (1.0 ± 1.3) | 0/8 |
| | **48** | 1/8 (*LDL) | 0/8 |
| **McIlvaine's buffer (pH 2.6)** | **2** | 0/8 | 0/8 |
| | **6** | 0/8 | 0/8 |
| | **24** | 0/8 | 0/8 |
| | **48** | 0/8 | 0/8 |
| **PBS (pH 7.4)** | **2** | 8/8 (2.9 ± 0.9) | 8/8 (2.5 ± 1.1) |
| | **6** | 8/8 (2.9 ± 1.0) | 8/8 (3.8 ± 1.0) |
| | **24** | 8/8 (3.5 ± 1.1) | 8/8 (3.0 ± 1.0) |
| | **48** | 8/8 (2.5 ± 1.2) | 8/8 (1.8 ± 1.0) |

*LDL = virus concentration below the lower detection limit of the virus titration ($log_{10}$ 1.75 $TCID_{50}$/ml).

4 manually homogenised replicate samples from each respective time point and buffer treatment, and the 4 matching replicate samples that were immersed in buffer immediately, were combined into a single group each (thus resulting in 8 replicates per time point per buffer). PAXgene Tissue System Fixative was effective at inactivating O/ALG/3/2014 in all 8 replicates with 2 h of incubation; one replicate of A/IRN/22/2015 after 2 h incubation was positive by virus isolation, but the titre was below the limit of detection of the titration assay (Table 1; Fig. 1A-B). The PAXgene Tissue System Stabiliser was unable to completely inactivate A/IRN/22/2015 in all replicates after up to 48 h of incubation, although an inactivation effect was noted from 24 h onwards as demonstrated by a drop in median titre (Fig. 1A). Conversely, the Stabiliser was able to inactivate O/ALG/3/2014 in 6 of 8 replicates after 2 h incubation and resulted in reduction of virus titre in the two remaining positive replicates below the limit of detection of the virus titration. McIlvaine's citrate-phosphate buffer was effective in completely inactivating virus in all replicates after 2 h of incubation. None of the buffers had any residual toxic effect on cells during virus isolation or virus titration.

The amount of viral RNA in each homogenate was quantified by RT-qPCR as described before (Horsington et al., 2020). Briefly, the viral RNA was extracted from 100 µl of sample homogenate with the NucleoMag VET kit (Macherey-Nagel, Düren, Germany) on a KingFisher Flex magnetic particle processor (Thermo Fisher Scientific, MA, USA). One-step RT-qPCR was performed using primers and a TaqMan probe targeting the 3D coding region of FMDV (Callahan et al., 2002; Rasmussen et al., 2003) using AgPath-ID One-Step RT-PCR reagents (Thermo Fisher Scientific, MA, USA). Reactions were performed on a CFX96 Real-Time PCR Detection System (Bio-Rad, CA, USA). All samples were tested in duplicate. Negative control samples were included at

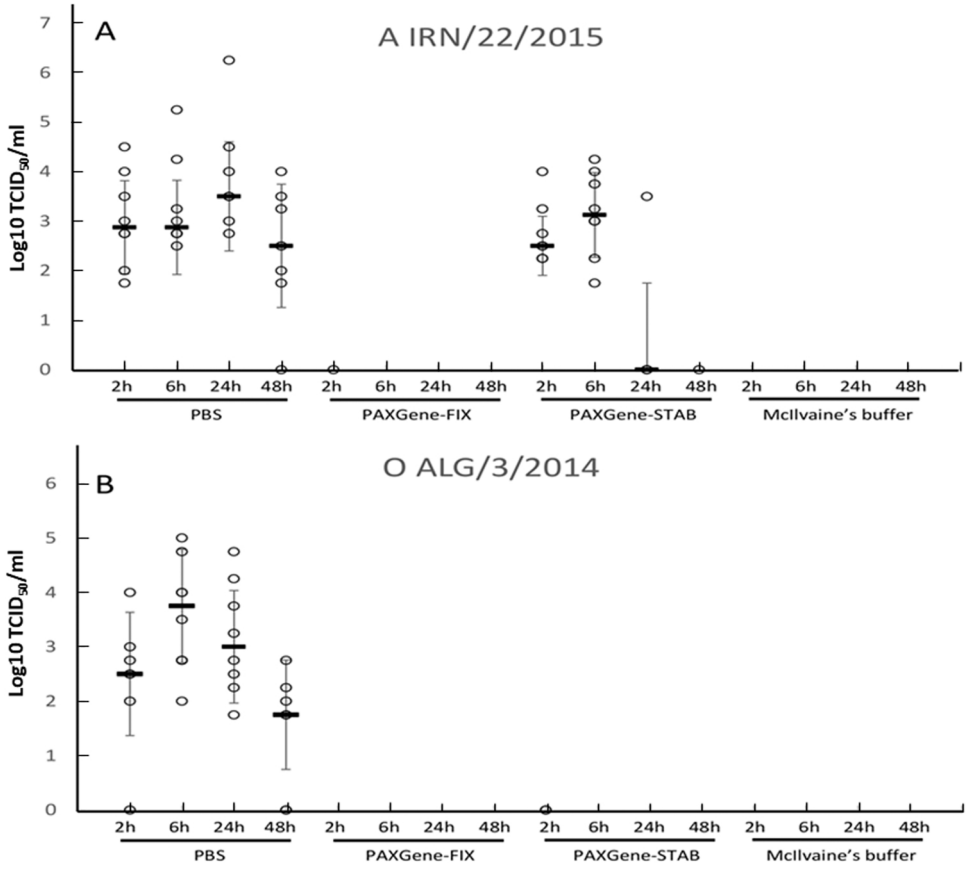


**Fig. 1.** Individual and median virus titres after incubation in buffers over time, as measured by virus titration. (A) A/IRN/22/2015 and (B) O/ALG/3/2014. Data points with a y-axis value of "0" represent samples that are VI positive but below the lower detection limit of the titration assay and are included only for visualisation. Where no datapoints are visible, this indicates that these samples were VI negative and therefore not subjected to virus titration.

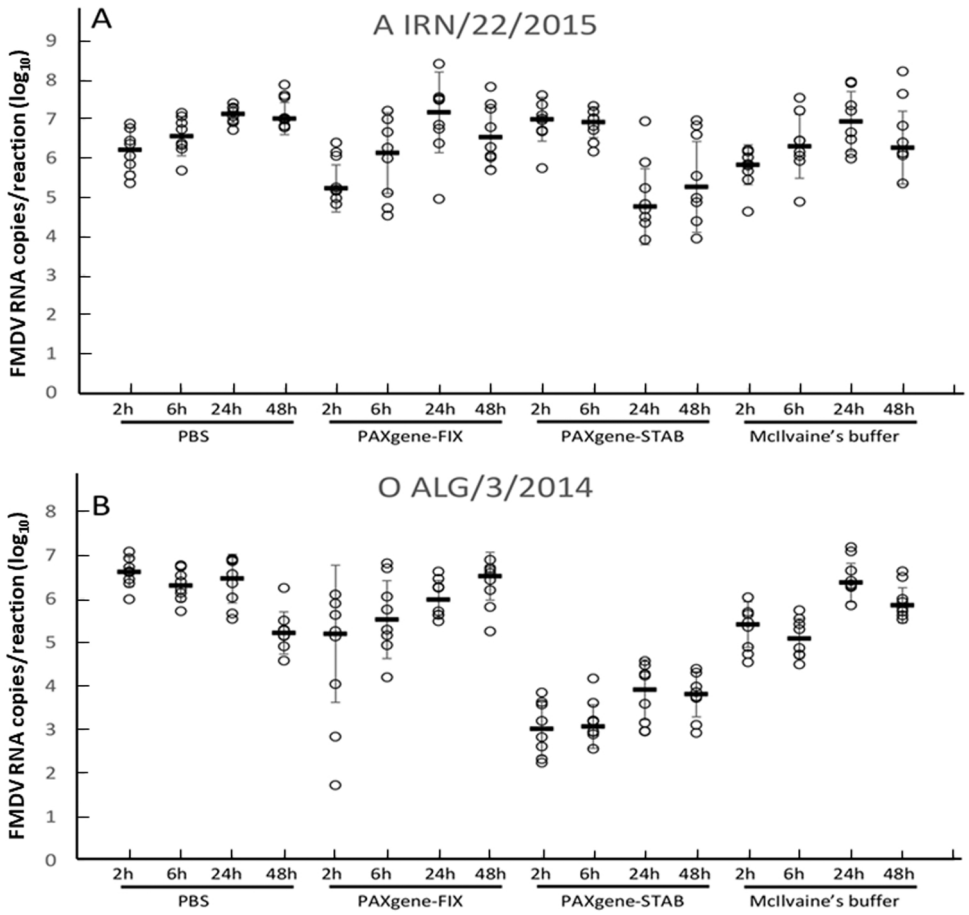


**Fig. 2.** Individual sample and mean FMDV RNA copy numbers after incubation in buffers over time, as measured by RT-qPCR. (A) A/IRN/22/2015 and (B) O/ALG/3/2014.

both the extraction and RT-qPCR steps. Heterologous spike-in RNA was used as an internal control (Hoffmann et al., 2006). Viral RNA was quantified using a standard curve derived from 10-fold serial dilutions of RNA of known concentration, from $10^7$ to $10^0$ copies/reaction.

Apart from an apparent reduction in viral RNA in O/ALG/3/2014 samples treated with PAXgene Tissue System Stabiliser at all time points compared to all other samples, there was no drastic effect on RT-qPCR RNA detection following incubation with the different buffers, up to at least 48 h (Fig. 2).

Sequencing of the VP1 capsid protein coding region (1D) of FMDV is widely used for determination of serotype, lineage and strain which aids in outbreak characterisation and vaccine strain selection (Dill and Eschbaumer, 2019; Horsington et al., 2020). RNA specimens extracted from the 24-h incubation samples (n = 64) were tested as follows. The VP1-coding region was amplified using primers FMD-3161-F and FMD-4303-R (Dill et al., 2017) with the Superscript III One-Step kit (ThermoFisher Scientific). The PCR products were analysed by agarose gel electrophoresis and a band of the expected size (~1.1 kb) was excised from the gel and purified with the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Sequencing reactions were set up using the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Darmstadt, Germany) for two representative samples of each treatment buffer and for each virus (n = 16). Consensus sequences from forward and reverse reads were aligned with the genome of the inoculum viruses and found to be identical (results not shown). This complements the RT-qPCR results in showing that viral RNA is sufficiently preserved to allow sequencing of the 1D region for epidemiological investigations following incubation in the buffers tested here for at least 24 h.

Transfection of extracted FMDV RNA was performed as described previously (Horsington et al., 2020) using Lipofectamine 3000 (Invitrogen), as per the manufacturer's instructions. The same 24-h RNA samples subjected to Sanger sequencing were utilised for transfection (two representative samples of each treatment buffer and for each virus, n = 16); a duplicate transfection (technical replicate) was performed for each sample. Briefly, for each sample two 1.5 ml microtubes containing 50 µl serum-free DMEM and 2 µl Lipofectamine 3000, and two 1.5 ml microtubes containing 50 µl serum-free DMEM, 1 µl P3000 and 6 µl RNA were prepared. The RNA/P3000 mixtures were added dropwise to the lipofectamine mixtures and incubated at RT for 10 min. Monolayers of $LFBK_{\alpha V\beta 6}$ cells (80–90% confluent) in 24-well cell culture trays were washed with PBS and 300 µl serum-free DMEM was added to each well. The RNA-lipofectamine mixtures were added dropwise to the wells and the plates were incubated for 15 min at 37°C with 5% $CO_2$. Following this, 500 µl DMEM with 5% FBS was added to each well and the plates were incubated for 24–48 h at 37°C with 5% $CO_2$. A further passage of the supernatants from all wells was performed using fresh monolayers of $LFBK_{\alpha V\beta 6}$ cells (80–90% confluent) in 24-well cell culture trays, to confirm virus growth in transfected wells showing CPE, or to potentially amplify virus from wells without apparent CPE. Cells of initial transfections and the further passages were observed for CPE and supernatants from all wells were tested by FMDV antigen ELISA to confirm virus rescue. Control wells inoculated with RNA with no lipofectamine and lipofectamine with no RNA were included in each plate in duplicate.

Recovery of virus following transfection and one passage was achieved in at least one technical replicate of all test samples (Table 2). Recovery of virus following transfection and one passage was confirmed by antigen capture ELISA. This further complements the RT-qPCR and sequencing results in demonstrating that full-length viral genomic RNA

**Table 2**
Transfection of RNA extracted from samples incubated for 24 h in respective buffers.

| A/IRN/22/2015 | | | | Initial transfection | | Further passage once in LFBK$_{\alpha v\beta 6}$ cells | |
|---|---|---|---|---|---|---|---|
| **Buffer** | **BR*** | **TR*** | **log$_{10}$ RNA copies/reaction** | **CPE** | **Antigen ELISA** | **CPE** | **Antigen ELISA** |
| PBS | 1 | 1 | 6.92 | + | + | + | + |
| PBS | 1 | 2 | | + | – | + | + |
| PBS | 2 | 1 | 7.30 | + | + | + | + |
| PBS | 2 | 2 | | + | + | + | + |
| PAXgene Fixative | 1 | 1 | 4.96 | + | – | + | + |
| PAXgene Fixative | 1 | 2 | | – | – | – | – |
| PAXgene Fixative | 2 | 1 | 7.52 | + | + | + | + |
| PAXgene Fixative | 2 | 2 | | + | + | + | + |
| PAXgene Stabiliser | 1 | 1 | 3.91 | – | – | + | + |
| PAXgene Stabiliser | 1 | 2 | | – | – | – | – |
| PAXgene Stabiliser | 2 | 1 | 4.35 | + | – | + | + |
| PAXgene Stabiliser | 2 | 2 | | – | – | + | + |
| McIlvaine's buffer | 1 | 1 | 6.66 | – | – | + | + |
| McIlvaine's buffer | 1 | 2 | | + | + | + | + |
| McIlvaine's buffer | 2 | 1 | 7.96 | + | + | + | + |
| McIlvaine's buffer | 2 | 2 | | + | + | + | + |
| **O/ALG/3/2014** | | | | | | | |
| PBS | 1 | 1 | 6.92 | + | + | + | + |
| PBS | 1 | 2 | | + | + | + | + |
| PBS | 2 | 1 | 6.90 | + | + | + | + |
| PBS | 2 | 2 | | + | + | + | + |
| PAXgene Fixative | 1 | 1 | 6.27 | + | + | + | + |
| PAXgene Fixative | 1 | 2 | | + | + | + | + |
| PAXgene Fixative | 2 | 1 | 5.64 | + | + | + | + |
| PAXgene Fixative | 2 | 2 | | + | + | + | + |
| PAXgene Stabiliser | 1 | 1 | 3.59 | + | + | + | + |
| PAXgene Stabiliser | 1 | 2 | | – | – | + | + |
| PAXgene Stabiliser | 2 | 1 | 4.57 | + | – | + | + |
| PAXgene Stabiliser | 2 | 2 | | + | + | + | + |
| McIlvaine's buffer | 1 | 1 | 7.09 | + | + | + | + |
| McIlvaine's buffer | 1 | 2 | | + | + | + | + |
| McIlvaine's buffer | 2 | 1 | 6.33 | + | + | + | + |
| McIlvaine's buffer | 2 | 2 | | + | + | + | + |

*BR = biological replicate; TR = technical replicate; CPE = Cytopathic effect; + = Positive outcome and - = negative outcome.

is adequately preserved after at least 24 h incubation in all buffers tested. The variable virus recovery from some samples, where transfection was only successful for one of two technical replicates, is likely due to lower RNA concentration in those samples caused by the inherent variability of virus concentration in biological samples. Samples with unsuccessful transfections and/or no CPE after passage had RNA concentrations around 2.0 log$_{10}$ lower than other samples (Table 2).

## 2. Discussion

In FMD-free countries, rapid on-site inactivation of FMDV in epithelial samples collected from suspect lesions will greatly enhance biosafety and biosecurity of laboratories that would be performing diagnostic tests on samples received during an outbreak or surveillance to establish freedom from disease. It will ensure safe transport, but more importantly safe handling of samples in laboratories that do not have biocontainment at an adequate level, without the risk of contamination. This safeguard is also important for endemic countries that may not have high-containment facilities, to lower any risks from potential virus escape. However, adequate sample preservation, particularly of the viral nucleic acid, is paramount to ensure accurate laboratory diagnosis and allow virus sequence characterisation for epidemiological investigations. In addition, the ability to successfully recover infectious virus following transfection once samples are forwarded to high-containment reference laboratories will greatly assist in additional virus characterisation and vaccine strain matching.

Previous studies focused on inactivation of different viruses in fluid matrices indicating the suitability of viral lysis buffers to inactivate FMD virus (Wood et al., 2020), Ebola virus (Alfson and Griffiths, 2018; Haddock et al., 2016), avian influenza virus (De Benedictis et al., 2007) and rabies virus (Wu et al., 2017) in samples such as cell culture supernatant, epithelial tissue suspension and milk samples. However, Ngo et al. (2017) observed that the inactivation of viruses by commonly used lysis buffers was unreliable across different viral families. In addition, the ability of the lysis buffers to penetrate tissues in a short period will be limited and impacted by factors such as temperature, freeze thawing cycles, size of sample, to name a few. To our knowledge this is the first study to show the effects of different inactivation buffers on FMD virus inactivation in tongue epithelium samples.

Sufficient penetration of inactivating agents into submerged tissue pieces is a concern for complete inactivation. We found that manual homogenisation of infected epithelial tissue, before incubation in the buffers tested here, did not have any noticeable effect on the ability of the buffers to inactivate the two viruses in the study. However, such an approach might be advantageous when using different inactivation preservation buffers, and when using larger pieces of tissue, but this needs to be further investigated.

The PAXgene Tissue System is a formalin-free reagent set that consists of two parts, the Fixative and Stabiliser, and is meant to be used as a two-step system to preserve and stabilise tissue morphology and nucleic acids. The manufacturer recommends fixation of tissue samples for at least 2 h, followed by transfer to the Stabiliser component for laboratory submission. However, such a two-step process would be cumbersome in the field where epithelial samples are likely to be collected under time pressure, and where multiple handling steps will increase risk of contamination. We therefore assessed the efficacy of the individual components of the system for virus inactivation and nucleic acid preservation. The Fixative component was effective in virus inactivation after as little as 2 h incubation, with only a single A/IRN/22/2015 sample not being fully inactivated at this earliest time point, but all O ALG/3/2104 samples being inactivated. The Fixative was also able to adequately preserve FMDV RNA for up to 48 h of incubation at room

temperature as demonstrated by RT-qPCR (and up to 24 h as assessed by sequencing and transfection), despite the samples not being transferred to Stabiliser as intended by the manufacturer. The Fixative component contains methanol, acetic acid and polyethylene glycol at pH 4, with the acid component likely responsible for virus inactivation, and methanol and PEG important for fixation of tissue structures (Warmington et al., 2000). It is unknown how incubation longer than 48 h would affect RNA quality.

Contrary to the effect of the Fixative, the PAXgene Tissue System Stabiliser component yielded variable results in terms of virus inactivation. This is not surprising, considering that this component is advertised to only stabilise biomolecules, and the manufacturer does not claim any fixation or virus inactivation properties. However, the Stabiliser was still able to inactivate virus in all but two O ALG/3/2104 samples following 2 or more hours of incubation. Interestingly the Stabiliser was not very effective for inactivation of A/IRN/22/2015 in epithelial tissue, with partial virus inactivation only occurring after at least 24 h of incubation at room temperature. The Stabiliser component also resulted in an apparent reduction in detectable RNA concentration. It is unclear, however, if this was due to lower viral concentration in the epithelium pieces used, or indicative of an incompatibility of the Stabiliser with the downstream RNA extraction protocol. Individual pieces of epithelial tissue can contain variable amounts of virus. Accordingly, the lower detected RNA concentration in O/ALG/3/2014 PAXgene stabiliser samples could be due to lower amount of virus in the epithelium pieces allocated to this treatment group. However, this is unlikely based on the equal allocation of epithelium pieces from each experimental animal to each treatment group. The lower concentrations might indicate incompatibility of the PAXgene Stabiliser component with the downstream RNA extraction process. Indeed, the manufacturer recommends a compatible PAXgene Tissue RNA kit that involves homogenisation of the fixed and stabilised tissue in a specific binding buffer. Regardless, the Stabiliser component would not be recommended for inactivation of FMD virus in epithelium tissues, due to poor inactivation and apparent poorer RNA recovery compared to other buffers tested.

McIlvaine's citrate-phosphate buffer (pH 2.6) was effective at inactivating both viruses within 2 h in small pieces of epithelial tissue (20–25 $mm^3$). It is probable that the time required could vary with bigger samples and with varying virus loads and needs further investigation. In addition, viral nucleic acid was adequately preserved to allow detection by RT-qPCR after up to 48 h of incubation, as well as successful VP1 sequencing and virus rescue by transfection after 24 h of incubation. The buffer is cheap and easy to produce and does not present a health risk to operators when preparing or handling it. The buffering component should also render it stable for storage by users in between farm visits. McIlvaine's citrate-phosphate buffer might therefore present an ideal option for collection of epithelial tissues on-farm during FMD outbreaks.

## 3. Conclusion

The data presented here improve upon our earlier study where RNAlater and RNA/DNA Shield were shown to require more extended period of incubation to achieve adequate virus inactivation (Horsington et al., 2020). As shown in this study with a limited number of isolates, inactivation might be dependent on virus strain, most likely impacted by virus capsid stability (Mateu, 2017), and therefore it might be advisable to expand the evaluation of McIlvaine's citrate-phosphate buffer to include a wider range of FMD viruses from different serotypes, in particular those that are currently circulating and presenting a risk of introduction into FMD-free regions.

## Declaration of Competing Interest

The authors have no competing interests to declare.

## Data availability

Data will be made available on request.

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