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Simple and reliable diagnostics of viral animal pathogens

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CONTENTS

CHAPTER I: INTRODUCTION

I. INTRODUCTION

In 2002, Earth Overshoot Day fell on September 19. As it is steadily and inexorably moving towards the beginning of the year, it was August 4 in 2021 and this year it took place on July 28 [1]. The date is based on calculations by the Global Footprint Network and indicates on which day the human population has exhausted all the natural resources that the Earth can regenerate in the respective year. This development is not expected to slow down, as the population continues to grow [2] and the demand for plant and animal foodstuffs, building materials, housing and industrial space continues to rise. This demand can only be met by steady expansion into remote areas and, thus, into the habitat of wild animals. The resulting contact between wild animals, domesticated animals and humans and the exploitation of formerly natural areas, is conducive to the transmission of wildlife and livestock pathogens [3–6]. In fact, many of today's most dreaded pathogens originated in wild animals. Among the most prominent examples are African swine fever virus [7], influenza A virus [8], Nipah and Hendra viruses [3], West Nile virus [8,9], or Ebola virus [10].

Together with the effects of climate change, which favor the spread of vector-borne diseases in particular [4], and the globalization of trade, we are confronted with a seemingly unstoppable increase in infectious diseases [11–13].

In the spirit of the One Health concept and with the knowledge that three quarters of all emerging diseases affecting humans originate in wildlife [13,14], it is essential to be able to perform safe and rapid diagnostics of animal pathogens at any time. The first step in this process is the collection and transport of the sample. If samples containing dangerous pathogens are shipped untreated, strict safety regulations apply, which make shipping very time-consuming and expensive [15]. Furthermore, continuous refrigeration of the samples is essential to preserve their integrity. Especially in remote regions, this often cannot be guaranteed, which can lead to a loss of sample quality [16,17] and a reduction of its diagnostic value [18]. If the sample is inactivated, the possible adverse consequences of the inactivation procedure for downstream diagnostic testing must be considered in advance.

In order to simplify the handling of viral samples without compromising safety or diagnostic capability, this work addresses (i) the feasibility of sending unrefrigerated samples on biosample collection cards via regular mail, the question of whether (ii) inactivated samples

containing single-stranded positive-sense viral RNA pose a safety risk, and (iii) the quality of standardized decentralized diagnostics for transboundary diseases such as foot-and-mouth disease.

CHAPTER II: REVIEW OF LITERATURE

II. Review of Literature

1. Inactivation vs. Stabilization

A sample containing viral pathogens should be as safe as possible. This requires the destruction of the viral structures necessary for infection. However, it is these same structures that can be crucial for diagnostics. To evaluate the balancing act between pathogen inactivation and stabilization of diagnostic targets, the common inactivation methods and their effects on viral structures, as well as the common detection methods and their prerequisites will be in focus.

1.1. Inactivation

The inactivation of viruses is based on the prevention of cell infection by the destruction of the viral structures it requires. In order to understand the different inactivation methods, the physiological processes of a successful cell infection must first be considered.

1.1.1. Cell Infection by Viruses

The viral cell infection mainly takes place in three steps: (i) binding of the virus to the host cell, (ii) injection of the genome and, depending on the virus, (iii) transcription, translation and replication of the viral genome to produce new virus particles [19]. The first two steps involve mainly the virus capsid [20,21] or, if present, the viral envelope [22] consisting of a lipid bilayer and glycoproteins [23]. In single-stranded positive-sense RNA viruses, the production of viral proteins can then proceed without further intermediate steps because the viral genome serves as a messenger RNA (mRNA) [23,24]. Viruses with other genome structures first have to produce translatable mRNA as part of their replication cycle. As soon as one of these steps can no longer be successfully carried out, the virus is inactivated [19,20]. Consequently, the main targets of viral inactivation are the capsid, the envelope if present, or the nucleic acid [22].

Characteristics of Single-Stranded Positive-Sense RNA

Viruses with a single-stranded positive-sense RNA genome represent a challenging situation for inactivation. The principle that inactivation is complete as soon as one of the infection steps cannot be carried out must be considered in more detail here. An RNA genome of positive polarity entails the decisive advantage that the production of viral proteins is not

dependent on the virus's own enzymes, which would have to be introduced into the cell together with the genetic material [25]. Instead, the viral genome itself serves as an mRNA [26], allowing direct translation at ribosomes [27,28]. The binding to ribosomes is mediated by an internal ribosome entry site (IRES) in the RNA [29]. The viral RNA-dependent RNA polymerase produced by this process can then synthesize complementary negative-sense RNA [27]. This is used as a template to produce new positive-sense RNA strands, which can then be assembled together with viral proteins to form new infectious virions [27,30]. Denaturation of the capsid and, if present, of the envelope, cannot achieve complete inactivation of positive-strand RNA viruses, since the naked viral genomic RNA is sufficient for infection [30,31]. Prerequisites for this are the successful introduction of the viral nucleic acid into a cell, as well as a good preservation of the RNA, since strand breaks in protein-coding or regulatory elements prevent successful translation [30]. Nevertheless, different research groups have successfully infected animals using naked full-length positive-sense viral RNA and transfection reagents [32–34]. In one case, this has been successful even without transfection reagent but by direct inoculation of the RNA into target organs [35]. Inactivation and stabilization of the sample are thus directly at odds here. As long as intact full-length positive-sense viral RNA is present in a sample, it must be regarded as potentially infectious. Without preserving the viral genome, however, a full diagnosis of the pathogen is no longer possible.

1.1.2. Inactivation Methods

Viral inactivation in the diagnostic field is achieved by physical, chemical and/or biological methods. In the following, only the methods that are most common and most relevant for this thesis will be considered in more detail.

	Method	Nucleic acid	Capsid	Envelope	Reference
Physical	Heat	+	+	+	[20,22]
	Desiccation	(+)	(+)	(+)	[36,37]
	UV irradiation	+	(+)	-	[20,22,38,39]
	Gamma irradiation	+	(+)	+	[40,41]
Chemical	Alcohol	-	(+)	+	[38]
	Acid	-	+	+	[42,43]
	Lysis buffer	-	+	+	[44]
	Surfactants	-	-	+	[45]
Biological	Microorganisms	+	+	?	[46,47]
	Purified enzymes (trypsin, pepsin, etc.)	+	+	+	[48,49]

Table 1: Overview of common inactivation methods in virological diagnostics and their targets. Abbrevations: (+), affected under certain conditions; +, affected; -, not affected; ?, unclear

PHYSICAL INACTIVATION

HEAT

Heat is one of the most reliable ways to inactivate microorganisms [50]. Depending on temperature and time, proteins are irreversibly denatured beginning at temperatures above 40°C [51–53]. At higher temperatures, this can destroy the capsids of non-enveloped viruses [20,54] and the envelopes of enveloped viruses [22,55]. In addition to altering antigenicity [56], heat treatment may lead to the disassembly of virus particles into nucleic-acid-free, or at least nucleic-acid-poor virus capsids and free RNA [57]. Antibodies for serological tests remain mostly unaffected at the commonly used inactivation temperature of 56°C, but are largely destroyed at 70°C [58,59].

Damage to nucleic acid has also been reported [22,60] and also increases with temperature. This arises in DNA mainly due to a changed secondary structure, since the original hydrogen bonds are destroyed by heating [61]. In the case of RNA, hydrolysis and strand breaks can result in cleavage of the molecule [61]. Replication of the virus is then impossible [22]. These denaturation processes start at a temperature of about 60°C for RNA [62].

DESICCATION

Desiccation can lead to several changes within the virus. Destabilization of the native folding of capsid subunits [36] is possible, as well as an altered interaction of the capsid with the genome [63]. This causes many viruses to release their genome [36]. In addition, enormous capillary forces act on the virus particles during drying, which can deform, or even disrupt them [36]. But the envelope and the nucleic acid can also be affected and exhibit phase changes that may be difficult to revert to the original state [37]. However, there are different levels of resistance to desiccation, with enveloped viruses usually being more susceptible than non-enveloped viruses [64,65]. The same is observed for RNA viruses compared to DNA viruses [66,67]. Thus, enveloped RNA viruses such as morbilliviruses [68], influenza A virus [69], henipaviruses [70], human immunodeficiency viruses [71,72] or human coronaviruses [73] can be rapidly inactivated by dry conditions. By contrast, African swine fever virus, which belongs to the enveloped DNA viruses, is more resistant to dry conditions. This was demonstrated by successful virus isolation of positive blood and tissue samples dried on filter paper and stored at 37°C for 2 months [74]. Non-enveloped viruses have very different

sensitivities to desiccation [71]. While hepatitis C virus was no longer infectious after drying and storage at room temperature for only 4 days [75], hepatitis A retained its infectivity in dry feces for 30 days [76] and poliovirus or rotavirus for 60 days [77]. In addition to the properties of the nucleic acid, environmental factors such as temperature, pH [78] and surface texture [79] play a significant role in the resistance of viruses to desiccation.

UV Irradiation

Especially UV-C irradiation with a wavelength of 200-280 nm [80–82] has very strong antiviral properties [80], affecting mainly the viral nucleic acid. The radiation is absorbed by the genome and induces the formation of pyrimidine dimers [83,84]. These photoproducts inhibit polymerases or cause misreading during translation, transcription or replication [85], and are thus considered lethal to the virus [86]. Nucleic acids are radiosensitive, whereas viral proteins are comparatively resistant [87]. Nevertheless, structural changes can also take place in the capsid, rendering it unable to protect the RNA [19,20,88]. The viral envelope does not seem to be affected [22]. The application of UV radiation is relatively uncomplicated, since UV lamps are easy to use, inexpensive and widely available [81]. A disadvantage of this method, however, is the poor penetration depth of the irradiation due to the extensive absorption in liquids and plastic materials [81]. The sample tube should, therefore, be opened [89] and the volume should be kept to a minimum [80].

Gamma Irradiation

Even higher frequency than UV irradiation is gamma irradiation. When interacting with an atom, its entire energy is transferred to an electron [90] resulting in ejection from its orbital [41]. This electron deficiency can move very rapidly [41] through an entire molecule (such as a protein or nucleic acid [91,92] where it has a structure-altering effect on the bonds [92]. Once too many bonds within the molecule are affected, this can lead to disruption of the capsid, altered geometry of the viral envelope including breakage, and degradation of the nucleic acid [40,41]. However, different proteins show different sensitivity [40] and the effect is highly dependent on dose [93] and temperature [41]. For example, serological testing can still be performed [94] because antibodies remain largely unharmed [95]. The advantage lies in the ease of handling, as the irradiation has an extremely high penetration [96] and thus entire packages of goods or samples can be treated unopened [95]. Nevertheless, the purchase and maintenance of such a system is expensive [41,97], strictly regulated, and requires special protection of employees [90].

CHEMICAL INACTIVATION

ALCOHOLS & KETONES

Representatives of these groups, such as ethanol, methanol and acetone, dissolve the lipids of the viral envelope [22]. Thus, they are effective inactivators for enveloped viruses [38]. Even so, due to the additional denaturing and coagulating effect of proteins [98], they can also be used against non-enveloped viruses [99]. The nucleic acid remains intact [22]. The efficacy of inactivation, however, strongly depends on the concentration and the type of alcohol [100]. For example, the benefit of isopropyl alcohol is limited to the inactivation of enveloped viruses only [100], while ethyl alcohol can be used very effectively against enveloped and some non-enveloped viruses [100].

ACIDS

A change in pH can cause charge modifications that affect the folding of proteins [101], resulting in denaturation [102]. In enveloped viruses, lowering the pH leads to an altered structuring of the glycoproteins in the viral envelope [103] and the nucleocapsid [42,104]. During the natural infection of a cell, this mechanism will induce the fusion of the viral membrane with the membrane of the endosome after the virus has entered the cell via endocytosis [105]. For inactivation, this effect is triggered prematurely, with the result that the virus has no longer the capacity of entering a cell. A similar effect is seen with non-enveloped viruses: here the capsid breaks down into its components [43,106,107] and releases the RNA [43,108,109]. An extremely acid-labile representative of this group is foot-and-mouth disease virus (FMDV), as it already dissociates at a pH slightly below neutrality [108,110]. In contrast, other viruses show a much higher resistance to acids down to a pH value of 3 [111,112].

LYSIS BUFFERS FOR NUCLEIC ACID EXTRACTION

Two frequently used products are AVL buffer (Qiagen, Valencia, CA, USA) and TRIzol LS Reagent (Life Technologies, Grand Island, NY, USA). Both contain chaotropic salts (guanidine isothiocyanate) [113]. These interfere with the hydrophobic interactions that support the secondary structure of proteins [114]. As a consequence, proteins in the capsid or viral envelope are denatured [44,115,116], while the nucleic acid is not affected as its stabilizing bonds are not hydrophobic. TRIzol LS Reagent additionally contains phenol [117], which belongs to the aromatic alcohols and also causes protein denaturation [118]. These products are primarily designed for the extraction of nucleic acid [44], but often also have an

inactivating component due to the aforementioned effects [113,119]. However, this effect is highly dependent on the sensitivity of the virus to the buffer used [44,120]. Inactivation can be improved in these cases by further treatment with heat or alcohol [44,121].

Surfactants

Surfactants lower the surface tension of the medium or the interfacial tension of solutions in mixed phases [45]. One representative of this group are soaps. They can inactivate enveloped viruses very effectively by affecting the integrity of the lipid-containing envelope through micelle formation [45]. Consequently, the function of the receptors in the envelope is disturbed which prevents the virus from binding to the host and successfully infecting a cell [45].

Biological Inactivation

Microorganisms

It has been shown that various microorganisms [46,122,123] can have antiviral effects. The underlying mechanisms are diverse. In addition to bacterial enzymes that attack viral proteins [47,124] and metabolites with an inactivating effect [46,47], the use of viral capsid proteins as substrates by some microorganisms has also been demonstrated [47].

Purified Enzymes

A variety of enzymes can be used to adversely affect viral structures. In particular, digestive enzymes such as trypsin [49] and pepsin show good inactivating action. During enzymatic treatment, hydrolysis of structures necessary for host cell binding [48], altered antigen structure [48] or destruction of peptide bonds [125] may occur.

1.2. Stabilization & Diagnostic Methods

For diagnostics to be as informative as possible and yet safe, a balance must be found between inactivation on the one hand and stabilization on the other. This chapter discusses various diagnostic methods and the degree of stabilization required for each.

The routine diagnosis of viral pathogens can be divided into virological and serological detection methods [126], which are based on the identification of various viral components. In the following, only the diagnostic methods relevant for this work will be explained in more detail.

	Method	Principle	Prerequisites	Reference
Virology	**Virus isolation**	- Propagation of viable viruses in a susceptible system - Detection by light microscope based on virus-typical cytopathic effect and/or specific immunostaining	Intact, infective virus	[127]
	Electron microscopy	- High-resolution visualization of pathogens after negative staining by electron irradiation using a transmission electron microscope	Intact virus or identifiable viral structures	[128]
	Endpoint polymerase chain reaction (PCR)	- Enzymatic amplification of a target region of viral nucleic acid using specific paired primers in repetitive temperature cycles - Detection by e.g. agarose gel electrophoresis	Short genome fragments	[129]
	Reverse transcription PCR (RT-PCR)	- Enzymatic synthesis of complementary DNA from an RNA template before endpoint PCR		
	Real-time quantitative PCR (qPCR or RT-qPCR)	- Detection of amplified genome segments in real time by fluorescence signal from a region-specific probe after cleavage by polymerase		
	Multiplex qPCR	- Detection of multiple genome segments in one reaction		
	Nested PCR	- After amplification of the target region, re-amplification of a smaller region within the previously amplified region		
	Sequencing	- Determination of the nucleotide sequence in a DNA molecule		
	Sanger sequencing	- Analysis of nucleic acid sequence of a specific genomic region - Carried out by PCR reaction with in addition to untreated nucleotides, fluorescently labeled di-deoxy nucleotides which lead to elongation stop - Separation of fragment pieces by capillary electrophoresis - Detection of color signals specific for nucleotides	Long genome fragments	[129]
	Next-generation sequencing	- Amplification of carrier-bound fragmented genome pieces (=libraries) - Parallel sequencing of spatially separated, clonally amplified DNA templates - Detection by means of fluorescence signals or hydrogen ions released by the DNA polymerase during incorporation	short genome fragments	[130]
	Transfection	- Recovery of viable viruses by introducing viral positive-sense RNA into the cytoplasm of cells in culture - Detection by light microscope based on virus-typical cytopathic effect and/or specific immunostaining	Intact full genome	[131]
Serology	**Immunoblotting**	- Separation of viral antigens after solubilization by gel electrophoresis - Transfer to a membrane and binding of antigens by specific antibody - Detection by means of enzyme-labeled secondary antibody which leads to a measurable color change due to substrate turnover	Intact antigens	[132]

Serology	**Enzyme-linked immunosorbent assay (ELISA)**	- Specific binding of antigen to antibody - Non-specific binding is eliminated by washing - Detectable change of optical density due to substrate turnover by enzyme-labeled secondary antibody		[129]
	Antigen-capture ELISA	- Binding of viral proteins to carrier-bound virus-specific antibodies - Binding of the complex by an enzyme-labeled secondary antibody	Intact antigens	
	Competitive ELISA	- Binding of serum antibodies/viral antigens to carrier-bound virus-specific antigens/antibodies - Competitive effect by addition of enzyme-labeled antibodies/antigens with the same paratope/epitope - Addition of substrate after washing - Color change is inversely proportional to the abundance of the target	Intact antigens/antibodies	
	Indirect ELISA	- Binding of serum antibodies to carrier-bound virus-specific antigens - Removal of unbound serum antibodies by washing - Binding of the complex by an enzyme-labeled secondary antibody specific to immunoglobulins	Intact antibodies	
	Blocking ELISA	- Binding of serum antibodies to virus-specific antigens during pre-incubation - Transfer to ELISA plate - Binding of the remaining, non-bound antigens to carrier-bound antibodies - Binding of the complex by an enzyme-labeled secondary antibody specific to the virus antigen	Intact antibodies	
	Immunofluorescence	- Detection by fluorescence microscope		[129]
	Direct	- Binding of viral proteins by fluorescence-labeled antibodies	Intact antigens	
	Indirect, for antigen	- Binding of viral proteins by unlabeled antibodies - Binding of the complex by immunoglobulin-specific fluorescence-labeled secondary antibody	Intact antigens	
	Indirect, for antibody	- Binding of serum antibodies to carrier-bound antigens, e.g. in infected cells - Binding of the complex by immunoglobulin-specific fluorescence-labeled secondary antibody	Intact antibodies	
	Haemagglutination inhibition test	- Specific binding of test virus by serum antibodies blocks hemagglutinin antigens - Detection by optical evaluation of hemagglutination after addition of erythrocytes	Intact antibodies	[133]
	Neutralisation test	- Specific binding and neutralization of test virus by serum antibodies - Detection by light microscopic evaluation of cytotoxic effect and/or specific immunostaining in cell culture	Intact neutralizing antibodies	[134]

Table 2: Overview of common diagnostic methods for viral pathogens. Listed are the principles and the requirements for successful detection of a sample.

1.2.1. VIROLOGY

VIRUS ISOLATION

Virus isolation has long been considered the gold standard for virus detection [135]. Propagation of virus from the sample material is carried out in cell culture [17], embryonated eggs [136] or laboratory animals [137]. After an incubation at 37°C, a light microscope is used to screen cultures for a cytopathic effect (CPE) indicative of virus replication [127]. The obtained isolate can then be further characterized [18,126,127,138,139] which can provide crucial information, especially for epidemiological surveillance [17]. However, virus isolation requires that the sample contains intact, infectious viruses necessitating stringent biosafety during transport and subsequent handling of the samples. Improper transport conditions such as long storage at high ambient temperatures, can lead to limitations in viral propagation [17,136]. Other constraints of virus isolation include the high time requirement [137], the fact that an adequate cell system is not available for every virus species [137], and that some viruses do not cause CPE or it is poorly visible [140].

REAL-TIME PCR

The development of the polymerase chain reaction (PCR) is considered a milestone in the diagnosis of infectious diseases [129]. The ability to detect pathogen-specific nucleic acid sequences rapidly, reliably, with high sensitivity and specificity has made it an indispensable and widely used diagnostic tool [126,129]. An informative fragment of viral nucleic acid is defined using a pair of primers – DNA oligonucleotides that bind the viral genome up- and downstream of the region of interest – and amplified during repetitive temperature cycles by a thermostable DNA polymerase extending the oligonucleotide primers [141]. The advantage of the method is the need to amplify only relatively short regions (ca. 100-150 bp) of the viral genome [142], provided they are long enough to be differentiated and visualized by agarose gel electrophoresis. Successful detection can therefore be achieved even if only a small amount of target is present in the sample [129] and/or if the genome of the pathogen has been highly fragmented, e.g. by irradiation [143]. Another major benefit is the speed of the method, which has been further increased by the introduction of real-time PCR [142], where the detection is based on specific [129] dual-labeled oligonucleotide probes binding between the primers which emit a fluorescence signal after cleavage by the exonuclease activity of the DNA polymerase [126,142].

SANGER SEQUENCING

The sequencing of nucleic acids represents a significant advance in molecular biology. It is now possible to characterize pathogens in more detail based on the sequence of the nucleotides of their genome, even in routine diagnostics [129]. This allows the collection of important epidemiological data such as virus lineage or serotype [144–146], pathogenicity markers [147] and possible transmission chains [148], ensuring effective animal disease control [149,150]. Sanger sequencing is based on the chain-termination principle. Using an oligonucleotide primer a defined segment of a nucleic acid is replicated by DNA polymerase. In addition to untreated nucleotides, fluorescently labeled di-deoxynucleotides are added to the reaction. When incorporated, these lead to an elongation stop and mark the DNA fragment with a dye specific to the di-deoxynucleotide. After the amplification, the fragments are separated according to their length by capillary electrophoresis and the fluorescence signal is measured. In this way, the nucleotide sequence can be deduced [129,151]. In order to be able to analyze as many phylogenetic markers as possible, genome samples that are as little fragmented as possible are desirable. With Sanger sequencing, under normal conditions the sequencing of fragments up to 850 bp in length is possible [152].

TRANSFECTION

This procedure allows the introduction of foreign DNA or RNA into a eukaryotic cell cytoplasm or nucleus [131,153]. In order to overcome the electrostatic repulsion between intact cell membranes and nucleic acid [131], different in vitro methods can be applied [131]. In the biological, virus-based transfection (also known as transduction), a recombinant virus infecting the host cell is used to cross the cell membrane and integrate the foreign genome into the cell [154–156]. Physical methods include, for example, direct injection and electroporation. In the latter, brief, high-intensity electrical pulses form temporary pores in the membrane, which allow nucleic acid to enter the cell [157]. In a lipid-based chemical approach, the negatively charged nucleic acid becomes enveloped by positively charged lipid aggregates and can thus be introduced into the cell, presumably via endocytosis [131]. In addition to its many uses in research [131], transfection can also be a powerful tool in diagnostics, as in the case of single-stranded positive-sense RNA viruses. This technique makes it possible to start a productive infection with only an intact whole genome without the need for an intact virion [31]. Replicative virus can thus be recovered from an inactivated sample.

1.2.2. SEROLOGY

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

ELISA is the most widely used immunological tool for the diagnosis of animal pathogens due to its sensitivity, reliability and versatility, and, at the same time, simplicity and rapidity [129,158]. Either antigens of the pathogen or antibodies directed against the pathogen can be detected semi-quantitatively [129]. In foot-and-mouth disease control, a competitive ELISA is often used for this purpose. Specific antigens (non-structural viral proteins) to which naturally infected animals have produced antibodies, but vaccinated animals have not, are bound to a solid phase (Paton, 2005). If present, antibodies from the sample compete with enzyme-conjugated detection antibodies for the binding sites on the immobilized antigen [129,158]. Increased binding of the labeled detection antibodies leads to increased color change by chromogenic or fluorogenic substrate turnover by the enzyme [159], which can be measured in a spectrophotometer. The measured color change is, therefore, inversely proportional to the amount of specific serum antibodies [129].

2. BIOSAMPLE COLLECTION CARDS

A simple method that ensures simultaneous inactivation and stabilization of a sample is the use of so-called biosample collection cards. The following section will take a closer look at how the cards walk this tightrope.

2.1. HISTORY/DEVELOPMENT

The idea of using dried samples for disease diagnosis probably originated with Alejandro Chediak in 1932, who diagnosed syphilis from a blood sample dried on a glass slide [160,161]. Zimmermann successfully adapted this method in 1939 by replacing the microscope slide with a filter paper [160]. Further applications from blood and faeces dried on filter paper were found in the fields of bacteriology and parasitology [162,163]. The most widely known use, however, is probably the Guthrie card. Blood from the pricked heel of newborns is applied to filter paper and examined for the presence of phenylketonuria-typical metabolites by a bacterial inhibition assay [164]. In the years to come, further metabolic screenings for sickle cell anemia and other hemoglobinopathies were performed, using dried blood spots [165], and the technique was finally adopted in the field of virology [166–169].

The advantage of using samples dried on filter paper is primarily simplicity. Only a small sample volume is required, the sample does not need to be processed, which means that no specific laboratory equipment is required at the collection point, and, once the sample has dried, it can be sent unrefrigerated in a sealed envelope via regular mail without any specific safety precautions. Thus, large field studies, in particular, can be carried out in remote areas with little logistical effort [165,170] and samples can subsequently be stored for the long term without complications [169].

2.2. Effectiveness

In addition to the basic principle of dehydration by the filter paper, the cards used today often have additional chemical coatings [171,172] that can lyse cells and denature proteins [173]. The released nucleic acid of the pathogen is subsequently immobilized by filter fibers [174] and protected from environmentally induced degradation, e.g. by oxidation, nucleases, UV irradiation or microbial overgrowth [172,175]. This allows the sample to be handled safely, to be sent in an envelope by regular mail without refrigeration, and to still be used for detailed diagnostics afterwards [174].

2.3. Inactivation

The inactivating effect of biosample collection cards has been confirmed for many different viruses. Card material spotted with virus suspension was macerated in liquid and subsequently incubated in cell culture or embryonated eggs to determine whether viable virus could be recovered. The available literature covers single-stranded positive-sense RNA viruses with envelope [176–178] as well as without envelope [179], single-stranded negative-sense RNA viruses with envelope [180–186], double-stranded RNA viruses without envelope [187–189] and a DNA virus also without envelope [190]. Since recovery was achieved in only one study [189], it can be assumed that the pathogens are sufficiently inactivated by the cards in most cases. This often required only 1 hour drying at room temperature [176,180–182].

2.4. Stabilization & Diagnostic Possibilities

While the pathogens are inactivated in most cases, their genome remains largely intact and allows a variety of diagnostic analyses to be performed [191].

2.4.1. VIROLOGY

VIRUS ISOLATION

Infectious bursal disease virus was successfully cultivated from card material in embryonated chicken eggs, according to Maw et al. [189]. However, Moscoso dealt with the same question and was unable to reisolate virus from the card [187].

REAL-TIME (RT)-PCR

Depending on the size of the excised piece of filter paper used in the extraction [178] and the storage conditions [180,192], pathogen detection by real-time (RT)-PCR can take place with only minor loss in sensitivity [188]. DNA could still be amplified after decades if the cards were stored at room temperature [74,193] . Even less favorable storage conditions, such as 37 °C and 60% humidity for 7 days, did not lead to any adverse effect compared to a reference sample stored at -20 °C [168]. RNA is considered to be generally less stable than DNA, also on the cards [66]. Here, genome detection was still possible after 8 months of storage at -20 °C [187], up to half a year at 4 °C [186] and several months at room temperature [177,179–181]. However, even in the case of an elevated temperature of 37 °C, it was still possible to detect many RNA viruses for several weeks [188,189], although with increasing degradation of the genome in some cases [192]. Detection was successful with a variety of sample matrices spotted on the cards, including blood, solid tumors, feather pulp [66], tissue [139] and allantoic fluid [194].

SEQUENCING

Few data are available about the extent to which the cards fragment the genome of the pathogens. However, there are various studies in which the viral genome was successfully sequenced [177,180,191,195]. Nevertheless, in some cases, worse results were obtained compared to the use of the original virus isolate [191]. Again, the amplifiable fragment length depends on the storage conditions. Thus, RNA fragments up to a length of 1200 nt could be amplified from cards after storage at 4 °C and up to 800 nt after 6 months [196].

TRANSFECTION

That intact whole genomes are also present on the cards was demonstrated by Biswal. They succeeded in the recovery of infectious FMD viruses via chemical transfection after storage of the card at 4 °C and RT for up to 7 weeks and at 37 °C for up to 6 weeks [197].

2.4.2. SEROLOGY

The use of cards in serology allows the direct application of whole blood instead of the otherwise necessary preparation of serum as sample material [74]. Furthermore, the sample volume is greatly reduced as only a few drops are required. Taken together, this simplifies the sample collection procedure, eliminates the need for further preparation and ensures uncomplicated transport [198,199].

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Compared to corresponding serum samples, the detection of specific antibodies from blood dried on filter paper showed in many cases only a slight decrease in sensitivity, if at all [74,198,200,201]. Detection of measles antibodies was still possible after 6 months of storage at 4°C, but not longer than 15-17 months [202]. Even at room temperature, the level of dengue antibodies remained stable for at least 28 days [192]. However, results for the samples stored at 37°C for the same period of time differed significantly [192]. HIV-1 antibodies could be stored for 6 weeks under tropical conditions without false-negative results [203]. Even after 20 weeks, there was no effect of storage conditions on the high-titre samples. Reliable detection of low-titre samples is considered more difficult [74,203].

FURTHER METHODS

Other serological methods such as haemagglutination inhibition [204,205] also allowed the use of dried blood samples, in some cases with 100% agreement to the corresponding serum sample [206]. Likewise, detection by Western blotting was successful [203].

3. DECENTRAL DIAGNOSTICS FOR FOOT-AND-MOUTH DISEASE VIRUS

Especially for high-impact pathogens and/or for those from which a country is considered free, safe and standardized diagnostics must be ensured. Notifiable animal diseases in Germany are determined by the Federal Ministry of Food and Agriculture [207] and currently include 54 diseases, 33 of which are caused by viruses [208]. The Friedrich-Loeffler-Institut (FLI), Federal Research Institute for Animal Health, is the national reference laboratory (NRL) for notifiable diseases [207]. To the extent that animal disease diagnostics are performed by other laboratories in Germany (e.g., state veterinary laboratories), the FLI provides scientific and technical advice and conducts periodic proficiency tests.

Until 2013, all laboratory diagnosis for FMDV was performed in the high-containment unit of the FLI, without participation by the state laboratories. After the catastrophic outbreak of FMD in the United Kingdom in 2001 [209], however, it became clear that one laboratory would not be able to cope with the enormous volume of samples that need to be tested in a large outbreak. At the same time, new diagnostic methods such as RT-qPCR assays and antibody ELISAs were developed, which do not require the use of infectious virus and can safely be carried out in standard diagnostic laboratories. Involving the state laboratories in the routine diagnosis of FMD (i.e., exclusion testing) builds and maintains capacity that will be critical in an outbreak response.

Germany has been free of FMD for over 30 years. Therefore, it is important to maintain and increase awareness of the disease among farmers, veterinarians and other stakeholders. FMD can be difficult to differentiate clinically from other diseases and conditions [210] and even certain nonspecific syndromes require FMDV exclusion [211,212]. Veterinarians need to be able to obtain rapid laboratory clarification when FMD is a possible differential diagnosis, but, previously, the submission of samples for FMD testing was carried out exclusively via the competent authority. It is assumed that, as in the case of other animal pathogens, many questionable cases were not sent for investigation [213,214], because farmers and veterinarians were afraid of adverse consequences [215]. For this reason, the submission of samples has been facilitated and FMDV exclusion testing (by real-time RT-qPCR) can now be carried out in the state laboratories without involving the authorities and without any restrictions being placed on the farm [207]. To maintain the necessary high diagnostic standard, the FLI provides the laboratories with guidance on diagnostic methods and conducts regular proficiency tests, wherein all participating state laboratories receive a blinded panel of inactivated FMDV samples to be analyzed by real-time RT-PCR and ELISA [207].

4. Use of Concept: Example of Transboundary Viruses

To maximize the utility of our study, we have included four highly relevant animal pathogens, that are as different as possible in their biochemical properties: RNA and DNA viruses with double-stranded and single-stranded genomes with and without a lipid envelope, viruses with high and low resistance to environmental influences, and viruses that are transmitted in different ways, such as by vectors. All of these viruses are listed in the WOAH Terrestrial

Animal Health Code as having a significant impact on the health of humans, domesticated and/or wild animals [216].

4.1. BLUETONGUE VIRUS

The causative agent of bluetongue disease belongs to the family *Reoviridae* and is the type species of the genus *Orbivirus* [217]. Its non-enveloped three-layered capsids contain a multisegmented linear dsRNA genome [25].

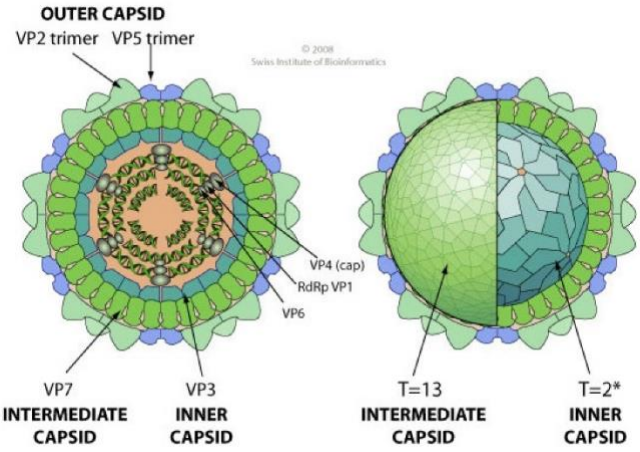

Figure 1. Structure of a bluetongue virus particle. For rights see Chapter X, Supplementary Material, Permission for Reproduction.

The virus is mainly transmitted by *Culicoides* midges [218] and infects domesticated and wild ruminants [219]. Clinically, however, it especially affects sheep [220], in which the disease can range from subclinical to fatal [221]. Cattle can act as reservoir hosts [218], but are also clinically susceptible to certain virus strains [220]. The clinical picture is defined by nasal discharge, increased salivation, erosions and hemorrhages of the nasal and oral mucosa, conjunctivitis and fever [218,222]. Originally considered a tropical disease [223] it continues to spread northward as a result of warmer temperatures [224], which provide suitable conditions for both endemic *Culicoides* species and species introduced via transport and imported goods to transmit the virus [224,225]. Due to the rapid spread [226,227], high losses in sheep production [226], and the costs associated with disease control [228], the bluetongue virus is considered a significant threat to animal production [226].

4.2. Foot-and-Mouth Disease Virus

Foot-and-mouth disease virus is an aphthovirus and belongs to the family of *Picornaviridae* [24]. The icosahedral capsid is not enveloped and contains a linear non-segmented single-stranded positive-sense RNA genome [229].

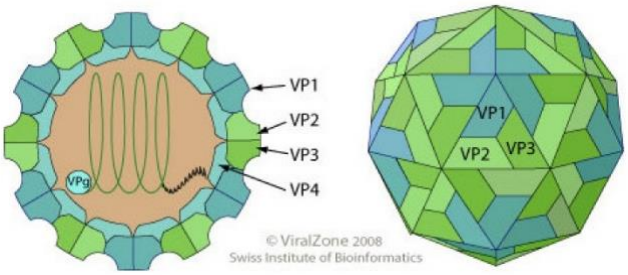

Figure 2. Structure of a foot-and-mouth disease virus particle. For rights see Chapter X, Supplementary Material, Permission for Reproduction.

Due to the very low infectious dose, the short generation time, the large amount of virus excreted by infected animals and the multitude of ways of transmission, FMDV is considered the most contagious disease of animals [230]. The most relevant mechanism of transmission is direct contact with infected animals and their secretions, excretions and exhaled air [229]. In addition, indirect transmission through the contaminated environment, fomites or animal products is possible, as is transmission via the wind under certain conditions [229]. Both domesticated and wild cloven-hoofed animals are affected [231]. Characteristic of the disease are lesions in the mouth area, on the feet and teats as well as non-specific signs such as strong salivation, lameness and fever [231]. Lethality depends on the age of the animals. In young animals, it can be very high since the heart is involved in the infection [231]. Older animals often recover, but there can be complications due to secondary bacterial infections [232,233]. Reduced weight gain and milk yield can lead to long-term losses in animal production [231]. Currently, many countries in Africa, the Middle East and Asia are affected and there are small remaining pockets of infection in South America [234]. However, due to increasing global trade and travel, there is a constant risk of reintroduction to FMDV-free countries [235]. Since an outbreak precipitates high costs for control measures and enormous trade restrictions, FMD is considered the most important constraint to the international trade in animals and animal products [231].

4.3. Peste des Petits Ruminants

Peste des petits ruminants is caused by a member of the genus *Morbillivirus* of the family *Paramyxoviridae* in the order *Mononegavirales*. The virus has a non-segmented single-stranded negative-sense RNA genome that is surrounded by a lipid bilayer envelope [236].

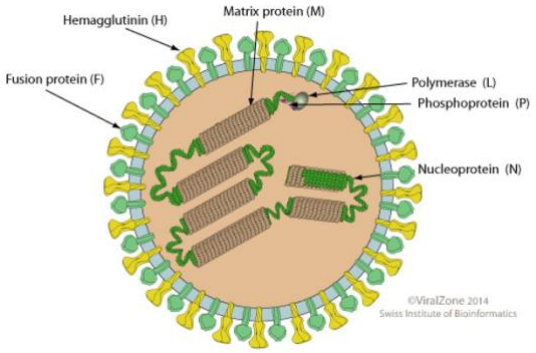

Figure 3. Structure of a peste des petits ruminants virus particle. For permission rights see Chapter X, Supplementary Material, Permission for Reproduction.

Due to the instability of the envelope against heat, UV irradiation, and dehydration [237], transmission occurs only in close contact between animals via infected excreta, aerosols [238], or through contaminated feed or water [239]. Mainly small ruminants are clinically affected [238,240]. Depending on the age and breed of the animals [241], the virus strain [242] and other factors [238,243], the lethality varies greatly, but can be up to 100% [244], mainly due to impairment of the respiratory and digestive tracts. The characteristic erosions on the eyes, nose and mouth area are accompanied by discharge and can occur in combination with respiratory problems or diarrhea. Abortions can also occur [238,243]. The disease was mainly found in West Africa, the Middle East and the Indian subcontinent [244–247] but is increasingly spreading [248–251]. Over 70 countries and 80% of the sheep and goat population in the world are now affected, while 50 other countries are considered at risk [252]. In endemic countries, the disease is one of the main problems for sheep and goat production [244,253,254] which is associated with large financial losses [252]. As it mainly affects smallholder farmers in poor rural regions, who obtain their food and income from animal husbandry, controlling peste des petits ruminants virus is an important component of the fight against poverty in these countries [243,250]. Not least for this reason, the OIE and the FAO decided in 2015 on a program for the global elimination of peste des petits ruminants virus until 2030 [252].

4.4. Lumpy skin Disease Virus

Another virus that causes an economically significant transboundary disease [255,256] is lumpy skin disease virus. It is a representative of the enveloped, double-stranded DNA viruses and belongs to the genus *Capripoxvirus* within the family of *Poxviridae* [257–260].

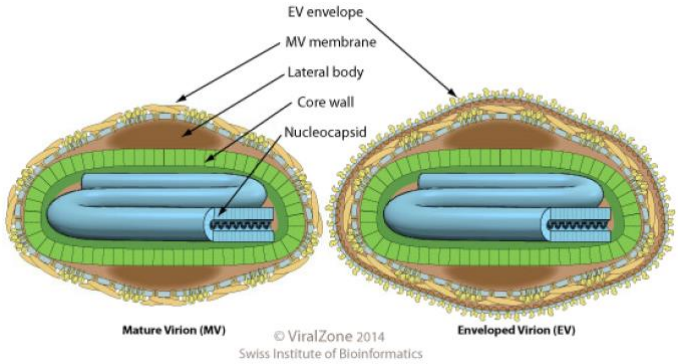

Figure 4. Structure of a lumpy skin disease virus particle. For rights see Chapter IX, Supplementary Material, Permission for Reproduction.

The most important route of transmission is via bloodsucking insects and arthropods [261]. Mosquitoes [260], stable flies [262] and ticks [263] are considered possible vectors. The disease can vary from inapparent to severe [259]. After initial fever and lymphadenopathy [259,261,264], characteristic skin nodules form due to the epithelial cell tropism of the virus [258] in most cases. They can occur sporadically or generalized on the entire outer skin, especially in regions with little hair, such as the head, genitals, perineum and limbs [259,265]. In severe cases, they can also be found on mucous membranes, such as the eye [264], respiratory tract [265], digestive tract [259] or other organs [256]. After healing, they leave permanent scars and lower the value of the hides [266]. Other long-term consequences may include lower milk yield and decreased growth [267], abortions [268] and temporary infertility of bulls [269]. Morbidity varies between 3-85% [270] and mortality is usually less than 10% [270] but has also been reported to be higher [267]. Possible factors influencing this are considered to be the virus isolate, the breed and health status of the animals, and the insect species that transmitted the virus [258,265]. Initially found only in Africa [271], the virus has spread through the Middle East [272] to Russia [273] and Europe [259,263] and thus poses a threat to surrounding countries. The main impact of an outbreak is not so much direct livestock losses as the months-long drop in production [264], restrictions on global trade, and the costs of control and treatment [258,274].

CHAPTER III: STUDY OBJECTIVES

III. STUDY OBJECTIVES

In the most remote regions of the world, an unknown multitude of potential pathogens are lurking, just waiting to be discovered. The SARS-CoV-2 pandemic shows us all too clearly the extent that such a pandemic can reach on a global scale. Sophisticated diagnostic methods for animal pathogens are available, but sampling in remote areas is limited by factors such as refrigeration and shipping. This work has its focus in improving both diagnostics and sample transport by analyzing the performance of an easy to use and safe matrix for sample transport, evaluating the risk of contagion associated with positive-sense viral RNA genomes and assessing the proficiency of state veterinary laboratories in Germany.

Objective I: Feasibility study of simplified sample collection and transport without compromising diagnostic utility.

Publication I

The use of biosample collection cards in the diagnosis of viral pathogens promises simpler sample collection and non-refrigerated transport via standard mail, for a wide range of diagnostic options. To what extent the method guarantees a safe inactivation in combination with a sufficient stabilization of the pathogens was examined in this work. In addition, the aim was to determine the range of diagnostic tests that are possible with the dried samples.

Objective II: Investigation of the infectivity of single-stranded positive-sense RNA and the associated risk of infection during sample transport.

Publication II

Viral genomes in the form of single-stranded positive-sense RNA, as in foot-and-mouth disease virus, can be infectious on their own. Thus, destruction of the protein capsid alone is not sufficient to prevent infection. The subject of analysis in this objective was whether inactivated FMDV samples can cause infections in susceptible animals. From this, practical conclusions can be drawn for the safe transport of samples containing inactivated foot-and-mouth disease virus.

Objective III: Verification of proficiency of decentralized foot-and-mouth disease virus diagnostics in Germany.

Publication III

It is one of the duties of the NRL for FMD at the Friedrich-Loeffler-Institut to promote adequate diagnostic capability at the regional laboratories. With the decentralized diagnostic strategy, the state laboratories carry out examinations for the exclusion of foot-and-mouth disease. The aim was to verify that the participating laboratories can reliably perform the necessary virological and serological tests.

CHAPTER IV: RESULTS

IV. RESULTS

The reference section of each manuscript is presented in the style of the respective journal and is not repeated at the end of this document. The numbering of figures and tables corresponds to the published form of each manuscript.

1. Publication I: Comparison of Biosafety and Diagnostic Utility of Biosample Collection Cards

Publication I

Comparison of Biosafety and Diagnostic Utility of Biosample Collection Cards

Hanna Keck, Michael Eschbaumer, Martin Beer, Bernd Hoffmann

Viruses

2022

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viruses

MDPI

Article

Comparison of Biosafety and Diagnostic Utility of Biosample Collection Cards

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Abstract: Six different biosample collection cards, often collectively referred to as FTA (Flinders Technology Associates) cards, were compared for their ability to inactivate viruses and stabilize viral nucleic acid for molecular testing. The cards were tested with bluetongue virus, foot-and-mouth disease virus (FMDV), small ruminant morbillivirus (peste des petits ruminants virus), and lumpy skin disease virus (LSDV), encompassing non-enveloped and enveloped representatives of viruses with double-stranded and single-stranded RNA genomes, as well as an enveloped DNA virus. The cards were loaded with virus-containing cell culture supernatant and tested after one day, one week, and one month. The inactivation of the RNA viruses was successful for the majority of the cards and filters. Most of them completely inactivated the viruses within one day or one week at the latest, but the inactivation of LSDV presented a greater challenge. Three of the six cards inactivated LSDV within one day, but the others did not achieve this even after an incubation period of 30 days. Differences between the cards were also evident in the stabilization of nucleic acid. The amount of detectable viral genome on the cards remained approximately constant for all viruses and cards over an incubation period of one month. With some cards, however, a bigger loss of detectable nucleic acid compared with a directly extracted sample was observed. Using FMDV, it was confirmed that the material applied to the cards was sufficiently conserved to allow detailed molecular characterization by sequencing. Furthermore, it was possible to successfully recover infectious FMDV by chemical transfection from some cards, confirming the preservation of full-length RNAs.

Keywords: biosample collection cards; FTA (Flinders Technology Associates) cards; Ahlstrom-Munksjö; Whatman; Copan; Macherey-Nagel; virus isolation; real-time RT-qPCR; sequencing; transfection; Lipofectamine 3000

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1. Introduction

The use of untreated cotton-based filter paper for mass screening of newborns for inherited metabolic diseases in the 1960s [1] was one of the first steps towards biosample collection cards. During the following years, the principle of blood samples dried on fiber-based material was also applied to other investigations [2,3]. This method allowed the use of smaller sample volumes for the detection of pathogens and space-saving storage at room temperature over a long period of time [4,5]. For some viruses, desiccation alone already has an inactivating effect [3], but for others this is not the case [6]. For this reason, additional coatings of the cellulose material have been established. In most cases these consist of chaotropic or anionic substances [7] and are able to lyse white blood cells [8], tumor cells [9], most types of bacteria [10,11], or viruses [12–15] and to denature proteins [16]. Nucleic acid storage cards that are treated with inactivating substances are often collectively referred to as Flinders Technology Associates (FTA) cards after the institution where they were originally invented in the 1980s, Flinders University in Adelaide, Australia [17]. Once the sample is applied to the card, the structure of the pathogen is disrupted and the nucleic acid is released [18]. The fibers of the matrix then capture the free nucleic acid

and preserve it [16,19,20]. Additionally, the now immobilized genetic material is protected by the coating substances from further degradation by nucleases, oxidases, or from UV damage [16,19,21,22]. As a result, a stabilized sample is obtained that does not need to be refrigerated [20,22] and can be shipped in a standard envelope via letter mail [13,23,24].

Originally developed for long-time preservation of DNA [1,22], the principle of the cards is now used for DNA and RNA in almost all analytical fields [25], including virology [5,7,26–28], bacteriology [29], parasitology [11,30], monitoring of mosquito-borne pathogens [31], genomic analyses [32,33], pharmacogenetics [34], forensics [35], and biobanking [18]. The array of sample matrices is correspondingly wide, ranging from body fluids to fresh tissue samples of humans, animals, or plants [26,36,37]. DNA stored on cards at room temperature was successfully amplified by PCR after several years [9,19,36,37]. Available data on the stability of RNA on cards usually cover several months [5,13,15,16,21], but in some cases only a few weeks [22,38]. Over these time spans, preserved RNA was found to be relatively resistant against the influence of temperature and humidity [12,20,22]. The utility of the recoverable RNA depends on the sample material, the type of nucleic acid (single-stranded or double-stranded), the storage duration, and the sensitivity of the assay used [38]. If RNA samples are to be stored for a long time, refrigeration to 4 °C or lower is recommended [16]. At temperatures of −20 °C or below, RNA samples on cards are stable for over a year [39].

The preserved samples are suitable for virological and serological analyses [40], but further characterization or culturing of the pathogens is constrained, because the direct isolation of infectious virus from the card is not possible [13,14,22,23]. For viruses with a single-stranded positive-sense RNA (+ssRNA) genome, however, the intact viral genome by itself is capable of replication. If the virus genome is introduced into permissive cells, it functions as an mRNA [41], which results in replication and ultimately the generation of new infectious virus particles. In the laboratory, this can be achieved by different transfection methods [42,43].

Previously published data were almost exclusively collected with the "FTA Classic" card by Whatman (now part of Cytiva, Marlborough, MA, USA). Meanwhile, there are many other manufacturers that also offer cards with various coatings for diverse use cases. In this paper, we use "biosample collection card" (BCC) as a generic term for all cards. We tested five other BCCs in addition to the FTA Classic card. Two standard filter papers, which are not intended for sampling, were also included to see if they are a viable alternative when no BCCs are available. Virus inactivation and nucleic acid stabilization were analyzed at different time points for a wide variety of viruses: bluetongue virus (BTV), family *Reoviridae*, double-stranded RNA genome, no envelope [44]; foot-and-mouth disease virus (FMDV), family *Picornaviridae*, single-stranded RNA genome, positive polarity, no envelope [45]; small ruminant morbillivirus (also known as peste des petits ruminants virus, PPRV), family *Paramyxoviridae*, single-stranded RNA genome, negative polarity, lipid envelope [46]; and lumpy skin disease virus (LSDV), family *Poxviridae*, double-stranded DNA genome, lipid envelope [47]. All are highly relevant animal pathogens that cause notifiable transboundary diseases.

2. Materials and Methods

2.1. Viruses and Cells

BTV-5 RSArrrr/05, FMDV A_{22}/IRQ/24/64, PPRV Nigeria 75/1, and LSDV Neethling V100 were used for the stabilization and inactivation experiments. Two additional FMDV isolates (A/IRN/08/2015 and O/FRA/1/2001) were used for the sequencing and transfection experiments. The cell lines and media for virus culture are listed in Table 1. For FMDV, BHK-21 cells were used for propagation and subsequent titration and LFBK-αVβ6 cells were used for the inactivation, stabilization, and transfection experiments.

Table 1. Cell lines and media used for virus culture.

Virus	Cell Line	FLI Cell Culture Collection No.	Culture Medium	Cell Count for Seeding [3]
BTV	BSR/5	RIE0194	MEM [1]	1.25×10^5
FMDV	BHK-21 clone Tübingen	RIE0164	MEM	1.25×10^5
FMDV	LFBK-αVβ6 [48]	RIE1419	DMEM [2]	1.5×10^5
PPRV	Vero dog SLAM [49]	RIE1280	MEM	1.25×10^5
LSDV	MDBK	RIE0261	MEM	1.0×10^5

[1] Minimal essential medium with Hanks' and Earle's salts and non-essential amino acids. [2] Dulbecco's modified Eagle medium. [3] Number of cells seeded per well of a 24-well culture plate to obtain an 80% confluent monolayer after 24 h. Cells in plates were incubated at 37 °C with 5% CO_2.

For continuous culture, the cells were propagated in the appropriate medium supplemented with 10% fetal bovine serum (FBS). For the preparation of a master stock per virus, the cells were infected as monolayers in 75 cm^2 cell culture flasks (Corning, NY, USA) without using FBS. After 2 h, medium with 10% FBS was added in equal amounts to reach a final serum concentration of 5%. This was followed by an incubation at 37 °C. For BTV, infected cultures were incubated for up to 5 days, for FMDV 3 days, for PPRV 4 days, and for LSDV 6 days. The optimal time for virus harvest was determined by close observation of the development of cytopathic effect (CPE).

For FMDV, the culture supernatant was clarified by centrifugation for 1 min at 2000× *g* and 4 °C and the pellet was discarded. For BTV, PPRV, and LSDV, the cell association of the viral particles was exploited to further concentrate the virus preparations. For this, the culture medium from several culture flasks was combined in a 50-mL conical tube and centrifuged for 15 min at 3000× *g* and 4 °C. The supernatant was discarded and the cell pellet was resuspended in 2 mL of MEM. All master stocks were aliquoted and stored at −80 °C. To determine their titers, one aliquot of each was thawed and titrated on the cells listed in Table 1. In addition, the viral genome loads of the stocks were defined by quantitative real-time PCR or RT-PCR.

Tissue culture plates (Corning) for the inactivation experiments (24-well plates) and transfections (6-well plates) were prepared one day in advance by seeding cells in medium with 10% FBS to obtain approximately 80% confluent monolayers on the day of inoculation. Before the cells were infected, the culture medium was replaced with fresh serum-free medium.

2.2. Cards and Filters

A total of six BCCs and two standard filter papers supplied by Ahlstrom-Munksjö (Helsinki, Finland), Whatman (Cytiva), Copan (Brescia, Italy), Macherey-Nagel (Düren, Germany), and VWR (Radnor, PA, USA) were used for the study. A detailed list of the manufacturers, products and catalogue numbers can be found in Table 2. The cards can be used with a multitude of biological samples [50–53] except for the Nucleic-Card, which is designed for blood and buccal cells only, and the NucleoCard by Macherey-Nagel, which is intended exclusively for blood samples [54]. Samples spotted on the cards can subsequently be used for a variety of genome analyses, although only the FTA Classic card from Whatman explicitly mentions diagnostics [51]. Copan refers to forensic use [53] and Ahlstrom-Munksjö additionally to biobanking [50], while the intended purpose given by Macherey-Nagel is long-term storage and subsequent examination by real-time PCR [54]. Only Whatman mentioned the stabilization of "nucleic acids" in general [51], while Ahlstrom-Munksjö [50], Copan [53], and Macherey-Nagel [54] refer exclusively to DNA. Nucleic acid stabilization is claimed for all cards [50,51,53,54] except the Human ID Bloodstain Card, whose untreated matrix was not designed for long-term storage [52]. In the case of GenSaver 2.0 [55], the FTA Classic card by Whatman [56] and Copan's Nucleic-Card [53], the prevention of microbiological growth during the long-term storage at ambient temperature is asserted. All cards in the study feature four circular collection

areas with a diameter of 2.5 cm, secured by a flip-down protective cover. The filters are standard uncoated laboratory filter papers, intended for the separation of fine solid particles from liquids.

Table 2. Biosample collection cards and filter papers used in the study.

Manufacturer	Product Name (Catalogue Number)	Code	Sample Type	Application	Special Features
Ahlstrom-Munksjö	GenSaver (8.564.0002.B-N)	A	Biological fluids	**DNA:** Forensic, Biobanking markets	
Ahlstrom-Munksjö	GenSaver 2.0 (8.566.0002.B-N)	A2	Biological fluids	**DNA:** Forensic, Biobanking markets	Prevents growth of microorganisms
Whatman	FTA Classic Card (WB120205)	W	Biological samples	**Nucleic acid:** Diagnostic, clinical applications, bacterial, viral, blood, plant, insect analysis, Genomic, Forensic...	Prevents growth of microorganisms
Whatman	Human ID Bloodstain Card (WB100014)	WB	Blood, bodily fluids	**DNA:** Short-term handling (collection and transport)	
Copan Flock Technologies	Nucleic-Card (4473977)	C	Blood, buccal cells	**DNA:** Forensic	Prevents growth of microorganisms
Macherey-Nagel	NucleoCard (740403.10)	MN	Blood	**DNA:** Long term storage, Real-time PCR analysis	
Whatman	Grade 1 filter paper (1001-110)	FV		Filtration	
VWR	Grade 413 filter paper (516-0815)	FW		Filtration	

2.3. Inoculation and Sample Collection

For each sample collection area of the cards, 125 µL sample material was spotted in the center. On the filter paper discs, collection areas of identical size to those of the cards had to be marked first. After spotting, cards and filter papers were air-dried in a biological safety cabinet (BSC) for at least 3 h. The dry inoculated cards and papers were placed in plastic bags inside sealed containers and kept at room temperature in the dark. Samples were recovered from the cards and papers after one day, one week, and one month, respectively. A quarter of each spot (which corresponds to 31.25 µL of applied sample) was cut out using sterile scissors and forceps and put in a 2 mL screw-cap tube with 1750 µL serum-free medium with antibiotics (1% Antibiotic-Antimycotic 100×, Thermo Fisher Scientific, Darmstadt, Germany; 0.4% Gentamicin/Amphotericin B 500×, Thermo Fisher Scientific) and a 4 mm stainless steel bead. The samples were macerated in a TissueLyser II (Qiagen, Hilden, Germany) for 3 min at 30 shakes/s. To separate the supernatant, the tubes were centrifuged at 3000× *g* for 5 min. About 150 µL of liquid was retained in the card matrix and 1600 µL was recovered for further experiments.

2.4. Inactivation Experiments

2.4.1. Preliminary Tests: Cytotoxicity and pH

The cards and filters were inoculated with serum-free medium instead of virus culture supernatant and then prepared as described above. Monolayers of all four cell lines were inoculated with 100 µL of a dilution series of the supernatant from the macerated card and filter material down to a 10^{-6} dilution. After incubation for 24 h at 37 °C and 5% CO_2, cytotoxic effects were evaluated under the microscope. For the GenSaver 2.0 card and Vero

dog SLAM (VDS) cells, the experiment was repeated with smaller dilution steps (1:10, 1:20, 1:30, etc., down to 1:100).

To determine the pH value of the card and filter macerates, one quarter of each card was processed as described above, but in distilled water instead of cell culture medium. The supernatant was then dripped onto a pH indicator strip (MQuant, Merck, Darmstadt, Germany) covering a range of pH values from 0 to 14. As a neutral control, the pH of the distilled water was also analyzed. The evaluation was performed visually based on the color change of the strip.

2.4.2. Virus Inactivation

The macerates of the spotted FTA Classic card and Nucleic-Card were diluted 1:10 in serum-free medium with antibiotics and then inoculated into cultures of all four cell lines. The same was carried out for the samples from GenSaver 2.0 cards, but the 1:10 dilution was only used for BHK-21, LFBK-αVβ6, and MDBK cells. For VDS cells, the pre-dilution was 1:20. All dilutions were taken into account to calculate the limits of virus detection and inactivation. No dilution was necessary for the remaining cards and the filters.

For each combination of card/filter and virus, 500 µL of the (diluted) macerates were added to 3 wells of a 24-well cell culture plate. The cell line and incubation time at 37 °C and 5% CO_2 was virus-specific (see above). The cultures were examined for CPE with a microscope and the plates were then frozen at −80 °C in sealed containers. The cells were lysed by freezing and thawing to recover the entire contents of the wells for a second culture passage. For the passage, 500 µL of the lysate were transferred to one day old cell monolayers in medium containing 5% FBS. Again, CPE was assessed after incubation at 37 °C and 5% CO_2. On each plate, two wells containing only cells in medium were used as a negative control and two wells with cells and 500 µL virus culture supernatant, diluted in the same way as the samples, were used as a positive control.

The final evaluation was carried out on a percentage basis. It was calculated how many of the tested replicates, per card, virus and time point, caused CPE in culture. For BTV, FMDV, and LSDV, 48 replicates per card or filter and time point were tested. For PPRV, there were 36 replicates.

2.5. Nucleic Acid Stabilization and Extraction

Nucleic acid stabilization for all viruses was assessed by quantitative real-time PCR or RT-PCR. The supernatants of the card and filter macerates were extracted using the NucleoMagVET kit (Macherey-Nagel) on the KingFisher Flex platform (Thermo Fisher Scientific) according to the manufacturers' instructions. A positive extraction control containing the original virus cell culture supernatant was included in the extraction.

Previously published assays were used for the viral genome quantification: Hofmann et al. for BTV [57], Callahan et al. for FMDV [58], Batten et al. for PPRV [59], and Bowden et al. [60] with a modified TaqMan probe according to Dietze et al. [61] in the case of LSDV. A heterologous internal control was included in all runs [62].

For the RNA viruses BTV, FMDV, and PPRV, the AgPath-ID One-Step RT-PCR Reagents (Thermo Fisher Scientific) were used for the RT-PCR. Here, the total reaction volume was 12.5 µL, including 1.25 µL nuclease-free water, 6.25 µL 2× RT-PCR buffer, 0.5 µL 25× RT-PCR enzyme mix, 1 µL primer-probe mix for the virus detection (with 7.5 pmol of each primer and 2.5 pmol of FAM-labeled probe), 1 µL EGFP primer-probe mix 4 for the internal control (with 2.5 pmol of each primer and 2.5 pmol of HEX-labeled probe), and 2.5 µL template RNA. Cycling conditions were as follows: 10 min at 45 °C, 10 min at 95 °C followed by 42 cycles of 15 s at 95 °C, 20 s at 56 °C, and 30 s at 72 °C. To improve primer binding to the double-stranded genomic RNA of BTV, the template RNA in the PCR plates was denatured at 95 °C for 5 min in a heating block, followed by snap freezing in liquid nitrogen. The master mix was then added to the frozen RNA. For LSDV, the QuantiTect Multiplex PCR NoROX Kit (Qiagen) was used with a total reaction volume of 12.5 µL containing 1.75 µL nuclease-free water, 6.25 µL 2× QuantiTect Multiplex PCR

NoROX Master Mix, 1 µL primer-probe mix for virus detection, 1 µL EGFP mix 4 and 2.5 µL template DNA. The temperature profile used was 15 min at 95°C, followed by 42 cycles of 60 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C.

Samples with a cycle of quantification (C_q) value > 40 were defined as negative. All assays were run in duplicate with the two resulting C_q values subsequently averaged. For each sample, a delta C_q value (ΔC_q) was determined. This was done by subtracting the averaged C_q value of the sample from the C_q value of the appropriate positive control (per card or filter type, virus and time point). Since several repetitions were performed, the mean value of the individual ΔC_q values was then determined for each sample. This corresponds to the increase in C_q value (i.e., decreased detection of viral nucleic acid) that results from applying the sample to the card or filter and re-extracting the nucleic acid, compared with analyzing the sample directly.

For BTV, FMDV, and LSDV, 20 replicates per card or filter and time point were tested. For PPRV, there were 18 replicates.

A virus-specific mean ΔC_q across all cards and filter papers was calculated to see which virus was stabilized and/or recovered most effectively. Finally, the average overall loss was calculated by averaging all ΔC_q values regardless of the card or filter type, time point, or virus used. The same was conducted with only the best-performing card types.

To evaluate the statistical significance of the results, the 95% confidence intervals of the mean ΔC_q values were calculated for each virus, card, or filter and time point using the following formula, where $\overline{x}$ stands for the sample mean, σ for the standard deviation, and n for the sample size:

$$\overline{x} \pm 1.96 \times {}^{\sigma}/_{\sqrt{n}}$$

The calculations were conducted with Microsoft Excel or with GraphPad PRISM for the visual representations, respectively. Non-overlapping confidence intervals were considered evidence of a statistically significant difference.

2.6. Extraction and Transfection of FMDV Samples

Virus culture supernatant of FMDV strains A_{22}/IRQ/24/64, A/IRN/08/2015, and O/FRA/1/2001 as well as homogenized epithelial tissue from tongue lesions were used for the transfection experiments. Pieces of vesicular epithelium (approximately 3 mm × 3 mm) from a bovine infected with FMDV A_{22}/IRQ/24/64 were homogenized in 400 µL serum-free DMEM, using a 4 mm stainless steel bead in the TissueLyser II (Qiagen) for 3 min at 30 shakes/s. After centrifugation at 3000× *g* for 5 min, the supernatant was obtained.

The cell culture and tissue supernatants were used to prepare a 10-fold dilution series in serum-free DMEM down to a dilution of 10^{-4}. A volume of 125 µL of the original supernatant and the 10^{-1} dilution were spotted on each card or filter. The tubes with the dilution series were stored at −80°C. The cards and filters were dried overnight in a BSC. The following day the spots were macerated as described above, but using only 400 µL of serum-free DMEM. RNA was extracted from the supernatants using TRIzol LS reagent (Thermo Fisher Scientific), which is an improved single-step RNA isolation method originally described by Chomczynski and Sacchi [63]. It was performed following the manufacturer's instructions. The RNA pellet was dried on a heating block for approximately 5 min at 30 °C. The contents of the reaction tubes used to spot the cards, as well as the previously unused 10^{-3} and 10^{-4} dilutions, were thawed and treated in the same way as the samples for use as a positive control. The extracted RNA was then immediately used for transfection.

Lipofectamine 3000 (Thermo Fisher Scientific) was used for the transfections. For each preparation, 1 µL of Lipofectamine 3000 reagent was diluted in 25 µL of serum-free DMEM and mixed gently without vortexing. In a second tube, 3 µL of RNA, 0.5 µL P3000 reagent and 25 µL DMEM were mixed in the same way. The two preparations were then combined dropwise and incubated for 10 min at room temperature. Fifty µL of the mixture was added to one well of a 6-well cell culture plate, again dropwise and without touching the surface. The following incubation of 15 min was carried out at 37 °C and 5% CO_2. Finally, the wells

were filled with 500 µL DMEM with 5% FBS and incubated at 37 °C and 5% CO_2 for two days. Two wells were used per sample. The remaining volume of eluate was used for real-time RT-PCR and subsequently stored at −80 °C. After the incubation, the monolayers were checked for signs of CPE under the microscope and the plates were then frozen at −80 °C. A passage and second reading of CPE was performed as described above. These tests were repeated at least two times for each virus strain.

2.7. Sequencing of FMDV Samples

RNA samples from cards that could not be successfully transfected were used for the sequencing analyses. The qScript XLT One-Step RT-PCR ToughMix (Quantabio, Beverly, MA, USA) and the previously published primers FMD-3161-F and FMD-4303-R were used to amplify the VP3- and VP1-coding regions of the FMDV genome with an amplicon size of 1143 bp [64]. The total reaction volume of 10 µL contained 0.6 µL of nuclease-free water, 5.0 µL 2× One-step Tough Mix, 0.4 µL qScript XLT OneSTep RT enzyme, 1 µL each of the forward and reverse primer at a concentration of 10 pmol/µL, and 2.0 µL template RNA. The temperature profile was 20 min at 48 °C, 3 min at 94 °C, followed by 40 cycles of 15 s at 94 °C, 30 s at 60 °C, 60 s at 68 °C, and finally 5 min at 68 °C. The PCR products were then run in an agarose gel. Bands of the expected size were excised and DNA was extracted with the QIAquick gel extraction kit (Qiagen) and sent to Eurofins Genomics (Ebersberg, Germany) for sequencing.

3. Results

3.1. Virus Propagation

The same master stock of each virus was used for all experiments. The titer of the BTV master stock was $10^{8.0}$ $TCID_{50}$/mL and it had a C_q value of 7.9, FMDV A_{22} Iraq was $10^{7.8}$ $TCID_{50}$/mL and C_q 12.5, FMDV A/IRN8/2015 was $10^{7.3}$ $TCID_{50}$/mL and C_q 12.3, FMDV O/FRA/1/2001 was $10^{5.3}$ $TCID_{50}$/mL and C_q 14.4, PPRV Nigeria 75/1 was $10^{6.5}$ $TCID_{50}$/mL and C_q 13.3, and LSDV Neething V100 was $10^{7.7}$ $TCID_{50}$/mL and C_q 13.7.

3.2. Inactivation Experiments

3.2.1. Cytotoxicity

No cytotoxicity was observed for the GenSaver, Human ID Bloodstain Card and NucleoCard macerates in any cell line. In contrast, all cell lines inoculated with the undiluted card macerates from GenSaver 2.0, FTA Classic and Nucleic-Card showed obvious signs of cytotoxicity. The cytotoxic effects were abolished when the macerates were diluted 1:10 or, in the case of GenSaver 2.0 and VDS cells, 1:20. The cytotoxicity data for all cards and filters are summarized in Table S1 in the Supplementary Material.

3.2.2. pH

The pH of the macerates of the Human ID Bloodstain card, NucleoCard, and the filter papers was determined to be approximately 5, the same as the pH of the distilled water. For GenSaver, GenSaver 2.0, FTA Classic, and the Nucleic-Card the pH was approximately 7. FMDV is particularly sensitive to even mildly acidic pH [65].

3.2.3. Virus Inactivation

Taking into account the dilutions required to avoid cytotoxicity, the main experiment demonstrated complete inactivation of at least 10^4 $TCID_{50}$ spotted on the card for all cards and filters and all viruses except PPRV (Figure 1). Due to the lower initial titer of the PPRV master stock, inactivation of a virus dose of 10^4 $TCID_{50}$ could only be shown for cards where no dilution of the macerate was required (GenSaver, Human ID Bloodstain Card, NucleoCard). For the samples that had to be pre-diluted, i.e., 1:10 for FTA Classic and Nucleic-Card and 1:20 for GenSaver 2.0, the inoculated virus dose (which was completely inactivated) was $10^{3.7}$ $TCID_{50}$ and $10^{3.6}$ $TCID_{50}$, respectively.

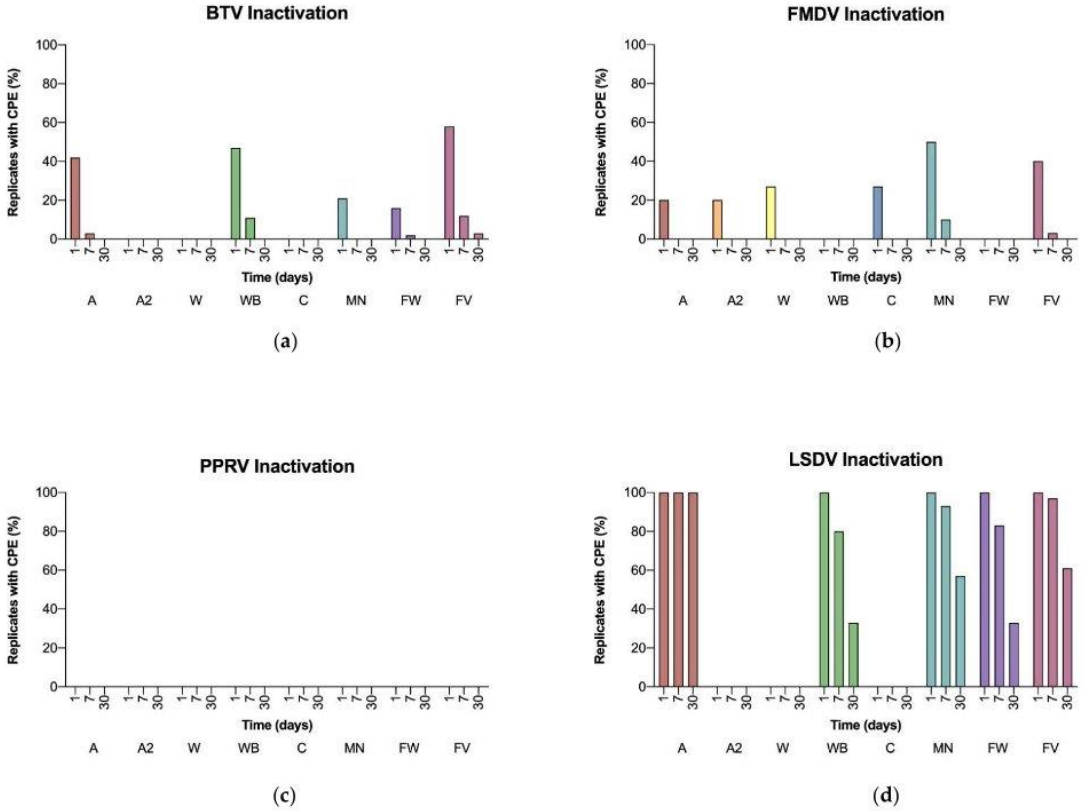

Figure 1. Virus inactivation on cards and filters during one day, one week, and one month of incubation. The percentage of all replicates still causing CPE in cell culture is shown for each virus, card, or filter and time. (**a**) BTV; (**b**) FMDV; (**c**) PPRV; (**d**) LSDV.

In summary, almost all cards or filters markedly reduced virus infectivity over time for almost all viruses. PPRV was rapidly inactivated by all cards and filters within less than one day. For BTV and FMDV, there often was residual infectivity after one day of incubation and the largest decrease in infectivity was seen between one and seven days. GenSaver 2.0, FTA Classic and Nucleic-Card inactivated BTV in less than one day and NucleoCard within one week of incubation. For GenSaver, Human ID Bloodstain Card and the Whatman filter paper, complete inactivation of BTV was demonstrated after 30 days of incubation. On the VWR filter paper, BTV remained infectious virus even after one month. FMDV was inactivated by the Human ID Bloodstain Card and the Whatman filter paper within less than a day. All other cards completely inactivated FMDV within a week, except for NucleoCard and the VWR filter paper, which required a month of incubation for full inactivation. The inactivation of LSDV was most challenging overall. While GenSaver 2.0, FTA Classic and Nucleic-Card completely inactivated LSDV within less than a day, this was not possible for GenSaver, Human ID Bloodstain Card, NucleoCard and the filter papers. They were unable to completely inactivate LSDV even after one month of incubation, and GenSaver did not show a reduction of infectivity at all (Figure 1).

3.3. Nucleic Acid Stabilization

Clear differences were also observed in the stabilization efficacy (Figure 2). On one hand, the stability of nucleic acids on a card or filter can be observed over time, demonstrating that the level of detectable virus genome remained approximately the same over time for all cards, filters, and viruses used.

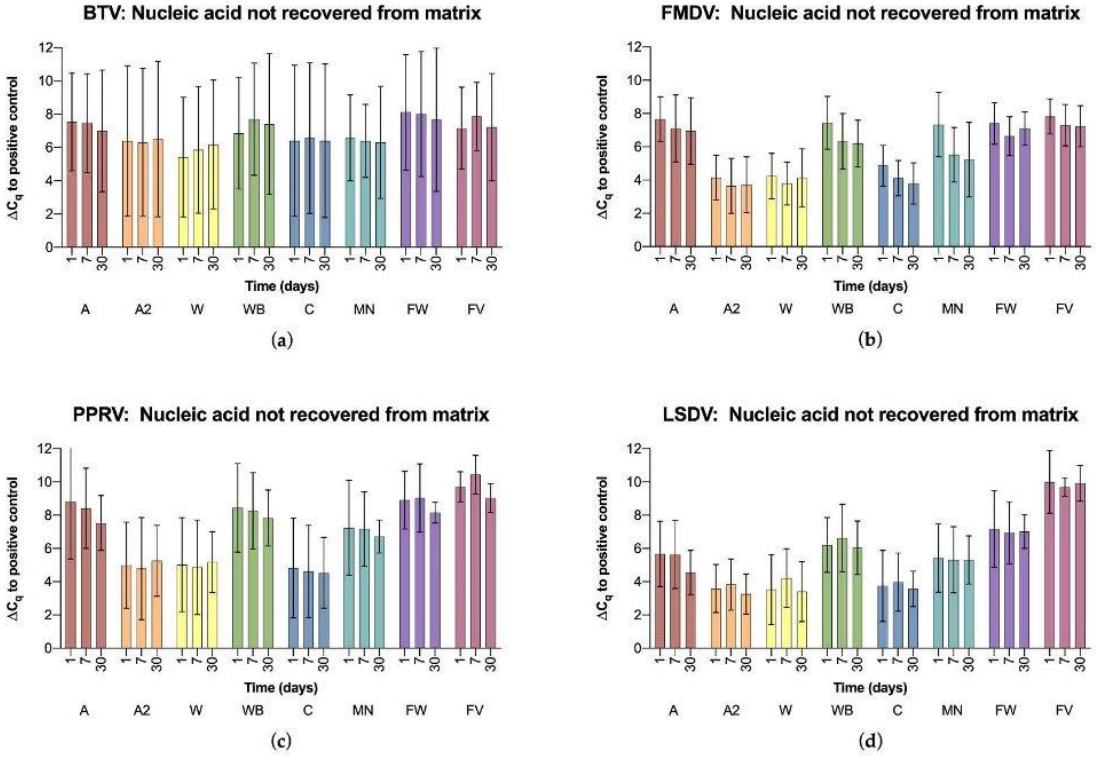

Figure 2. Stabilization and recovery of nucleic acid from cards and filters analyzed by real-time (RT)-PCR after one day, one week and one month of incubation, showing the mean ΔC_q values for each virus, sample time point, and card with their 95% confidence intervals indicated by the error bars. ΔC_q values were calculated between the eluted nucleic acid and the virus preparation used for spotting (positive control). (**a**) BTV; (**b**) FMDV; (**c**) PPRV; (**d**) LSDV.

On the other hand, the amount of viral RNA or DNA that can be eluted from the cards or filters can be compared to the original liquid sample, revealing a wide range of ΔC_q values. Among the specimens with the highest average ΔC_q values for all viruses, i.e., the greatest loss of nucleic acid, were the filter papers. The cards with the lowest average ΔC_q values, i.e., the best recovery of nucleic acid from all viruses, were GenSaver 2.0, FTA Classic and Nucleic-Card. Their mean ΔCq value was 4.6, whereas the mean ΔCq value for all cards and filters was 6.3. Between virus groups, the best recovery was seen for the double-stranded DNA genome of LSDV, with a mean of all ΔC_q values of 5.6, followed by FMDV with 5.8 and BTV with 6.6. The highest loss was found for PPRV with a mean ΔC_q value of 7.1.

The significance of the results was evaluated by calculating the 95% confidence intervals of the mean ΔC_q values. The widely overlapping confidence intervals indicated that there were no significant differences between the cards and filters in the stabilization of BTV. For FMDV, PPRV, and LSDV, on the other hand, the cards A2, W and C showed significantly better results, especially in comparison with the filter papers FW and FV.

3.4. Transfection

Successful recovery of infectious FMDV from spots of virus culture supernatant was possible for GenSaver 2.0, the FTA Classic card, Nucleic Card, and NucleoCard (Table 3). Across all experiments, transfection was successful up to a maximum C_q of 27.0 for card or

filter samples and 32.6 for positive controls (original liquid samples). Detailed information is presented in Table S2 in the Supplementary Material.

Table 3. Transfection results by card or filter type, virus strain and sample dilution, showing the number of successful transfections compared to the total number of replicates. The average C_q value of the samples for which the transfection was successful is given in parentheses.

Product Name	Virus Strain and Dilution					
	FMDV A_{22} Iraq		FMDV A/IRN/8/2015		FMDV O/FRA/1/2001	
	Undiluted	10^{-1}	Undiluted	10^{-1}	Undiluted	10^{-1}
GenSaver	0/3	0/3	0/2	0/2	0/2	0/2
GenSaver 2.0	1/3 (21.2)	1/3 (26.7)	2/2 (18.5)	1/2 (22.9)	2/2 (20.5)	0/2
FTA Classic card	1/3 (21.2)	1/3 (25.8)	2/2 (20.0)	1/2 (24.2)	2/2 (21.8)	0/2
Human ID Bloodstain Card	0/3	0/3	(0/2)	(0/2)	0/2	0/2
Nucleic-Card	2/3 (21.0)	1/3 (24.7)	2/2 (19.6)	1/2 (26.8)	1/2 (24.2)	0/2
NucleoCard	2/3 (20.9)	0/3	1/2 (19.0)	0/2	0/2	0/2
Filter paper, Whatman	0/3	0/3	0/2	0/2	0/2	0/2
Filter paper, VWR	0/3	0/3	0/2	0/2	0/2	0/2

For the epithelial homogenate from a tongue lesion of an animal infected with FMDV A_{22} Iraq, the C_q value of the RNA extracted directly from the undiluted liquid sample was 14.8. This was the only RNA that could be successfully transfected. No infectious virus was recovered from the RNA extracted from higher dilutions of the liquid sample (with C_q values of 18.6, 23.1, 27.3, and 31.2) nor from any of the RNA extracted from cards spotted with the epithelial homogenate.

3.5. Sequencing

Failure to recover infectious virus by transfection indicates the absence of intact full-length viral genomes. For these samples, it was analyzed whether the fragmentation of the RNA still allowed PCR amplification and sequencing. This did not reveal any differences between the cards. The 1143 bp fragment of the VP3- and VP1-coding regions was successfully amplified from all eluates (see Table S3 in the Supplementary Material). The sequences obtained from the cards matched the previously determined sequences of the used virus strains.

4. Discussion

4.1. Sample Collection and Extraction

Biopsy punches are often used to excise card material for nucleic acid extraction [26,42,66–68]. Cross-contamination is not considered a problem for this method [67] since the card fibers do not adhere to the punch [36]. Nevertheless, using a "clean" punch [36] or disinfecting the punch between samples is recommended [7,23]. It has not been evaluated whether residual disinfectant can affect subsequent samples. In our experience, the punched-out discs are awkward to manipulate and are so light that they are often blown away by the laminar air flow in the biosafety cabinet when trying to transfer them to reaction tubes. Therefore, we decided to use a fresh set of reusable sterile scissors and forceps for each sample, as described by several previous publications [6,11,14,21].

4.2. Inactivation and Stabilization

Clear differences were seen between the various cards and filters in terms of their inactivation and stabilization efficacy. GenSaver 2.0, FTA Classic, and Nucleic-Card delivered the best results. They were able to inactivate almost all tested viruses within one day at a spotted virus dose of at least 10^4 $TCID_{50}$, except for the FTA Classic card requiring one week of incubation for the inactivation of FMDV. The critical dose of 10^4 $TCID_{50}$ is based on

the guidelines for testing virucidal disinfectants, where a reduction of infectivity by 4 $\log_{10}$ (or 99.99%) is required [69]. The complete inactivation of a virus dose of $10^{3.6}$ $TCID_{50}$ (as demonstrated for PPRV) still represents a reduction of 99.975%. All tested cards and filter papers inactivated PPRV within less than a day, which is in line with the expected low tenacity of enveloped single-stranded negative-sense RNA viruses [46]. Since the sample with the shortest incubation time was taken after 24 h, no statement can be made about how quickly the cards and filters can actually inactivate PPRV. Further tests with shorter incubation times could be carried out to determine this. When using the other cards for FMDV or BTV, an incubation period of at least a month is required to ensure complete inactivation. Careful selection of the card to use is especially important with DNA viruses such as LSDV, which are much more stable than RNA viruses in samples spotted on storage cards [26].

Most cards or filters were able to keep RNA and DNA stable over a long period of time. The recovery of nucleic acid from the cards was most effective for the DNA virus, which gave generally low ΔCq values compared with the original sample. For the RNA viruses, a considerably smaller fraction of the viral nucleic acid was recovered from the cards, confirming the findings of earlier studies [66]. The lower stability of RNA is also cited as a reason for this. Overall, the cards with the best inactivation efficacy, GenSaver 2.0, FTA Classic and Nucleic-Card, also performed well in the stabilization experiments.

For the positive control, 100 µL of pure virus culture supernatant was used. This corresponds to more material than would be present in a quarter of the spot on a card or filter. It is intended to reflect the best case, where the original liquid sample can be tested directly. In contrast, the cards or filter papers reflect the situation in the field, where the sample must be preserved for storage or transport to the laboratory.

In summary, the cards that prevent microbiological growth also produce the best inactivation and stabilization results. We can only speculate about possible additives that make the inactivation and stabilization so much better, as the companies do not provide precise information about the formulation of the chemical coating. For this reason, we have been reluctant to write too much about the causes of the very different results for the cards, because we are simply not aware of them.

If the samples eluted from these cards are to be used in cell culture, however, they must be diluted to avoid damage to the cells. Remarkable are the results of the plain filter papers, which did reasonably well in both experiments. This shows that the drying on absorbent paper alone already has considerable inactivating and stabilizing effects [3–5,24,70] which are entirely incidental to the intended application of these products.

It should be noted that in this study, with the exception of the FMDV-positive tongue lesion, we used only cell culture supernatant as a sample material. This, of course, does not correspond to sampling in practice. It remains unclear to what extent the results can be transferred to other sample matrices. We assume that this is possible for aqueous samples with low protein content (e.g., saliva). However, for sample materials with high protein content (e.g., whole blood), it is conceivable that the inactivation and stabilization performance may be impaired.

4.3. Transfection and Sequencing of FMDV Genomes

A similarly heterogeneous picture emerged from the attempts to recover intact viral genomes from the cards and filters. Transfection was only attempted with RNA eluted one day after spotting on the cards. With GenSaver 2.0, FTA Classic, and Nucleic-Card, transfection of the 10^{-1} dilution was successful for FMDV A/IRN/8/2015 and A_{22} Iraq, but FMDV O/FRA/1/2001 was only successfully transfected from the undiluted RNA. One possible reason for this is the over 100-fold lower initial titer of the latter, which conceivably corresponds to a lower number of full-length viral genomes.

In summary, with high-titer samples and the right card, a sufficient number of full-length genomes can be recovered to allow successful transfection. Once again, GenSaver 2.0, FTA Classic, and Nucleic-Card performed better than the other cards. To obtain optimal

results, sample processing and transfection should be carried out as quickly as possible after the arrival of the cards in the laboratory [38]. PCR amplification and sequencing of the VP1-coding region, on the other hand, was possible with all cards and filter papers. In our study, this was only attempted with cards spotted the previous day, but it has been reported that sequencing was still possible after storing cards at temperatures of 41 °C for 15 days [38].

4.4. Use of Biosample Collection Cards in Diagnostics

Improper collection or storage of liquid samples can quickly lead to degradation of nucleic acid or overgrowth by microorganisms [71,72], potentially compromising the diagnostic value of the sample [12]. Biosample collection cards offer an inexpensive solution for collecting and sending samples that is widely available and requires little preparation. Due to the ease of shipping via regular mail without refrigeration or strict biosafety requirements [13,23,24], the cards have a clear advantage in terms of logistical effort during sample collection as well as during transport and subsequent storage. Especially in situations with limited resources and infrastructure, this can result in a considerable increase in sample quality and quantity. Especially for large-scale mass surveys, the cards can be used advantageously [2]. Due to the small amount of samples needed, puncturing an ear vein is often sufficient for sampling [73]. This saves materials and time and makes handling animals much less stressful. Additionally, no additional laboratory equipment is required to process the collected samples before shipment.

It must be noted, however, that the inactivating effect of the cards is restricted to the collection areas intended for the samples. Contamination of the protective envelope must be avoided. As demonstrated in our study, DNA viruses in particular will not be inactivated by contact with absorbent paper alone.

However, if the sample is applied properly, the risk of transmission during transport is minimized by three levels. The fibers of the card spots immobilize the sample after initial drying [16]. This represents the first level. The flip-down protective cover can be considered the second level. Finally, the sample is shipped in a sealed envelope and is again shielded from the environment, which represents the third level [74].

The biological risk can be further reduced by heating spotted cards to 70 °C for 30 min [75], immersing them in 0.2% acetic acid for 15 min [76], or by treatment with phenol [77]. It is assumed that this will not affect downstream real-time PCR analysis or sequencing, but may cause increased degradation of full-length genomes. Further studies are necessary to determine the diagnostic utility of this approach.

5. Conclusions

The performance of the tested cards varied considerably, with GenSaver 2.0, FTA Classic, and Nucleic-Card delivering the best results for inactivation, stabilization, and nucleic acid recovery.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/v14112392/s1. Table S1: Cytotoxicity data. Table S2: Transfection results. Table S3: RT-PCR amplification of the VP1-coding region.

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Data Availability Statement: Summarized data from the study are included in the manuscript and the supplementary material. Raw data for the individual experiments will be provided by the authors upon justified request.

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Conflicts of Interest: The authors declare no conflict of interest.

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2. Publication II: Full-Length Genomic RNA of Foot-and-Mouth Disease Virus is Infectious for Cattle by Injection

Publication II

Full-Length Genomic RNA of Foot-and-Mouth Disease Virus is Infectious for Cattle by Injection

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Full-Length Genomic RNA of Foot-and-Mouth Disease Virus Is Infectious for Cattle by Injection

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Abstract: Safe sample transport is of great importance for infectious diseases diagnostics. Various treatments and buffers are used to inactivate pathogens in diagnostic samples. At the same time, adequate sample preservation, particularly of nucleic acids, is essential to allow an accurate laboratory diagnosis. For viruses with single-stranded RNA genomes of positive polarity, such as foot-and-mouth disease virus (FMDV), however, naked full-length viral RNA can itself be infectious. In order to assess the risk of infection from inactivated FMDV samples, two animal experiments were performed. In the first trial, six cattle were injected with FMDV RNA (isolate A_{22}/IRQ/24/64) into the tongue epithelium. All animals developed clinical disease within two days and FMDV was reisolated from serum and saliva samples. In the second trial, another group of six cattle was exposed to FMDV RNA by instilling it on the tongue and spraying it into the nose. The animals were observed for 10 days after exposure. All animals remained clinically unremarkable and virus isolation as well as FMDV genome detection in serum and saliva were negative. No transfection reagent was used for any of the animal inoculations. In conclusion, cattle can be infected by injection with naked FMDV RNA, but not by non-invasive exposure to the RNA. Inactivated FMDV samples that contain full-length viral RNA carry only a negligible risk of infecting animals.

Keywords: foot-and-mouth disease virus; safe sample transport; single-stranded positive-sense RNA; TRIzol extraction; naked RNA; infectivity; RNA transfection; lipofectamine; self-transfection; BHK cells

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1. Introduction

Diagnostic samples can contain large amounts of infectious disease agents and can be a source of contagion. Inactivating agents or treatments can be used at the point of care to mitigate the risks associated with these samples without compromising their diagnostic utility. For many infectious diseases, the target of first-line diagnostic tests is pathogen-specific nucleic acid. Therefore, inactivating agents such as alcohols or acids, which denature proteins and dissolve lipids, but do not destroy nucleic acids, are often preferred.

Acid treatment is particularly suitable for foot-and-mouth disease virus (FMDV) [1], a high-consequence agricultural pathogen that causes vesicular disease in domestic and wild ruminants and swine. FMDV is an aphthovirus of the *Picornaviridae* family. Like all picornaviruses, FMDV is a non-enveloped virus with a non-segmented single-stranded RNA genome of positive polarity [2]. Unlike cellular mRNA, the viral RNA is not capped, but contains an internal ribosome entry site that allows its direct translation in host cells [2]. Therefore, purified FMDV RNA can initiate an infection in the absence of viral proteins, but in the natural infectious cycle the viral capsid is required for adhesion to the host cell and insertion of the RNA into the cytoplasm [3].

The FMDV capsid readily disassembles at low pH, leading to a rapid loss of infectivity [1]. Accordingly, citric acid is highly effective at inactivating FMDV in tissue samples [4] and on lateral flow devices (LFDs) [5] used for pen-side diagnosis of FMD. Acid-treated samples and LFDs can then be submitted for laboratory analysis under less strict biosafety conditions [6]. Nevertheless, both studies confirmed that these specimens still contain full-length viral RNA, from which infectious virus can be recovered by chemical transfection of permissive cell cultures. It is unknown, however, if animals that come in contact with the full-length viral RNA—accidentally or deliberately—are similarly at risk of FMDV infection.

Several research groups have demonstrated that animals can be infected by naked single-stranded positive-sense genomic RNA of various viruses when transfection reagent is added to the RNA before inoculation [7–9]. For porcine reproductive and respiratory syndrome virus it was shown that a productive infection can also be initiated by the injection of viral RNA alone [10].

To investigate the risk of FMDV infection from inactivated diagnostic samples, in this study cattle were exposed to naked FMD RNA by injection and by non-invasive mucosal deposition.

2. Materials and Methods

2.1. Cells and Viruses

2.1.1. Cell Culture

Two cell lines were used for the cultivation of FMDV: BHK-21 cells (clone "Tübingen") (RIE 194, Collection of Cell Lines in Veterinary Medicine, FLI, Greifswald, Germany) grown in minimal essential medium (MEM) with Hank's and Earles' salts and non-essential amino acids and LFBK-αVβ6 cells [11] (RIE 1419), for which Dulbecco's modified Eagle medium (DMEM) was used. Both media were supplemented with 10% fetal bovine serum (FBS) during routine culture.

2.1.2. Susceptibility of Cells to Free Viral RNA

To prevent false-positive results in the virus isolation, it is essential that infection of the cell culture is only caused by intact virus particles and there is no spontaneous uptake of free viral RNA by the cells. Accordingly, both cell lines were tested for their self-transfection ability. Two identical 96-well PCR plates were prepared with 200 µL FMDV suspension (strain A_{24} Cruzeiro, approximately 10^7 $TCID_{50}$/mL) in each well. The plates were then exposed to lengthwise temperature gradients (i.e., with columns of 8 replicate wells heated to the same temperature) of 55–75 °C and 75–95 °C for 10 min in a thermocycler. The heat-treated samples were brought to room temperature, transferred to a tissue culture plate containing either BHK-21 or LFBK-αVβ6 cells, then incubated for 3 days at 37 °C and 5% CO_2, and subsequently evaluated for cytopathic effect (CPE). A second experiment was completed in the same way, but half of the replicates at each temperature were treated with 5µL RNase A (200 µL/mL) after heating and before cell culture inoculation (BHK-21 cells only).

2.1.3. Selection of Virus Isolates

Three isolates of FMDV, namely A/IRN/8/2015 (lineage A/ASIA/G-VII), A_{22}/IRQ/24/64 (also known as "A_{22} Iraq", lineage A/ASIA/A_{22}), and O/FRA/1/2001 (lineage O/ME-SA/PanAsia), were propagated on BHK-21 cells. The virus preparations were analyzed for infectivity by titration on LFBK-αVβ6 cells and for FMDV genome content by RT-qPCR. To test their transfectability, the virus preparations were serially diluted 10-fold in serum-free DMEM down to a final dilution of 10^{-5}. Total RNA was extracted from the dilutions and the extracted RNA was analyzed by RT-qPCR and transfection as described below.

2.1.4. Preparation of Virus Stocks

Master stocks of FMDV A_{22}/IRQ/24/64 were prepared in BHK-21 cells grown in MEM supplemented with 5% FBS. In a 75 cm^2 cell culture flask with 16 mL culture medium, 50 µL of a previous preparation of A_{22}/IRQ/24/64 were used to inoculate a 90% confluent cell monolayer. About 80% CPE was detected after one day of incubation at 37 °C. After freezing and thawing, the virus suspension was clarified by centrifugation at 1000× *g* for 5 min, aliquoted and stored at −80 °C. Virus and viral RNA content were quantified by end-point titration on LFBK-αVβ6 cells and RT-qPCR.

2.1.5. RNA Extraction and In Vitro Transfection

RNA was extracted from cell culture supernatants using TRIzol LS Reagent (Thermo Fisher Scientific, Waltham, MA, USA) as directed by the manufacturer. The transfections for the preliminary tests and the positive control were performed with Lipofectamine 3000 (Thermo Fisher Scientific). Per well, 1 µL of Lipofectamine 3000 reagent was diluted in 25 µL of DMEM and mixed gently without vortexing. In parallel, 3 µL of extracted RNA, 0.5 µL of P 3000 Reagent, and 25 µL of DMEM were mixed the same way. The two preparations were combined and incubated for 10 min at room temperature, before 50 µL per well were added dropwise to a 24-well plate with near-confluent LFBK-αVβ6 cells. Care was taken not to touch the cell monolayer. After 15 min of incubation at 37 °C, 500 µL of DMEM supplemented with 5% FBS were added per well. CPE was evaluated after 2 days at 37 °C with 5% CO_2.

2.1.6. RT-qPCR

FMDV RNA was quantified with an RT-qPCR assay targeting the 3D coding region [12], using AgPath-ID One-Step RT-PCR Reagents (Thermo Fisher Scientific) with 2.5 µL of template in a total reaction volume of 12.5 µL.

2.2. Material for Inoculation

2.2.1. Assessment of RNA Degradation by Atomization

Before the animal experiment, it was tested if nebulization of FMDV RNA leads to significant fragmentation, as had been observed for large DNA molecules [13]. Half of the pooled RNA extracted from an FMDV A_{22}/IRQ/24/64 culture was serially diluted in nuclease-free water and transfected into LFBK-$\alpha_V\beta_6$ cells using two replicate wells per dilution. The other half was nebulized using a syringe-mounted atomization device (MAD Nasal, Teleflex, Morrisville, NC, USA). Nebulization was performed in a biological safety cabinet and the nebulized RNA was collected in a 50 mL conical tube for dilution and transfection. After 2 days of incubation at 37 °C with 5% CO_2, the transfected cells were examined for CPE.

2.2.2. Preparation of RNA Inoculum

To prevent the viral RNA from being damaged by freezing and thawing and obtain as many intact full-length viral genomes as possible, it was extracted immediately before inoculation of the animals in each experiment. For the first experiment, the pooled RNA of 18 replicate extractions was diluted 1:5 in nuclease-free water. For the second experiment, the pooled RNA was also diluted 1:5, but half of the 1:5 dilution was then further diluted to provide an adequate volume for intranasal administration by atomizer. For this purpose, 1.2 mL of the diluted pooled RNA were added to 11.4 mL of nuclease-free water. The freshly extracted and diluted RNA was transported to the animal room on ice. No transfection reagent was added to the viral RNA for any of the animal inoculations.

The leftover RNA inoculum was brought back to the laboratory on wet ice for confirmatory tests.

2.2.3. Negative Control: Exclusion of Virus Contamination

To confirm that it did not contain any intact viral particles, 50 µL of the RNA inoculum without transfection reagent were added to an intact monolayer of LFBK-αVβ6 cells and incubated at 37 °C for 3 days. The test was carried out in three replicates. After 3 days, the cultures were frozen and thawed and the cell lysate was passaged onto fresh LFBK-αVβ6 cells. After another 3 days of incubation, the cultures were examined for CPE.

2.2.4. Positive Control: Demonstration of Full-Length Viral RNA

To confirm that intact full-length FMDV genomes were present in the inoculum, it was serially diluted in nuclease-free water and transfected into LFBK-αVβ6 cells using lipofectamine as described above. The viral RNA content of the inoculum was quantified with the FMDV 3D RT-qPCR assay. A serial dilution of an in vitro transcribed FMDV RNA standard was included to construct a calibration curve and calculate genome copy numbers.

2.3. Animal Experiments

2.3.1. Animals and Ethics Statement

Six Holstein-Friesian cattle were used for each trial. The animals were brought to the BSL4vet facility of the FLI Riems one week before the start of the trial to ensure proper acclimation. In the first experiment, the animals were between 5 and 8 months of age. In the second experiment, they were between 3 and 4 months.

For the inoculations and clinical exams, the animals were kept off feed overnight and sedated by intramuscular injection of 0.3 mg xylazine per kg body mass into the hind quarters. The sedation was reversed by intramuscular injection of 0.03 mg atipamezole per kg body mass.

All work with animals occurred after ethical review and in compliance with local, state, and national animal welfare regulations. The experimental protocol was filed with the State Office for Agriculture, Food and Fisheries of Mecklenburg-Vorpommern (LALLF M-V), the competent authority for animal experiments conducted at FLI Riems (file no. 7221.3-2-82-026/17). The animals were handled in accordance with all applicable European and German guidelines for the use of experimental animals.

2.3.2. Experimental Design

The first animal experiment was designed to clarify whether it is at all possible to cause an FMDV infection in cattle with naked RNA. Accordingly, the most reliable route of inoculation was used: injection into the epithelium of the tongue [14]. The tongue of the deeply sedated animals (ear tag numbers 248, 249, 251, 260, 262, and 263) was pulled out of the mouth and the 1:5 diluted RNA was injected as superficially as possible with a fine hypodermic needle until a small bleb was formed around the tip of the needle. Four injections were made and 100 µL of RNA was injected at each site, but it is not possible to say with certainty how much of the inoculum remained in the tongue epithelium [15].

In the second experiment, less invasive routes of inoculation were used. Half of the 1:5 diluted RNA (200 µL per animal) was dripped onto the tongue of the sedated animals. The tongues were inspected before application of the RNA to make sure that there were no visible breaks in the epithelium. For each animal, 2.1 mL of the further diluted RNA preparation was applied to the nasal mucosa by spraying into a nostril using a syringe-mounted atomizer (MAD Nasal, Teleflex). The stepwise application of the total volume of RNA was timed to coincide with inspiration.

2.3.3. Monitoring and Sample Collection during the Trials

Rectal body temperature was documented daily during the acclimation and experimental period. After inoculation, the animals were examined each day for signs of FMD. A clinical score was calculated by evaluation of the general attitude, feed intake, body temperature, and gait (see Table S1 in the Supplemental Material for details on the evaluation criteria).

Serum and saliva samples were collected immediately before inoculation and daily thereafter. An additional saliva sample was collected immediately after inoculation. Blood was drawn from the jugular vein into collection tubes with clotting activators (Kabevette, Kabe Labortechnik, Nümbrecht, Germany). Saliva was collected by swabbing the oral cavity with a human vaginal tampon (o.b., Johnson & Johnson, Neuss, Germany) to quickly obtain a large quantity of fluid. Samples were transferred back to the laboratory on wet ice, centrifuged immediately for 10 min at 2000× *g* and 4 °C, then aliquoted and stored at −80 °C until further analysis.

2.3.4. Necropsy

At the end of the trial or when a humane endpoint was reached, the animals were deeply sedated as described above and then euthanized by intravenous injection of an overdose of sodium pentobarbital (Release, WdT eG, Garbsen, Germany). Samples of the lesions on the tongues and in the interdigital spaces were taken at necropsy and placed in 10% formalin for histopathological examination.

For determination of the viral RNA content, small pieces of vesicular epithelium (2 mm × 2 mm) from the same sites were collected in a screw-cap tube without fixative.

2.4. Sample Processing

2.4.1. Virus Isolation and RT-qPCR in Ex Vivo Samples

The collected serum and saliva samples were checked for the presence of infectious virus by isolation on LFBK-αVβ6 cells. For this purpose, 12.5 cm^2 tissue culture flasks with 90% confluent cell monolayers were inoculated with 50 µL of sample material. The assay was performed in duplicate. After incubation for 3 days at 37 °C, the flasks were evaluated for CPE and frozen at −80 °C to disrupt the cells. After thawing, 1 mL of the lysate was passaged onto fresh LFBK-αVβ6 monolayers, incubated, and again evaluated for CPE.

The tissue samples collected for RT-qPCR were homogenized in 500 µL serum-free DMEM with a 4 mm stainless steel bead at 30 shakes/s for 3 min in a Tissue Lyser II (Qiagen, Venlo, The Netherlands). The homogenate was centrifuged for 2 min at 20,000× *g* at 4 °C, the supernatant was collected and used for RNA extraction with the QIAamp Viral RNA Kit (Qiagen).

The viral RNA content of the serum and saliva samples, as well as of vesicular fluid collected from lesions and homogenized vesicular epithelium, was quantified with the 3D RT-qPCR assay.

2.4.2. Sequence Analysis

The serum samples of all animals from day 2 of the first experiment were used for FMDV sequence analysis. RNA was extracted using the QIAamp Viral RNA Kit (Qiagen). The VP1 coding region was amplified using the previously published primers FMD-3161-F and FMD-4303-R [16] with the qScript XLT One-Step RT-qPCR ToughMix (Quanta, Beverly, MA, USA). The PCR product was purified by agarose-gel electrophoresis. Bands of the expected size (1.2 kbp) were excised, the DNA was extracted and sent to Eurofins (Ebersberg, Germany) for Sanger sequencing.

2.4.3. Histopathology

Histological sections of all tissue samples taken during the first animal experiment were prepared. These were each stained with hematoxylin and eosin (H.E.) as well as with an in-house polyclonal rabbit anti-FMDV antiserum (1:2000). Adjacent tissue sections treated with pre-immune serum served as a negative control.

Since there were no FMDV-positive ex vivo samples and no conspicuous lesions in the second animal experiment, no histological examination was performed.

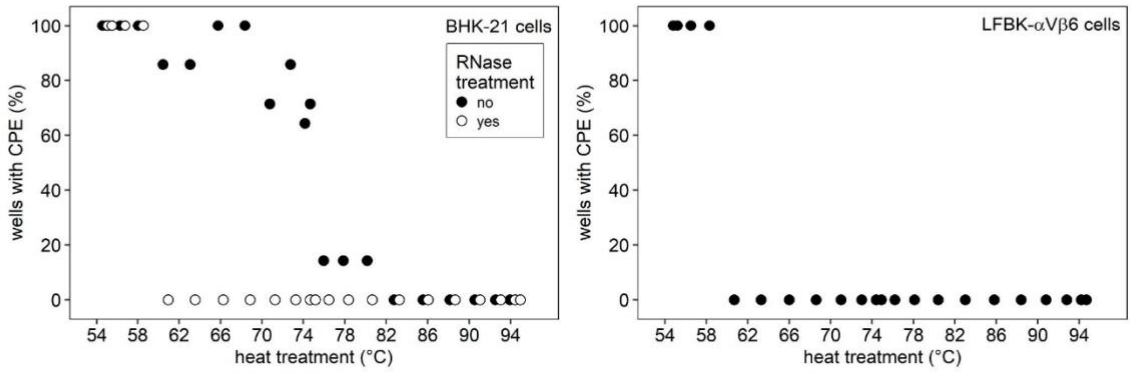
2.5. Statistical Analysis

For the statistical analysis, the animal inoculations were considered Bernoulli trials with a binary outcome (infection/no infection). Accordingly, each animal experiment comprised six independent observations, from which 95% confidence intervals for the proportion of infection were calculated using Jeffreys' method as implemented in the Epitools suite (https://epitools.ausvet.com.au/ciproportion) (accessed on 21 July 2022).

3. Results

3.1. Susceptibility of Cells to Free FMDV RNA

Without RNase treatment of the heated virus suspension, there was a clear difference between LFBK-αVβ6 and BHK-21 cells. After heating to about 60 °C for 10 min, the virus suspension no longer caused infection in LFBK-αVβ6 cells. In BHK-21 cells, on the other hand, CPE did still occur up to a temperature of about 80 °C (Figure 1). When RNase A was added to the heat-treated virus suspension before inoculation of BHK-21 cells, there no longer was any difference between the cell lines and no CPE was seen for any sample heated to at least 60 °C for 10 min.



(**a**) (**b**)

Figure 1. Inoculation of cell cultures with heat-treated FMDV. For each treatment temperature, the percentage of replicate wells that developed CPE 72 h after inoculation with the previously heated virus suspension is shown: (**a**) heat-inactivated virus preparation on BHK-21 cells, with and without RNase treatment after heating; (**b**) heat-inactivated virus preparation on LFBK-αVβ6 cells.

3.2. Virus Stocks for Animal Experiments

The preparations of FMDV A/IRN/8/2015, A_{22}/IRQ/24/64, and O/FRA/1/2001 had virus titers of $10^{7.3}$, $10^{8.4}$, and $10^{5.3}$ $TCID_{50}$/mL with C_t values of 12.3, 12.5, and 14.4, respectively. The transfection was successful for O/FRA/1/2001 down to a dilution of 10^{-2}, for A/IRN/8/2015 to 10^{-3}, and for A_{22}/IRQ/24/64 to a dilution of 10^{-4}, which corresponded to a C_t value of 26.5. Based on these results, A_{22}/IRQ/24/64 was selected for the animal experiments, because it provided the largest margin of safety for successful transfection.

The master stock of A_{22}/IRQ/24/64 used in the second animal trial had a virus titer of $10^{7.8}$ $TCID_{50}$/mL with a C_t value of 14.4.

3.3. Assessment of RNA Degradation by Atomization

The comparison of untreated and previously nebulized RNA showed no difference in transfectability. For both preparations, transfection was successful in at least one of two replicates down to a dilution of 10^{-4}.

3.4. Confirmatory Tests of RNA Inoculum

The RNA preparations used to inoculate the animals had FMDV C_t values of 13.7 in the first experiment and 13.3 in the second, corresponding to about 10^{11} copies of FMDV genome in each animal dose.

Cultures of LFBK-αVβ6 cells inoculated with the RNA preparations without transfection reagent did not exhibit any CPE in either the first or second animal experiment.

With lipofectamine, the RNA inoculum from the first experiment was successfully transfected into LFBK-αVβ6 down to a dilution of 10^{-3}. For the second experiment, in vitro transfection of the inoculum was successful down to a dilution of 10^{-4}.

3.5. Clinical Findings

In the first animal experiment, excessive salivation was observed in all animals 24 h after inoculation. In addition, two animals exhibited an increased body temperature of more than 39.5 °C (see Figure 2). Cursory examination of the oral cavity without sedation revealed conspicuously raised areas on the tongue epithelium of 4 of the 6 animals. The following day, 48 h after inoculation, all 6 animals were sedated for a comprehensive clinical exam. Clearly visible lesions were found on the tongues of all animals, corresponding to the areas of injection, but also on the upper gums. Vesicles, some of which were still intact, were found in the interdigital spaces of all animals. One animal (no. 248) was euthanized immediately, while the remaining 5 cattle received anti-inflammatory analgesics and necropsy was scheduled for the next day.

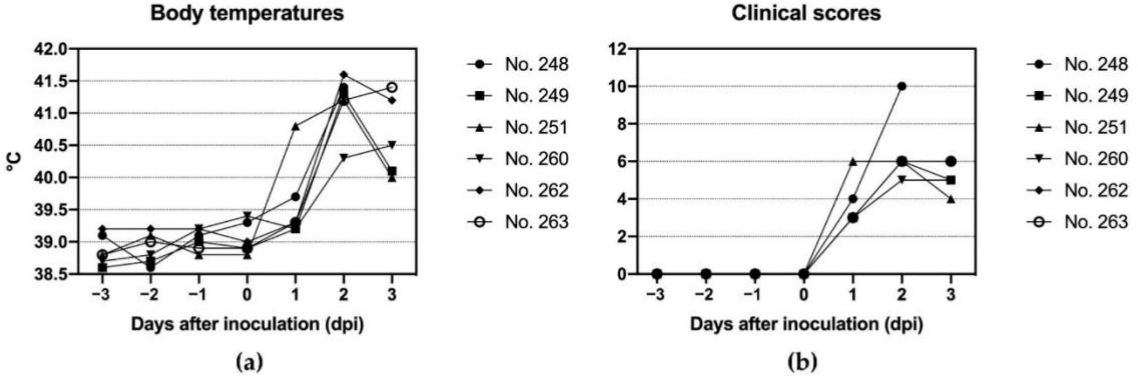


Figure 2. Clinical findings in the first animal experiment after injection of naked FMDV RNA into the tongue: (**a**) rectal body temperatures; (**b**) clinical scores. See Tables S1 and S2 in the Supplemental Material for the raw data summarized in this figure.

In contrast, no clinical signs of FMD were seen in any of the 6 cattle in the second animal experiment.

3.6. Virus Isolation

All samples taken on days 1 and 2 after inoculation in the first animal experiment were positive in the virus isolation. No positive samples were found in the second experiment.

3.7. FMDV RT-qPCR

No FMDV genome was detected in the serum and saliva samples collected before infection in the first animal experiment. Beginning on the first day after inoculation, however, a steady increase in the viral genome load was observed in serum and saliva (see Figure 3). The homogenized vesicular material of the tongue lesions had an average C_q value of 16.6 in the FMDV RT-qPCR.

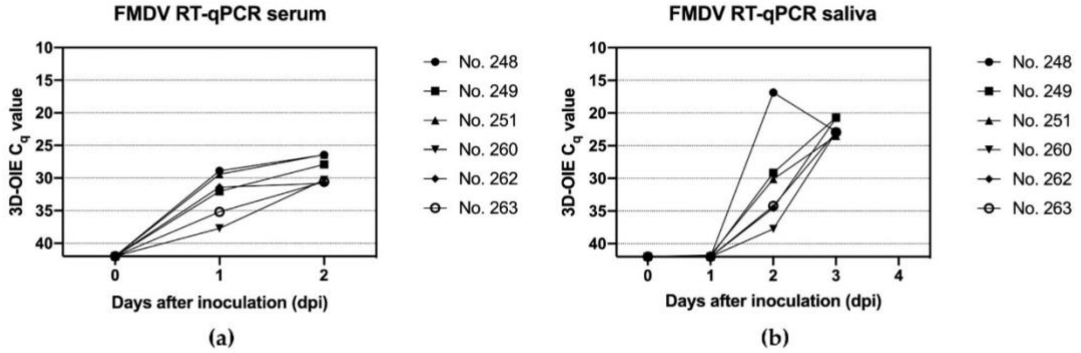


Figure 3. FMDV RT-q-PCR results after injection of naked FMDV RNA in the first animal trial: (**a**) saliva samples; (**b**) serum samples. See Table S3 in the Supplemental Material for the raw data summarized in this figure.

In the second animal experiment, no FMDV genome was detected in any sample.

3.8. Sequencing

The viral sequence recovered from the samples of the first animal experiment matched the database sequence of FMDV A_{22}/IRQ/24/64 to at least 99.7%.

3.9. Necropsy

The clinical findings of the first animal trial were confirmed during necropsy (see Figure 4). The tongues of all animals showed extensive, mostly superficial, but occasionally deeper lesions of the epithelium. Lesions were also found on the lip, gums, and hard palate as well as in the interdigital spaces. Several vesicles in the interdigital space were still intact at the time of necropsy and vesicular fluid was recovered.

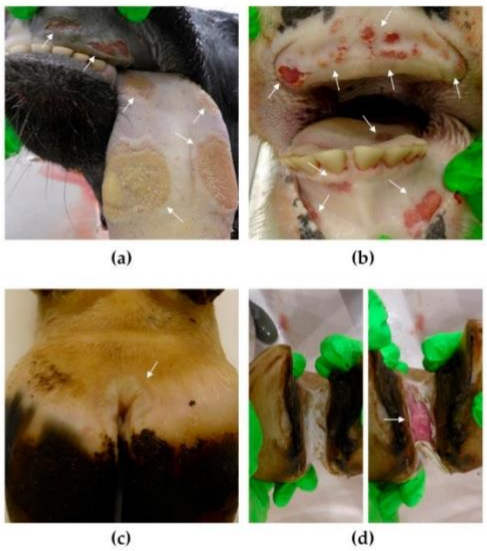


Figure 4. Postmortem findings after injection of naked FMDV RNA into the tongue. All six cattle in the first animal trial developed severe clinical signs of FMD and pathognomonic findings were made at necropsy (arrows): (**a**) lesions on the tongue, corresponding to the areas of injection, and on the upper gums; (**b**) lesions on the tongue, the upper and lower gums; (**c**) large, mostly intact lesion in the interdigital space; (**d**) the true extent of the lesion is revealed after manipulation.

No lesions of this kind were found in the second animal experiment.

3.10. Histology

Vesiculoulcerative tissue damage can be seen in the H.E. staining in all samples of the first animal experiment (Figure 5). This coincides with specific staining that indicates the presence of FMDV antigen in the cells.

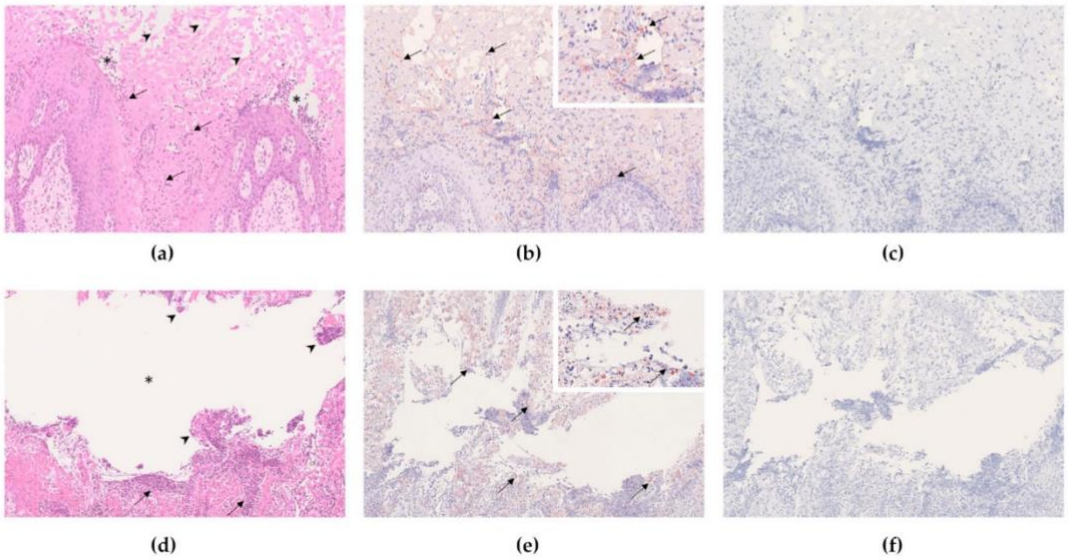


Figure 5. Pathohistological findings after injection of naked FMDV RNA into the tongue in the first animal trial ((**a**–**c**), tongue, 5× magnification; d–f, interdigital space, 10× magnification). (**a**) Animal 249, tongue, H.E. staining. The lingual mucosa shows characteristic vesiculopustular lesions containing neutrophils and sloughed epithelial cells (asterisk). The adjacent mucosa is infiltrated by neutrophils (arrows) and reveals clear intercellular spaces between swollen and degenerating keratinocytes (arrowheads). (**b**) Animal 249, tongue, anti-FMDV immunohistochemistry. Associated with the lesions as described in a), FMDV-antigen-positive cells (red–brown signal, arrows) were detected throughout the lingual mucosal epithelium. The inset shows positively stained cells at higher magnification. (**c**) Animal 249, tongue, pre-immune serum immunohistochemistry (negative control). To confirm the specificity of the anti-FMDV antibody staining, tissue sections were incubated with pre-immune serum. Unspecific binding was excluded as no positively labeled cells were detected. (**d**) Animal 260, interdigital space, H.E. staining. Overview of a large vesicle in the interdigital cleft. A large intraepidermal space (asterisk) is filled with neutrophils and degenerate keratinocytes (arrowheads). The adjacent epidermis is severely infiltrated by neutrophils (arrows) intermingled with degenerating keratinocytes. (**e**) Animal 260, interdigital space, anti-FMDV immunohistochemistry. Numerous cells within the lesion were FMDV-antigen positive (arrow). The inset shows positively stained cells at higher magnification. (**f**) Animal 260, interdigital space, pre-immune serum immunohistochemistry. As described for (**c**), unspecific binding of the FMDV antibodies was ruled out as no positive signal was detectable with the pre-immune serum.

3.11. Statistical Analysis

In the first experiment, the proportion of animals in which the injection of FMDV RNA into the tongue led to infection was 100%, with a lower bound of the 95% confidence interval of 67%.

In the second animal experiment, none of the animals developed FMD, corresponding to an observed proportion of infection after non-invasive exposure to FMDV RNA of 0%. The upper bound of the 95% confidence interval was 33%.

4. Discussion

4.1. Self-Transfection of BHK-21 Cells

The critical temperature for FMDV inactivation is about 60 °C [1]. Above this threshold, the viral capsid is denatured and infectivity is abolished. This is in contrast with the observation that BHK-21 cells can become infected by virus suspensions that were heated to 80 °C. Since this was prevented by the addition of RNase A, we assume that the infection is caused by the viral RNA that is released when the capsid is destroyed. BHK-21 cells appear to take up RNA spontaneously from the surrounding medium. In a cell culture environment, optimal conditions for the cells in terms of nutrients, temperature, partial CO_2 pressure, and ambient pH prevail, and the cellular membrane of BHK-21 cells may have become increasingly permeable over the course of many passages. This can be beneficial in certain applications, e.g., for the rescue of infectious virus in reverse genetics.

However, the potential for "self-transfection" must be kept in mind when setting up virological assays. When BHK-21 cells are used for virus titrations, CPE is not only induced by intact virions but also by naked RNA and it is conceivable that slightly higher titer readings are obtained compared to other cells. Self-transfection can also lead to divergent results in inactivation tests of FMDV, which are carried out to assess the degree of stability of the virus particle when exposed to chemicals, heat, or radiation. The treated virus preparation is inoculated into cell culture and the cultures are evaluated for the development of CPE [1,17–20]. When no CPE develops after two or more passages, the sample is considered sufficiently inactivated. If the used method of inactivation does not sufficiently degrade the viral genome, however, using BHK-21 cells for the test readout can underestimate the extent of virus inactivation.

We did not see any indication of self-transfection with LFBK-αVβ6 cells. Based on this observation and on their high sensitivity for FMDV infection, we used these cells exclusively for all tests of the RNA inoculum and the samples from the animal experiments.

4.2. Infectivity of Full-Length FMDV RNA

The first experiment clearly demonstrated that cattle can be infected with FMDV by a superficial injection of naked viral RNA into the tongue. The occurrence of lesions in the interdigital space indicates that the infection became generalized within less than two days. The calculated inoculation dose of 10^{11} FMDV genomes is an upper bound, because the RT-qPCR assay does not distinguish between whole genomes and viral RNA fragments, as long as they contain the part of the 3D-coding region targeted by the PCR.

Certainly, the large amount of viral genome from a preparation selected for high transfectability created optimal conditions for successful infection. The rapid course of disease—no slower than after inoculation of infectious FMDV [21]—suggests that a significantly lower amount of injected RNA could also cause infection, but we did not attempt to determine a minimal infectious dose. This would be of limited use, since variables such as the quality of the RNA preparation, the proportion of full-length genomes in the total viral RNA, the choice of FMDV strain, the number of inoculation sites, the anatomic location of the inoculation, and the route of application will also have a major influence on the course of infection.

When conceiving of this study, we were not aware of any prior work where FMDV RNA was injected into animals without a transfection reagent. It has since been brought to our attention that there was in fact such a study in the 1950s [22], which also found that naked RNA extracted from vesicular epithelium of cattle infected with FMDV O_2 Spain, A_5 Eystrup, or C_1 Tölz was infectious for cattle and guinea pigs.

The non-invasive application of FMDV RNA in the second experiment, i.e., instillation on the tongue and intranasal nebulization, did not result in infection despite the very high amount of viral RNA. This is probably because an intact cellular membrane of epithelial cells efficiently prevents the entry of naked RNA under natural conditions. Saliva also contains a large number of different ribonucleases [23,24]. These play an important role

in host defense [25] due to their antiviral properties, including [26] but not limited to the degradation of free RNA [27,28].

However, as soon as the barrier of at least one cell is breached, e.g., if it is nicked by a hypodermic needle, a single intact full genome that is introduced to the cytoplasm can cause a productive FMDV infection. For this to occur, it is important that the damage is just large enough for the RNA to gain entry to the cell, but not so large that the cell dies. While a similar injury can be caused by chewing on a jagged piece of plastic from a damaged LFD, it must be emphasized that this scenario is highly contrived and seems very unlikely.

The main risk of spreading FMDV over longer distances certainly lies elsewhere, such as in trade with infected animals or contaminated materials [29]. Based on our results, it can be concluded that the risk of FMDV transmission through the shipment of properly inactivated samples is negligible and it is reasonable to treat these samples as exempt from most dangerous goods regulations. However, to further reduce the risk of deliberate diversion of the material or its inadvertent release to the environment, such shipments should only be handled by reliable carriers and should be tracked closely while in transit [6].

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/v14091924/s1, Table S1: Body temperatures; Table S2: Clinical scores. Table S3: FMDV RT-qPCR results.

Author Contributions: Conceptualization, B.H., M.B. and M.E.; methodology, B.H. and M.E.; validation, H.K. and M.E.; formal analysis, H.K. and M.E.; investigation, H.K., B.L., J.S.-E. and M.E.; writing—original draft preparation, H.K.; writing—review and editing, H.K., B.L., B.H., J.S.-E., M.B. and M.E.; visualization, H.K., J.S.-E. and M.E.; supervision, M.E.; resources, M.B.; project administration, M.E. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The animal study protocol was filed with the State Office for Agriculture, Food and Fisheries of Mecklenburg-Vorpommern (LALLF M-V), the competent authority for animal experiments conducted at FLI Riems (file no. 7221.3-2-82-026/17).

Informed Consent Statement: Not applicable.

Data Availability Statement: All quantitative data from the study are available in the Supplementary Material.

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Conflicts of Interest: The authors declare no conflict of interest.

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3. Publication III: Proof of Proficiency of Decentralized Foot-and-Mouth Disease Virus Diagnostics in Germany

Publication III

Proof of Proficiency of Decentralized Foot-and-Mouth Disease Virus Diagnostics in Germany

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Brief Report

Proof of Proficiency of Decentralized Foot-and-Mouth Disease Virus Diagnostics in Germany

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Abstract: A proficiency test was performed to verify that the regional veterinary laboratories in Germany can provide reliable foot-and-mouth disease virus (FMDV) diagnostics. Overall, 24 samples were to be analyzed for FMDV-specific nucleic acids by real-time RT-PCR, and 16 samples had to be tested by ELISA for antibodies against non-structural proteins of FMDV. For both methods, a range of dilutions of the original materials (inactivated FMDV vaccine or convalescent serum from infected animals, respectively) was prepared, and negative samples were included as well. All 23 participating laboratories were able to detect FMDV genome down to a dilution of 1:100,000 of the vaccine preparation. Even at a dilution of 1:1,000,000, FMDV genome was detected by more than half of the participants. With the antibody ELISA, all sera were correctly identified by all participating laboratories. No false-positive results were returned with either method. All participating laboratories were found to be fully proficient in FMDV diagnostics.

Keywords: foot-and-mouth disease virus; proficiency test; ring trial; diagnostics; real-time RT-PCR; ELISA; exclusion diagnostics

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1. Introduction

For the first time in history, 10 years have passed without a single case of foot-and-mouth disease (FMD) in the European Union (EU). However, the disease still circulates in countries close to the EU and in many other places in Africa, the Middle East, and Asia [1].

FMD virus (FMDV) is most likely to be spread by the movement of infected animals [2], but the virus can also be transmitted indirectly, with animal products such as milk and meat posing a considerable risk [3]. This was demonstrated by the 2001 outbreak in the United Kingdom, which began in a holding where contaminated food waste of unknown origin was fed to pigs [4]. Apart from animals and their products, the virus can also be spread by contaminated vehicles, clothing, and other fomites [3,5].

Increasing global trade and travel pose a significant risk of reintroduction of FMDV to the EU and Germany. Effective control can only be achieved by early recognition of the disease [5–9]. In the event of an incursion of FMD, its rapid and reliable diagnosis is therefore of paramount importance. There is a concern, however, that clinically unclear cases are not reported by veterinarians or farmers for fear of the possible consequences [10–12]. To lower the threshold for these reports and increase diagnostic capacity, a decentralized non-discriminatory exclusion testing scheme has been established in Germany and other countries. Formally declared FMD suspicions must be investigated by an official veterinarian, and samples are sent to the designated national reference laboratory (NRL) as outlined in Article 54 of Regulation (EU) 2016/429 of the European Parliament and of the Council of 9 March 2016 ('EU Animal Health Law'). In cases that do not rise to the level of an FMD suspicion but where FMD is to be excluded as a possible differential diagnosis, practitioners can submit samples to state laboratories for real-time RT-PCR (RT-qPCR) testing without the involvement of the competent authority and without restrictions placed on the affected farm [13–15].

Besides virological detection, the tasks of the state laboratories also include serological tests. These do not play a big role in FMD exclusion testing but will be essential if the FMD-free status of Germany is to be regained after an outbreak. In the case of emergency vaccination not followed by slaughter, serological testing of large cohorts of animals must prove that there is no evidence of infection in the vaccinated population [16]. It is therefore critical to reliably differentiate between infected and vaccinated animals (DIVA).

During the production of DIVA-capable FMD vaccines, the virus is replicated in cell culture, chemically inactivated, and the non-structural proteins (NSP) produced by the infected cells are then removed. In theory, the final formulated vaccine only contains viral capsids (i.e., structural proteins) and denatured viral RNA but no NSP [17]. Vaccinated animals have antibodies only against the structural proteins present in the vaccine. By contrast, infected animals have antibodies against structural proteins but also antibodies against the NSP produced during viral replication in the host. In practice, however, there are two concerns: the NSP removal may be incomplete, leading to eventual seroconversion after repeated vaccinations (risk of false-positive results), and the extent of viral replication in vaccinated animals may be very limited, leading to weak seroconversion and poor clinical sensitivity of the serological tests on the level of an individual animal (risk of false-negative results).

In summary, the state laboratories must be able to perform rapid and reliable virological and serological examinations for FMD, in extreme cases also on a very large scale. At regular intervals, the NRL carries out a ring trial for the state laboratories to confirm their diagnostic proficiency. Each of the 23 participating laboratories was provided 24 samples for analysis by RT-qPCR for FMD-specific nucleic acids and 16 samples for detection of antibodies to non-structural proteins of FMDV by ELISA. The samples were prepared and distributed by the NRL, and the results returned by the participating labs were analyzed and compared to assess their diagnostic capability and the performance of the assays. A high level of diagnostic proficiency must be maintained by the state laboratories to reliably detect a reintroduction of FMD to Germany, effectively contain an outbreak, and quickly regain FMD-free status.

2. Materials and Methods

A total of 23 virology panels consisting of 24 samples and 19 serology panels consisting of 16 samples were prepared. The volume of each sample was 500 µL.

For detection of FMDV genome by RT-qPCR, the starting materials were commercial inactivated whole-virus FMD vaccine preparations against several serotypes. The SAT2 vaccine was diluted by 1:100,000 to a target threshold cycle (C_t) of 35, the A Argentina vaccine was diluted by 1:10,000 (C_t 28), and A_{24} Cruzeiro was diluted by 1:1000 (C_t 22). In addition, a tenfold dilution series of an O_1 Manisa/O-3039 vaccine was prepared from 1:100 to 1:1,000,000 in fetal bovine serum (FBS). FBS was also used for the negative samples. Multiple replicates were included for all samples of the serotype O dilution series, but the SAT2, A Argentina, and A_{24} vaccines were represented by only one sample. From the dilution levels 1:100, 1:1000, and 1:10,000 of the O_1 Manisa/O-3039 vaccine, three identical aliquots were provided. Of the samples with lower RNA concentrations (dilutions 1:100,000 and 1:1,000,000), a total of four aliquots were provided. Each panel included four aliquots of FMDV-free FBS (see Table 1).

Table 1. Sample panel for virological testing.

ID	Content	ID	Content	ID	Content
P01	O 1:100	P09	O 1:10,000	P17	O 1:1,000,000
P02	O 1:100	P10	O 1:100,000	P18	FMDV-negative serum
P03	O 1:100	P11	O 1:100,000	P19	FMDV-negative serum
P04	O 1:1000	P12	O 1:100,000	P20	FMDV-negative serum
P05	O 1:1000	P13	O 1:100,000	P21	FMDV-negative serum
P06	O 1:1000	P14	O 1:1,000,000	P22	SAT2 1:100,000
P07	O 1:10,000	P15	O 1:1,000,000	P23	A Argentina 1:10,000
P08	O 1:10,000	P16	O 1:1,000,000	P24	A_{24} Cruzeiro 1:1000

In the 16 samples of the serology panel, antibodies against FMDV were to be detected by ELISA. Three different sera were provided undiluted. One was obtained from a bovine vaccinated against FMDV A22 Iraq and subsequently infected with A Iran 96. It was collected on day 32 post infection. Another bovine serum was collected from an animal infected with FMDV A Iran 99 28 days after infection. To include a different host species, the serum of a goat infected with FMDV SAT2 Egypt 2014 was used. This serum was collected after 21 days.

In addition to the undiluted sera, a dilution series of an antibody-positive serum in FBS was prepared. Serum from a bovine infected with FMDV O_1 Manisa was collected on day 21 post infection and diluted, as shown in Table 2. Two identical replicates of each dilution step except 1:8 were included in the panel. The 1:8 dilution was represented by four replicates, FMDV antibody-negative serum was included three times, and there was one aliquot of each of the undiluted sera (see Table 2). All FMDV sera were tested to confirm the absence of detectable FMDV RNA and were heated to 56 °C for two hours to inactivate any residual virus.

Table 2. Sample panel for serological testing.

ID	Content	ID	Content	ID	Content
E01	Serum O_1 1:2	E06	Serum O_1 1:8	E11	FMDV antibody-free serum
E02	Serum O_1 1:2	E07	Serum O_1 1:8	E12	FMDV antibody-free serum
E03	Serum O_1 1:4	E08	Serum O_1 1:8	E13	FMDV antibody-free serum
E04	Serum O_1 1:4	E09	Serum O_1 1:32	E14	Serum A_{22}
E05	Serum O_1 1:8	E10	Serum O_1 1:32	E15	Serum A Iran 99
				E16	Serum SAT2

Each sample in each of the 43 panels was randomly assigned a unique sample number. The sample IDs shown in the tables were not disclosed to the participants. The participants were told which samples were part of the virology or serology panel but were not made aware of the identity of any of the samples or the number of replicates per sample group. Even though the samples were not infectious, the laboratories were encouraged to treat them as field samples and to process them the same way as they would a routine submission.

The sample panels were shipped unrefrigerated to the participating laboratories. Most participants received the samples the following day, and the remainder received them on the second day after dispatch. To confirm that unrefrigerated shipping did not interfere with the analysis, additional virology and serology panels were retained at the NRL. One set was stored at ambient temperature and another at −20 °C. The panels stored at ambient temperature were analyzed after 5 days and the results were compared to the panels that had been kept frozen.

A total of 23 laboratories took part in the proficiency test, including state and federal veterinary diagnostic laboratories in Arnsberg, Aulendorf, Bad Langensalza, Detmold, Dresden, Erlangen, Fellbach, Frankfurt (Oder), Freiburg, Gießen, Hamburg, Hannover,

Karlsruhe, Koblenz, Krefeld, Kronshagen, Leipzig, Neumünster, Oberschleißheim, Oldenburg, Rostock, Saarbrücken, and Stendal. All participants are accredited by DAkkS, the national accreditation body for the Federal Republic of Germany.

It was left up to the participants to decide whether they wanted to receive only the virology or serology panel or both. Virological testing was performed by all participants and all used the FMDV 3D RT-qPCR assay [18] recommended by the OIE and the EU reference laboratory for FMD. Furthermore, 12 laboratories also used an IRES assay [19] that had in the past been recommended by the NRL but is now being phased out. Serological testing was carried out by 19 participants using the ID Screen FMD NSP Competition ELISA. This kit is currently held in the national FMD diagnostics bank, which was established to ensure the supply of serological tests for Germany in case of an outbreak. The most recent tender for the bank was awarded to ID Vet in 2018. For use in the proficiency test, one test kit was provided free of charge to each state laboratory that opted to receive the serology panel.

In addition to testing the provided samples, the laboratories were asked to voluntarily report the number of virological and serological exclusion tests for FMD for each of the preceding four years (2017–2020) and provide details about their exclusion testing scheme. To verify the diagnostic specificity of the ELISA, the laboratories were requested to use the remaining reactions from the provided kits to test other samples from their routine caseload.

3. Results

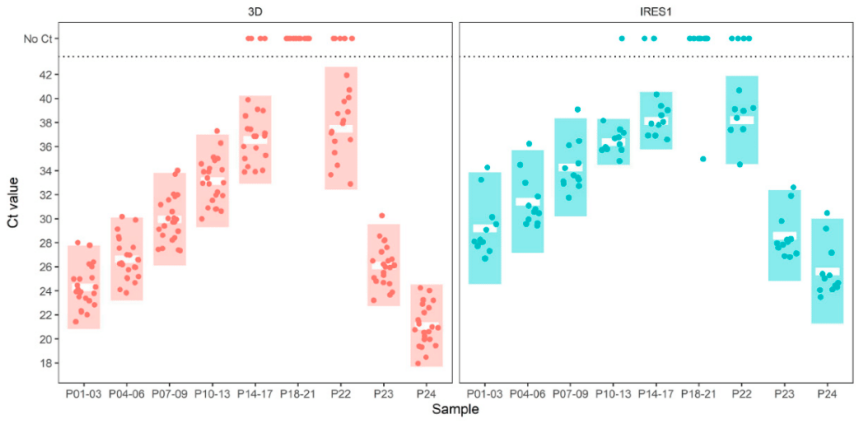
3.1. Detection of FMDV Genome by RT-qPCR

3.1.1. 3D-OIE Assay

Testing of the panels stored at the NRL confirmed the stability of the samples in the virology panel during shipping at ambient temperature. Even after 5 days at ambient temperature, C_t values had only increased by 1.3 on average compared to the frozen samples.

Up to a dilution of 1:10,000 (P07–P09; see Figure 1), all samples were correctly identified by all 23 participants using the 3D-OIE assay. At the next higher dilution of 1:100,000 (P10–P13), all but two laboratories were still able to identify all positive samples, and all laboratories returned a positive result for at least one of the four replicates of this dilution. At the 1:1,000,000 dilution (P14–P17), 60% of participants obtained positive results for all samples, 16% detected at least out of the four replicates as positive, but another 16% did not return a positive result for any of the four replicates.

Similarly, the more strongly positive samples P23 and P24 were detected reliably by all participants. Sample P22, which was the weakest sample in the virological panel, was correctly identified by 76% of the laboratories. All negative samples were correctly identified by all participants. Overall, the best results in terms of low C_t values and high clinical sensitivity were achieved with manual RNA extraction using the QIAmp Viral RNA Kit (QIAGEN, Venlo, The Netherlands) or automated extraction using the NucleoMag VET Kit (Macherey-Nagel, Düren, Germany) with a KingFisherFlex magnetic particle processor (Thermo Fisher Scientific, Waltham, MA, USA), followed by RT-qPCR with the SuperScript III One-Step RT-PCR System (Thermo Fisher Scientific).



(**a**) (**b**)

Figure 1. PCR results. The mean value of all results of each participant for a group of samples is shown as a single filled circle. The mean of means for all participants for each group of samples is represented by a white bar. The colored columns indicate the twofold standard deviation of the mean of means. (**a**) 3D-OIE assay; (**b**) IRES1 assay. See Table S1 in the Supplemental Material for the raw data summarized in this figure.

IRES1 Assay

The results obtained by the 12 laboratories using the IRES1 PCR were roughly similar to the 3D-OIE assay, but with reduced sensitivity, especially at the higher dilutions. Up to and including the 1:10,000 dilution (P07–P09), all positive samples were detected by all participants. Starting at the 1:100,000 dilution (P10–P13), however, some laboratories could no longer correctly identify some or all of the replicates. At the 1:1,000,000 dilution (P14–P17), only 23% of the participants returned positive results for all four replicates. The negative samples were identified correctly, with the exception of one laboratory, which evaluated one of the four replicates as positive.

Comparison between Assays

To analyze the spread of the PCR results obtained by the participants, the two-fold standard deviation was calculated (see Table 3). There is a clear difference between the two PCR assays. With the 3D-OIE assay, only 9% of all results returned by the participants lie outside this range, compared to 13% with the IRES1 assay. (Negative results are included in this calculation).

Table 3. Overview of the PCR results. Indicated for each sample group are the mean value and the twofold standard deviation of the results submitted by all participants.

P-No.	Dilution	C_t Value 3D-OIE		C_t Value IRES1		ΔC_t Value IRES1—3D-OIE
P01–P03	1:100	24.3	±3.5	29.2	±4.7	4.9
P04–P06	1:1000	26.6	±3.4	31.4	±4.3	4.8
P07–P09	1:10,000	30.0	±3.9	34.3	±4.1	4.3
P10–P13	1:100,000	33.1	±3.8	36.4	±1.8	3.3
P14–P17	1:1,000,000	36.6	±3.7	38.2	±2.4	1.6
P18–P20	Negative	Negative		Negative		/
P22	Undiluted	37.5	±5.1	38.2	±3.7	0.7
P23	Undiluted	26.1	±3.4	28.6	±3.8	2.5
P24	Undiluted	21.1	±3.4	25.6	±4.4	4.5

3.1.2. Detection of FMDV Antibodies Using the ID Screen FMD NSP Competition ELISA

Testing of the panels stored at the NRL confirmed the stability of the samples in the serology panel during shipping at ambient temperature. On average, after 5 days of storage, the S/N% ratios between the unrefrigerated and frozen samples differed by 2.1 percentage points, which had no impact on the interpretation of the ELISA.

All 19 participants receiving the serology panel correctly identified all samples (see Figure 2). All negative samples were also correctly evaluated as negative. A strong positive result was obtained for the 1:2 dilution (E01/02). With increasing dilution, the signal becomes weaker until it is finally below the cut-off at 1:32 (E09/10).

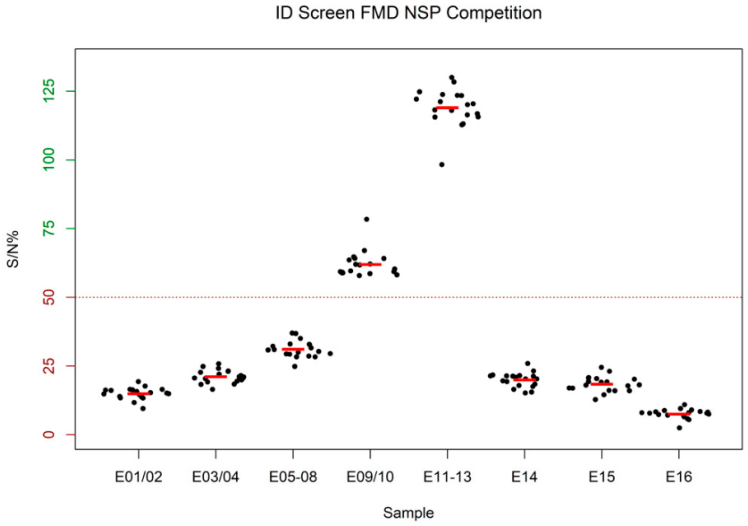


Figure 2. Results of the ID Screen FMD NSP Competition ELISA. The mean value of all results of each participant for a sample group is shown as a single filled black circle. The mean of means for all participants for each sample group is represented by a red bar. Samples with an optical density less than or equal to 50 % of the optical density of the negative control (S/N% $\leq$ 50) are considered positive. See Table S2 in the supplemental material for the raw data summarized in this figure.

3.1.3. Additional Specificity Data for the FMDV NSP Antibody ELISA

Eleven of the participating laboratories provided ELISA data for additional samples from their routine caseload. These data were also collected using the ID Screen FMD NSP Competition ELISA. With nearly 50% of the samples, the dominant animal species was cattle, followed by 20% small ruminants and approx. 30% pigs (domestic and wild boar). Some zoo animals were represented, such as six samples from Bactrian camels, two samples from okapi and Kirk's dik-dik, and one sample from a bison. A total of 2040 measurements were submitted, of which 2028 were negative and 12 were positive (Details on the positive samples can be found in Table S3 in the supplemental material). Ultimately, all positive results were considered false positives, and the overall specificity of the ELISA was calculated as 99.41%.

3.1.4. Exclusion Testing for FMD

Since Germany has been free of FMD for over 30 years, the disease has become increasingly obscure for many farmers and veterinarians. There are a wide variety of infectious and non-infectious conditions that can cause clinical signs similar to FMD, such as stomatitis, foot lesions, or lameness. When FMD cannot be ruled out based on the clinical presentation alone, a laboratory investigation is required. In Germany, samples can be submitted for FMD exclusion testing without any restrictions being placed on the farm. In order to get an indication of the acceptance of the exclusion testing scheme, the

participating laboratories were asked to report their activities for the previous four years (see Table 4).

Table 4. Total number of FMD tests at regional laboratories from 2017 to 2020.

Test	Year	Samples
FMDV RT-qPCR	2017	607
	2018	378
	2019	385
	2020	211
FMDV NSP antibody ELISA	2017	37
	2018	82
	2019	75
	2020	38

In 45% of the laboratories, exclusion tests for FMD are only carried out when specifically requested by the client. The remaining 55% also initiate tests on their own accord, e.g., if the case history includes clinical signs congruent with FMD. Of the submissions for the exclusion testing scheme, 44% come from official veterinarians, followed by 32% from practitioners and 24% from other facilities, such as universities or border control points at airports, which submit confiscated animal products. The costs are usually borne by the laboratory or by the state animal disease fund. If the samples come from necropsies carried out at the regional laboratories, the cost for laboratory tests is included in the fee to be paid by the animal owners. No fees are charged for necropsies mandated by an official veterinarian.

4. Discussion

4.1. Virology

Overall, all participating laboratories successfully detected FMDV RNA by RT-qPCR in all sample groups. In some laboratories, problems became apparent at lower RNA concentrations. This was the first national FMD ring trial that included many weakly positive samples with several replicates per dilution. In previous ring trials in Germany, smaller sample panels were used, and the participants were asked to prepare their own non-blinded serial dilutions [20]. It is expected that highly positive samples, such as fresh lesion material, can be reliably detected by RT-qPCR in any laboratory. However, not all samples conceivably collected from a suspect case of FMD contain high levels of RNA. As with many other contagious diseases, the amount of virus and thus viral RNA excreted is low early after infection, rises to a peak roughly coinciding with pronounced clinical signs, and then falls as the host's immune response increases [21]. Samples taken very early or very late or samples from old lesions may therefore contain only small amounts of FMDV RNA. It is open to debate, however, what level of analytical sensitivity is necessary to ensure adequate diagnostic sensitivity. Does it need to be pushed to the limit in a free country? Does a false negative result for a single sample with a low viral load necessarily lead to a significant delay in the detection of an outbreak? When considering this question, it is first necessary to look at the situation in the field. The veterinarian is usually consulted only in advanced clinical cases or when several animals are already affected. Even legal mandates intended to promote early detection rely on clusters of clinical cases within 7-day periods [13]. It is thus unlikely that samples are taken too early in infection. Sampling too late or from mature lesions with low virus load can be avoided by a comprehensive examination of the entire epidemiological unit rather than only the most obviously affected animal. Increasing the awareness of FMD among farmers and practitioners and encouraging submissions to exclusion testing programs will likely be more beneficial than attempting to further increase the analytical sensitivity of already very sensitive diagnostic tests. In any case, it is

important to conduct a thorough assessment of the costs, benefits, and risks of diagnostic tests in light of their intended application. The relative importance of sensitivity and specificity can vary greatly depending on the purpose of the test, e.g., exclusion testing in a free country versus surveillance to demonstrate freedom from FMDV infection after an outbreak.

As it was, the negative samples included in the virology panel were intended to detect cross-contamination between positive and negative samples during sample processing as well as a contamination of the laboratory environment by PCR products or positive control material. Future proficiency trials, however, should also include material from other vesicular disease viruses such as Senecavirus A or vesicular stomatitis viruses to better demonstrate the specificity of the RT-PCR assays used by the participating laboratories.

4.2. Serology

All participating laboratories correctly identified all positive samples, demonstrating adequate sensitivity of NSP antibody detection. Likewise, all negative samples were detected as such. However, as with the virology panel, the material used for the negative samples, commercial fetal bovine serum, was not ideal. The ELISA kits in the German diagnostic reagent bank are intended to be used for post-vaccination surveillance. To make sure that there will not be an unacceptably high rate of false-positive results when testing a vaccinated population, future proficiency trials should include sera from vaccinated but not infected animals as the negative sample set.

Beyond the negative samples provided by the NRL, the participating laboratories tested samples from their routine caseload. All samples originated from Germany. In this sample set, the specificity of the ELISA was calculated to be 99.41%. In the calculation, all positive results were considered false positives. Five of the twelve initially positive samples were negative in further tests. Another five were strongly hemolytic and bacterially contaminated plasma samples collected from wild boar carcasses, where the positive ELISA result was considered unreliable due to the poor sample quality. While there was no resolution or explanation for the two remaining positives, they were also considered to be spurious. This assumption is grounded in the fact that Germany has been free of FMD since 1988, and no outbreaks of FMD were reported anywhere in Europe during or after the study period.

In general, the quickest and easiest way to resolve a false positive result is to repeat the test after heat inactivation of the samples at 56 °C for 30 min, which can reduce many unspecific reactions [22]. If this does not succeed, it can be helpful to re-test the samples with a different assay [23]. The choice of the test to be used in this case is difficult, though. It has been shown that the two most common competitive NSP antibody ELISAs, ID Vet NSP Competition and PrioCHECK FMDV NS, can be used interchangeably [22]. So how does one interpret a positive result in one test and a negative result in the other? In this situation, neutralization tests are also unlikely to be useful since they are serotype-specific and less sensitive than most ELISAs.

If the problem cannot be resolved with the available sample material, it may be advisable to obtain a fresh sample from the animal in question (and possibly other animals from the same herd), which also avoids issues related to low sample quality. Bacterial contaminants can produce antibody-binding proteins that cause unspecific reactions [24].

It is possible, however, that the positive reaction will persist even with repeated sampling. For swine vesicular disease virus, between 1 and 3 of 1000 pigs are so-called "singleton reactors", which are serologically positive in the absence of any evidence of previous infection [25]. Similar phenomena were described for other viruses, such as bovine herpesvirus, where they are caused by cross-reactions between related viruses [26].

Ultimately, the final interpretation of each case must consider all available clinical, serological, virological, and epidemiological evidence.

4.3. Exclusion Testing

In the exclusion testing scheme, RT-qPCR plays a much larger role than NSP antibody ELISAs (1581 vs. 232 tests between 2017 and 2020) because FMD exclusion tests are usually prompted by acute clinical observations or suspicious post-mortem findings. Only a few hundred samples per year are submitted for FMD exclusion testing in Germany, which is very low compared to the number of cattle and pigs (approx. 11.3 million and 26 million) [27]. Most samples are still sent in by official veterinarians and not by practitioners. Here, further outreach could create more awareness of how important exclusion testing is and that there are no adverse consequences for the animal owner.

5. Conclusions

All participating laboratories are fully proficient in FMD diagnostics. FMDV antigen detection by RT-qPCR is routinely used for FMD exclusion tests. The number of exclusion tests is at an acceptable level but should be further increased to safeguard the freedom from FMD in Germany and the EU.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/v14051098/s1, Table S1: Virology data returned by the participants. Table S2: Serology data returned by the participants. Table S3: Field samples with false positive results in the NSP ELISA.

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CHAPTER V: DISCUSSION

V. DISCUSSION

The trade-off between the safest possible sample and the most comprehensive subsequent detection of the pathogen is an inevitable part of diagnostics. The further spread of high-impact pathogens must be prevented, but at the same time it is desirable to perform as wide a range of diagnostic tests as possible to gather essential information for disease control. A good understanding of the viral structures affected by the various inactivation methods, as well as knowledge of which viral components are the targets of which detection methods, is a prerequisite for this. Different groups of viruses can often be inactivated effectively by simple means. While enveloped viruses are inactivated by just one drop of detergent, a non-enveloped virus such as FMDV is not affected by this at all. However, it in turn is rapidly inactivated by very small amounts of acid. If virus rescue or whole genome sequencing is to be performed, other inactivation methods should be considered than for simple molecular detection, for which only short fragments of nucleic acid are required. Inactivation by gamma irradiation, for example, is very suitable for sera in which antibodies are subsequently to be detected. Especially in laboratory medicine, this method is useful since entire packages can be irradiated unopened. Nonetheless, this treatment is technologically challenging to implement and makes it impossible to carry out whole-genome assays afterwards. Overall, sample systems must be simple but effective, while allowing a variety of subsequent diagnostic tests.

Objective I: Feasibility study of simplified sample collection and transport without compromising diagnostic utility. → Publication I

In a comparative study, four different viruses, diverse in their particle structure and genome organization, were applied to six different biosample collection cards and two untreated filter papers. The cards and filters were then stored at room temperature for up to 30 days and analyzed for their inactivating and stabilizing efficacy.

Most of the cards and filters used achieved a satisfactory reduction of infectivity for almost all viruses after just one day. However, the results strongly depended on the cards or filter types as well as on the storage time and the virus. For example, the representative of the enveloped DNA viruses, lumpy skin disease virus, showed little or no reduction in some cards even after 30 days.

Due to the fact that the inactivation of the DNA virus was not achieved by all cards and filters to the necessary level, and also some cards and filters did not achieve this with RNA viruses, it is still advisable to consider the sample material on the card as potentially infectious in the first instance [196]. In diagnostics, this may well be used as an advantage for virus isolation and closer characterization. But what can be said about biosafety? The risk of disease transmission during sample transport is minimized on three levels and can be considered very low overall [196,275]. The first level consists of immobilizing the sample by drying into the fibers of the cards [185]. Second, the sample fields are secured by a flip-down protective cover. The last barrier consists of the sealed envelope in which the cards are shipped which additionally prevents it from exposure to the environment [275]. Care must be taken to ensure that the specimen is applied exclusively to the designated collection areas on the card, as otherwise the chemical additives cannot act on the sample and the protective cover may not cover it entirely. As long as the immobilization of the sample is not reversed by contact with liquids, contamination of the environment by the pathogens applied to the cards is very unlikely. Accidental contamination due to leaking sample tubes is therefore not possible [199,276]. Furthermore, the transmission route of the applied viruses plays an important role in the assessment of the risk posed by the cards during transport. Nevertheless, if there is clear evidence of the presence of one or more etiologic agents, the packaging and labeling of the cards must adhere to dangerous goods regulations [275] or additional inactivation procedures should be undertaken [189].

Samples spotted on cards or filter paper can be used for a variety of virological and serological tests [277]. In our experiments, the amount of nucleic acid that can be recovered from the card and detected by real-time RT-PCR, within a virus and a card or filter type, remained approximately constant over 30 days. This is true for the RNA viruses as well as for the DNA virus. However, compared to the original virus culture supernatant that was applied to the card, on average an increase by 6 C_q values (corresponding to a 64-fold reduction of nucleic acid or an unrecoverable fraction of around 98%, respectively) was observed. This value varied greatly within a range of approximately 3 to 10 C_q values depending on the card and filter used as well as the type of viral nucleic acid. There was an average loss of 6.5 C_q values for the RNA viruses and 5.6 C_q values for the DNA virus. Another study on the release of nucleic acid from biosample collection cards came to a similar conclusion [278]. Here, the average loss for the RNA virus was 9-11 C_q values and for the DNA virus approximately 5-7 C_q values [278]. Hence,

the effect occurred more pronounced with RNA there as well. One possible explanation for this is the more complex secondary and tertiary structure of RNA, which may render it more difficult to release from the fiber meshwork of the cards [278]. Furthermore, due to its biochemical structure, RNA is considered intrinsically less stable than DNA [67]. Nevertheless, the lower resistance to environmental influence is probably mainly due to the activity of nucleases [67]. Thus, a different degree of protection of RNA from nucleases by the different cards could explain the dependence of the effect on card types in our work. Another consideration is the amount of sample used. Depending on the manufacturer and sample matrix, approximately 125µl of sample is applied per collection area. In our case, only a quarter of the spot was used for further diagnostics. Many other studies use a punch to obtain small discs, usually 3 mm in diameter [197,278–280]. Depending on the study, approximately one to three discs are used for one subsequent analysis [197,278–280]. In most cases, the sample amount is thus much smaller compared to the amount used in a conventional extraction from a liquid sample (MN, 2018). Of course, this has advantages in sampling, since one needle prick e.g. on the ear is often enough to collect sufficient sample material, which can facilitate the performance of large studies by reduced stress for the animals, easier handling and cost savings in the materials [199,276]. Furthermore, no laboratory equipment such as centrifuges are required for sample preparation [199]. However, the detection from a smaller volume requires a more stringent validation of the downstream diagnostics in order to avoid compromising sensitivity and specificity [160]. One option is to punch out more discs [178] or discs with larger diameters for elution. However, studies have shown that conventional PCR is not significantly affected by disc size in the range of 0.3-5mm^2 [74]. One advantage of the smaller discs is certainly that this allows a larger number of tests from only one sample spot.

Our results are in contrast to many other studies that have reported only small losses in PCR sensitivity [281,282]. In this context, the sample material applied to the cards must also be taken into account. Using blood, for example, biosample collection cards may even have a positive effect on the amplification of nucleic acid, since PCR inhibitors such as proteins, hemoglobin, and iron are increasingly less eluted with time, whereas this effect does not apply to nucleic acid [283]. Another study came to a similar conclusion, but attributed the improved amplification of the nucleic acid to a successful prior removal of the inhibitors by washing the discs [284], which, again, has no influence on the immobilized nucleic acid [277]. Our study

used the supernatant of virus cultures, which gives optimal results in PCR, but is not a sample type commonly encountered in diagnostics.

Detection by real-time RT-PCR requires only relatively small fragments of the genome, whereas longer fragments must be present for sequencing and complete genomes are needed for transfection. Depending on the cards and filters used, all three procedures were successfully performed for FMDV, which allows a broad characterization of the pathogen in the same way as non-inactivated samples. The range of diagnostic possibilities is therefore not necessarily reduced by the use of the cards. However, the transfection experiments were all performed after only one day of storage at room temperature to test the feasibility per se. It would have to be further evaluated to what extent a longer storage at unfavorable temperatures could increase the fragmentation of the genome or cause other difficulties for downstream diagnostics. These have been reported, for instance, for samples with low antibody levels. Prolonged storage had a negative effect on the OD ratio [203], which can impair seroconversion studies, for example [203].

Objective II: Investigation of the infectivity of single-stranded positive-sense RNA and the associated risk of infection during sample transport. → Publication II

Considering that single-stranded positive-sense RNA is infectious in principle, the risk of FMDV transmission from inactivated samples needed to be assessed. For this purpose, groups of six cattle each were exposed to naked FMDV RNA by different routes in two animal experiments. There are many different possible routes of infection of FMDV, both natural and artificial. In cattle, needle inoculation of the virus into the epithelium of the tongue is considered the most sensitive [285]. Other routes such as intranasal, intratracheal, via the tonsillar sinus, via a retropharyngeal spray, intramuscular, intraperitoneal or intravenous injection, and even artificial insemination have been used experimentally in cattle and to some extent in sheep [285]. In pigs, intradermal inoculation into the coronary band and oral application are also mentioned [285]. The choice of injection into the tongue as the mode of application in the first trial was intended to demonstrate whether infection is at all possible under the most favorable conditions. Since this proved successful, the second experiment was conducted to test more realistic transmission paths in practice. For this purpose, the naked genome of FMDV was applied to six cattle by dripping it on the tongue and nebulizing it into the nose

during inhalation. That no productive infection occurred in any of the cattle supports the assumption that the epithelium provides an adequate barrier to the entry of nucleic acids and successfully prevents cell infection.

Thus, epithelial injury is the basis for successful infection with FMDV by injection of naked viral nucleic acid into the tongue. In agricultural practice, such tongue injuries can be caused in cattle by a variety of other diseases [286] or mechanically by, for example, feeding coarse or thorny forage [229,287]. Other common external injuries can occur in pigs from being kept on hard ground, in dairy cows from teat injuries caused by milking machines, or in sheep as a result of foot rot [229]. Nevertheless, these are probably of little significance for the transmission of FMDV via biosample collection cards, since there is no realistic scenario where domestic animals on farms are exposed to the cards. Of greater practical importance may be the exposure of free-ranging domesticated or wild animals. Due to the urban and peri-urban husbandry of ruminants in some regions, foraging can take place in roadside trash piles. It is conceivable that the resulting injuries to the oral mucosa could allow infection with FMDV from an ingested biosample collection card. Misdelivered cards that end up in the environment therefore represent a potential risk of infection. With our experiments, we have shown that injury is a prerequisite for infection and that the viral nucleic acid cannot make its way into the cell either orally or nasally without it. Even if the adsorbed sample is mobilized by adverse conditions, particularly high humidity, there is no risk of transmission in the absence of injury, even if the air contaminated with viral RNA is inhaled. In order to exactly reproduce the realistic case, further studies are conceivable in which the cards are mixed with moist and coarse feed in whole or in small pieces.

The risk of infection could also be tested in other animal species, like goats, sheep or pigs. Due to its foraging behavior, goats certainly have a greater potential to ingest a card on the side of the road than, for example, a cow. Goats are considered to be more selective eaters than cattle [288], nevertheless, the diet of free-ranging goats in urban areas also includes scrap paper, posters on walls, clothing and market waste [289]. Sheep avoid dry material when leaves, stems, and moist feedstuffs are available [288]. Wild boar will seek out and consume garbage, which sometimes makes up the main part of their diet in urban environments [290]. Pigs are considered less susceptible to airborne FMDV [291] but more susceptible to oral infection [285]. In contrast, oral infection of cattle with FMDV is considered difficult [292].

However, the main modes of transmission of FMD virus over long distances certainly are different. The major role is played by trade in infected animals, contaminated vehicles and other equipment as well as animal products [233].

In addition to the different routes of application and different host species, another interesting follow-up experiment would be to determine the minimum infectious dose of viral RNA. In our experiments, a very high initial dose was used to test the possibility of infection per se. Now a fine-grained approach with different dilutions could follow.

These results highlight the dilemma of diagnosing infections with single-stranded positive-sense RNA viruses. Even if samples have been inactivated, they must be considered potentially infectious due to the presence of viral genome. What represents a potentially critical risk in sample transport and preparation, brings a wide range of possibilities in subsequent diagnostics, as detailed investigations can still be performed in the absence of intact viral particles. Overall, however, the risk of FMDV transmission from an inactivated diagnostic sample is considered low. To further mitigate the residual risk, it is recommended to send such samples in a way that allows tracking and tracing [293].

Objective III: Verification of proficiency of decentralized foot-and-mouth disease virus diagnostics in Germany. → Publication III

The involvement of the regional veterinary laboratories in FMDV diagnostics requires regular evaluation of the sensitivity and specificity of the virological and serological methods used. In the proficiency test described here, 23 laboratories participated, which were to analyze 24 samples by real-time RT-PCR for FMDV-specific nucleic acid and/or 16 samples by ELISA for antibodies against non-structural viral proteins. In addition, information was requested from participants on the number of virological and serological exclusion tests performed each year and other details about their exclusion testing scheme.

Only diagnostic methods that do not require handling infectious virus can be performed at the state laboratories. Genome detection by real-time RT-PCR is considered to be of similar or higher sensitivity than virus isolation [294] and is ideal for the initial detection of FMDV due to its speed and sensitivity [295]. For serological testing, the commercially available ID Screen FMD NSP Competition ELISA kit was used. The detection of antibodies to non-structural viral

proteins is intended to screen vaccinated animals for infection, which is crucial in the case of a vaccine-to-live control strategy. When designing the sample panel, importance was attached to ensuring that, in addition to strongly and moderately positive samples, very weakly positive samples were also present in several replicates. Since there is little doubt that very strongly positive samples can be reliably detected with existing methods, the objective of this study was to determine whether borderline positive samples can also be detected as such.

The serological detection of antibodies against FMDV non-structural proteins was successful in all participating laboratories.

For virological detection of FMDV, several assays are available. WOAH and the EU reference laboratory recommend primer and probe sets for the detection of the 3D region according to Callahan [296] and for the detection of the 5'UTR region according to Reid [297]. In addition to the 3D RT-qPCR, several participating laboratories used the IRES1 assay by Oem [298], which had in the past been recommended by the NRL. These laboratories have been advised that the sensitivity of the IRES1 RT-qPCR for some strains of FMD has now been found to be significantly lower than that of the 3D RT-qPCR. Therefore, this method may only be used in combination with the 3D assay as a confirmatory test. If only one method can be performed, preference must be given to the 3D RT-qPCR. With the 3D assay, all participants were able to successfully detect FMDV RNA. Sporadic difficulties occurred only in the samples with low viral RNA concentration. The extent to which very weak positive samples should be part of a proficiency test in a country that has FMD-free status, and whether this is considered necessary at all, can be debated.

Such low-level samples can be found either at the very beginning or at the end of an infection as well as in old lesions [299]. Usually, a veterinarian is only called in if there are severe clinical signs, which generally coincides with high levels of virus excretion. If the presence of FMD is to be ruled out by laboratory diagnostics, all clinically conspicuous animals must first be sampled. If there are lesions in the mouth area, on the feet or teats, material should be obtained from these.

Based on the suggestions of the European Commission for the Control of FMD (EuFMD) and the NRL for FMD, samples should generally be taken from 20 conspicuous animals from the affected epidemiological unit in order to clarify clinical signs. If fewer than 20 animals showed clinical signs, animals that have or had direct contact with the animals showing the signs

should also be sampled. However, sampling more than 20 animals from the same epidemiological unit is generally not useful. If the unit concerned comprises fewer than 20 animals in total, all animals should be sampled [210]. If these recommendations are followed, a false-negative result and thus a missed disease outbreak due to samples taken too early or too late is unlikely, and the rapid and reliable detection of strong positive samples takes priority.

The situation is different after an outbreak when proof of freedom from FMD is required. Here, both sensitivity and specificity play a much more significant role. Furthermore, the inclusion of lowly positive samples into the panel allows the identification of possible difficulties in the analysis which would not have been detectable in the range of high positive samples. By comparing the performance of all participants, laboratories having difficulty detecting weakly positive samples can identify problems and take corrective action if necessary. In real-time RT-PCR, for example, the choice of extraction methods or PCR kits can have a tremendous impact on the performance. The picture of the underperforming kits in the proficiency test was very heterogeneous, as many different kits were used for extraction and RT-PCR. In many cases, a particular combination of extraction kit and PCR chemistry was used by only one laboratory, which is why we did not want to pass judgment about the performance of a particular product based on the results of only one or a few participants. To obtain more meaningful data, a larger study would need to be conducted, but this is outside of the remit of the NRL.

Validation of test methods involves not only the initial determination of sensitivity and specificity, but also the regular verification of these parameters [300]. In this context, the validity of the test results must be constantly ensured by internal and external quality control [301]. In countries that are considered free of certain diseases, this is often difficult to carry out. The ring trial provides a good opportunity to perform this validation, as well as to practice the general handling of such critical samples in the participating laboratories. In the event of an outbreak, laboratories are faced with masses of samples, making a smooth, well-rehearsed diagnostic process crucial.

Another important aspect is raising and maintaining awareness of FMD. The state laboratories represent the first important instance of FMD exclusion testing. More than half of the participating laboratories reported that they perform exclusion tests independently of

requests by the client if the case description lists clinical signs suggestive of FMD. Since the last FMD outbreak in Germany occurred more than 30 years ago [302] and Germany, as well as the EU, are officially considered FMD-free [303], however, awareness of this disease is receding further and further into the background.

At the same time, however, the policy of non-vaccination and the open borders within Europe have created a huge naive cattle population, which is always exposed to the risk of reintroduction of FMD due to increasing globalization and movement of animals and animal products, both legally and illegally [304]. Early detection is therefore essential to allow an effective outbreak response. To achieve this, awareness of the typical clinical signs of FMD, such as initial high fever of around 40°C, increased salivation and lesions in and around the mouth, on the udder and in the interdigital spaces, must be maintained at all levels, from farmers to veterinarians to the laboratory staff [232]. One contribution to this is the recurrent proficiency test, which draws the attention of the laboratories to the still current relevance of the disease and the ever-present risk of a new introduction.

State laboratories thus play an important role in the control of FMD, as they will be the first to receive samples and carry out the exclusion tests. An outbreak has extreme consequences, such as trade restrictions and high costs for control and eradication [305]. Only early detection can minimize the consequences [301,306]. Therefore, it is important to maintain the capacity of laboratories by giving them the opportunity to validate their assays for sensitivity and specificity and ensure smooth operations in the event of an outbreak.

Altogether, the three publications discussed here provided the opportunity to look at different aspects of disease control and gain new insights. Safe, unrefrigerated sample transport, especially of samples containing single-stranded positive-sense RNA, is of great importance, especially in endemic regions. In contrast, the verification of standardized decentralized FMD diagnostics is more important in disease-free areas.

CHAPTER VI: SUMMARY

VI. SUMMARY

Ongoing globalization in the trade of goods and animals, increased human mobility, global warming, and the ever-increasing exploitation of formerly remote areas are leading to an increase in animal pathogens that can spread across continents in a very short time. This underscores the need for the development of methods that allow safe sample collection and transport while providing a broad range of diagnostic methods for rapid and standardized detection of viral pathogens with high diagnostic specificity and sensitivity. This cumulative thesis focuses on effective disease control, considering various aspects from sample collection and transport to sample processing, testing and test evaluation.

To possibly simplify sample collection and transport, the suitability of various biosample collection cards was tested. Their inactivating and stabilizing efficacy on different viruses and the impact on downstream diagnostic procedures were investigated. Depending on the virus, almost all cards and filters showed complete inactivation within a few days. Based on these samples, various diagnostic methods such as real-time (RT)-PCR, sequencing or transfection can subsequently be performed. Despite the fact that complete inactivation was not observed for all viruses, the cards are still suitable for sampling and shipping, especially in remote regions where refrigeration is not possible, as the overall risk of transmission is low.

Furthermore, the infectivity of single-stranded positive-sense viral RNA was investigated to assess the risk of transmission by an inactivated sample of a virus from this group. In a first animal experiment in cattle, it was shown that the full-length genome by itself can initiate a productive infection if it is injected into the lingual epithelium. In a second experiment, the naked RNA was applied by nebulization into the nose and by dropping onto the tongue, without epithelial injury. This did not lead to infection. Inactivated samples of viruses with such a genome orientation must therefore in principle be regarded as infectious, but the risk of transmission associated with these samples can be considered low.

Finally, the proficiency of German state veterinary laboratories was evaluated with regard to FMD diagnostics. This involved the analysis of 24 samples for FMDV-specific nucleic acid by real-time RT-PCR and 16 samples for antibodies against nonstructural proteins of FMDV by ELISA. All participants demonstrated adequate proficiency, even though the virological

detection of weakly positive samples caused sporadic problems for some laboratories. However, in an FMD-free country, this does not pose a threat to effective disease control.

The purpose of this work is to contribute to simpler yet safer animal disease diagnostics. This is especially important in remote areas, where lack of refrigeration is often the limiting factor for successful sample submission. The quality of animal disease diagnostics in general is high but it faces new challenges due to climate change and increasingly intensive contact with wildlife. In this context, research into new methods for sampling and transport, assessment of risk associated with inactivated samples of single-stranded positive-sense RNA viruses and quality assurance of decentralized diagnostics represent an important contribution. In the long run, however, it is even more important to address the underlying causes. In the spirit of One Health, increased efforts must be made to rein in climate change and the inexorable spread of humans into previously untouched regions. Perhaps it will even be possible to push back Earth Overshoot Day towards the end of the year again.

1. ZUSAMMENFASSUNG

Die stetige Globalisierung im Handel von Waren und Tieren, der immer einfacher werdende Tourismus auch über lange Strecken, die Klimaerwärmung und die stetige Neuerschließung von entlegenen Gegenden führt zu einem Mehraufkommen von Tierseuchenerregern, welche sich innerhalb kürzester Zeit Kontinente übergreifend ausbreiten können. Dies unterstreicht die Notwendigkeit der Entwicklung von Methoden, die eine sichere Probenentnahme sowie einen sicheren Probentransport ermöglichen und gleichzeitig eine breite Palette von Diagnosemethoden für den schnellen und standardisierten Nachweis viraler Erreger mit hoher diagnostischer Spezifität und Sensitivität bieten. Diese kumulative Doktorarbeit beschäftigt sich mit der effektiven Tierseuchenkontrolle, indem von der Probennahme über den Probentransport bis hin zur Probenverarbeitung, Testung und Auswertung verschiedene Aspekte betrachtet werden.

Als mögliche Erleichterung in Bezug auf Probennahme und Transport wurde in einer ersten Studie die Tauglichkeit von sog. Biosample Collection Cards getestet. Untersucht wurden deren inaktivierende und stabilisierende Wirkung auf verschiedene Viren, sowie die anschließend noch durchführbaren diagnostischen Verfahren. Virusabhängig erfolgte bei fast

allen Karten und Viren eine vollständige Inaktivierung innerhalb weniger Tage. Auf Basis dieser Proben waren anschließend eine Vielzahl an diagnostischen Methoden wie real-time (RT)-PCR, Sequenzierung oder Transfektion möglich. Trotz der nicht bei allen Viren vollständigen Inaktivierung eignen sich die Karten dennoch vor allem in entlegenen Gebieten sehr gut zur Probennahme und -versendung, da eine Kühlung nicht erforderlich ist und das Risiko einer Seuchenverschleppung durch die Karten insgesamt als gering eingestuft wird.

Des Weiteren wurde die Infektiosität von einzelsträngiger viraler RNA positiver Polarität untersucht. Hierbei sollte eine Aussage darüber getroffen werden, inwieweit ein Übertragungsrisiko von inaktivierten diagnostischen Proben dieser Virusgruppe ausgeht. In einem Tierversuch konnte im Rind gezeigt werden, dass das „nackte“ virale Genom alleine in der Lage ist, bei Injektion in die Zunge eine produktive Infektion auszulösen. In einem zweiten Experiment, in dem die RNA lediglich in die Nase gesprüht bzw. auf die unverletzte Zunge aufgetropft wurde, kam es nicht zu einer Infektion. Inaktivierte diagnostische Proben von Viren dieser Gruppe müssen also prinzipiell als infektiös angesehen werden. Das Risiko einer Seuchenverschleppung durch die inaktivierten Proben kann aber als gering angesehen werden.

Weiterhin wurde die diagnostische Kapazität der veterinärmedizinischen Untersuchungseinrichtungen der Bundesländer hinsichtlich der Diagnostik des Maul- und Klauenseuche-Virus (MKSV) überprüft. Dazu sollten die teilnehmenden Labore 24 Proben mittels real-time RT-PCR auf MKSV-spezifische Nukleinsäure untersuchen, sowie 16 Proben mittels ELISA auf Antikörper gegen Nichtstrukturproteine von MKSV. Dies gelang allen Teilnehmenden, wobei der virologische Nachweis von niedriglastigen Proben einigen Laboren Probleme bereitete. In einem MKS-freien Land stellt dies aber keine für die Tierseuchenbekämpfung relevante Einschränkung dar.

Durch diese Arbeit soll ein Beitrag zur einfacheren und dennoch sicheren Tierseuchendiagnostik geleistet werden. Vor allem in abgelegenen Gebieten ist dies von großer Bedeutung, da dort die fehlende Kühlkette oft der limitierende Faktor für eine erfolgreiche Probensammlung ist. Die Diagnostik von Tierseuchenerregern befindet sich insgesamt auf einem hohen qualitativen Niveau, sieht sich aber durch den Klimawandel und dem immer intensiver werdenden Kontakt mit Wildtieren vor neue Herausforderungen gestellt. In diesem Zusammenhang stellt die Bewertung neuer Methoden zur Probennahme

und -transport, die Risikobewertung von inaktivierten diagnostischen Proben von Viren mit einzelsträngigen RNA-Genomen positiver Polarität, sowie die Qualitätssicherung bei der dezentralen Diagnostik einen wichtigen Betrag dar. Langfristig noch bedeutender ist allerdings das Bestreben, intensiver an den auslösenden Faktoren zu arbeiten. Im Sinne von „One Health“ sollte ganz allgemein versucht werden, den Klimawandel und die fortschreitende Ausbreitung des Menschen in bisher unangetastete Regionen zumindest zu verlangsamen und somit den „Earth Overshoot Day“ vielleicht sogar wieder etwas in Richtung Jahresende zu verlagern.

CHAPTER VII: REFERENCES

VII. REFERENCES

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CHAPTER VIII: APPENDIX

VIII. APPENDIX

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3. LEGAL PERMISSIONS

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CHAPTER IX: ACKNOWLEDGMENTS

IX. ACKNOWLEDGMENTS

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