Towards a more efficient control of foot-and-mouth disease outbreaks

I n a u g u r a l d i s s e r t a t i o n

zur

Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften (Dr. rer. nat.)

der

Mathematisch-Naturwissenschaftlichen Fakultät

der

Ernst-Moritz-Arndt-Universität Greifswald

vorgelegt von

Veronika Dill

geboren am 05.08.1986

in Flensburg

Greifswald, 30.11.2017

Dekan: Prof. Dr. rer. nat. Werner Weitschies

1. Gutachter: PD Dr. rer. nat. Rainer G. Ulrich
2. Gutachter: PD Dr. med. Andi Krumbholz

Tag der Promotion: 08.03.2018

“It is not the place of science to insist on explanation – but only to observe, in hopes that the explanation will manifest itself.”

- Lawrence Stern in *Voyager*, Chapter 62, Diana Gabaldon -

Contents

List of abbreviations

Abbreviations

(+) ssRNA	single stranded RNA of positive polarity
aa	amino acid
ACFM	animal-component-free media
BHK	baby hamster kidney
BHK21C13	BHK21, clone 13
BHK-2P	BHK21C13-2P
BRAV	Bovine rhinitis A virus
BRBV	Bovine rhinitis B virus
CHO	Chinese hamster ovary
CPE	cytopathic effect
ERAV	Equine rhinitis A virus
EC	extracellular
ECM	extracellular matrix
FBS	fetal bovine serum
FMD	Foot-and-mouth disease
FMDV	Foot-and-mouth disease virus
h	hour
HS	heparan sulfate
HSPG	heparan sulfate proteoglycan
IC	intracellular
IRES	internal ribosome entry site
JMJD6	Jumonji C-domain containing protein 6
MEM	Eagle’s Minimum Essential Medium
MKS	Maul- und Klauenseuche
MKSV	Maul- und Klauenseuche Virus
NSP	non-structural proteins
OIE	Office International des Epizooties / World Organization for Animal Health
ORF	open reading frame
RGD	arginine-glycine-aspartic acid

RNA	ribonucleic acid
RT-PCR	reverse transcription-polymerase chain reaction
S	Svedberg unit
SAT	Southern African Territories
$TCID_{50}$	50% tissue culture infectious dose
UTR	untranslated region
VNT	virus neutralization test
VP	virus protein
VPg	viral genome-linked protein
WRLFMD	World Reference Laboratory for FMD

Summary

Foot-and-mouth disease virus (FMDV) is a positive-sense RNA virus of the family *Picornaviridae* that comprises of seven serotypes and is distinguished by a high contagiosity with the ability of rapid spread. Strategies for abatement and control are based on an early detection, quick initiation of retaliatory actions and mass vaccinations. Therefore, aim of the study was the development of a fast and easy method for genome sequencing as well as an investigation into the causes, why some cell lines that are mainly used for vaccine production, are resistant towards FMDV infection. Finally, adaptive sequence changes in different cell culture systems and associated effects on particle stability and immunogenicity were examined.

In case of an outbreak it is of major importance to detect and rapidly characterize the circulating virus isolate to choose an appropriate vaccine to minimize the viral spread. In addition, comprehensive genome analysis of the outbreak strain provides information about the origin of the virus and allows molecular epidemiology. A universal primer set, covering most parts of the open reading frame of the viral genome, was developed to perform quick sequence analyses, independently of the viral serotype (Paper I). Especially in endemic regions, vaccination of susceptible animal species is the main action to combat foot-and-mouth disease (FMD) in an acute outbreak situation as well as a preventive measure. Reasons, why some baby hamster kidney (BHK) cell lines are resistant towards an infection with FMDV, were examined in a second study that narrowed down the cause for this phenomenon to an impaired attachment of the virus to the cell surface. Furthermore, an alternative approach could be developed to successfully adapt the virus to the resistant vaccine-production cell line by using a FMDV-sensitive “wet-nurse” cell line (Paper II). Adaptive changes in the capsid-coding region of the viral genome caused through cultivation and passaging of the virus in different BHK cell systems were the topics of the third study. It was shown that capsid alterations are rather serotype-specific and dependent on the cell line used than influenced by the cell media. Viral titers and neutralization profiles of the adapted isolates were not affected compared to the original viruses (Paper III).

Overall, this work expanded our knowledge on the control and eradication of FMD and will support the global effort to combat the disease.

Zusammenfassung

Das Maul- und Klauenseuche-Virus (MKSV) ist ein positiv-strängiges RNA-Virus aus der Familie der *Picornaviridae*, das sich in sieben Serotypen unterteilt und sich durch eine sehr hohe Kontagiosität für Klauentiere mit der Fähigkeit zur rasanten Ausbreitung auszeichnet. Strategien zur Bekämpfung und Kontrolle basieren auf einer frühzeitigen Erkennung akuter Infektionsausbrüche, schneller Einleitung von Gegenmaßnahmen und Massenvakzinierungen. Ziel dieser Arbeit war es daher, eine schnelle und einfache Methode zur Genomsequenzierung zu entwickeln sowie die Gründe zu untersuchen, warum einige Zelllinien, die hauptsächlich zur Impfstoffherstellung eingesetzt werden, sich als resistent gegenüber Infektionen mit MKSV erweisen. Des Weiteren wurden adaptive Sequenzveränderungen in verschiedenen Zellkultursystemen untersucht und die damit verbundenen Auswirkungen auf die Partikelstabilität und Immunogenität.

Im Falle eines Ausbruchs ist die schnelle Detektion und Charakterisierung des zirkulierenden Virusisolates von großer Bedeutung, um einen passenden Impfstoff auszuwählen, der die weitere Ausbreitung des Virus eindämmt. Zusätzlich geben umfassende Sequenzanalysen des Ausbruchstammes Auskunft über dessen Herkunft und ermöglichen molekularepidemiologische Untersuchungen. Um solche Sequenzierungen schnell und einfach durchführen zu können, wurde ein universelles Primerset entwickelt, das den überwiegenden Bereich des offenen Leserahmens im Virusgenom abdeckt und Serotyp-unabhängig einsetzbar ist (Paper I). Des Weiteren zählt die Impfung empfänglicher Tierarten zu den Hauptpfeilern der Maul- und Klauenseuche (MKS)-Bekämpfung, sowohl im Falle eines akuten Ausbruchsgeschehens als auch als vorbeugende Maßnahme in Endemiegebieten. Baby Hamster Kidney (BHK)-Zelllinien sind dabei die üblicherweise zur Impfstoffproduktion verwendeten Zellen. Gründe, warum sich einige BHK-Zelllinien als resistent gegenüber einer Infektion mit MKSV erweisen, wurden in einer zweiten Studie auf die Virusanheftung an die Zelle eingrenzt. Zudem wurde ein alternativer Lösungsweg entwickelt, wie das MKSV mittels einer MKSV-sensitiven „Ammenzelle“ erfolgreich auf eine resistente Produktionszelllinie adaptiert werden kann (Paper II). Darüber hinaus wurden in einer dritten Studie adaptive Änderungen in der kapsidkodierenden Region des Virusgenoms durch Kultivierung und Passagierung des Virus in verschiedenen BHK-Zellsystemen untersucht. Es zeigte sich, dass auftretende Kapsidveränderungen serotypspezifisch zu sein scheinen und durch die verwendete BHK-Zelllinie beeinflusst werden, während das verwendete Zellmedium eine

untergeordnete Rolle spielt. Virustiter und Neutralisationsprofil der adaptierten Virusisolate unterschieden sich nicht von denen der Ausgangsisolate (Paper III).

Zusammenfassend erweitert diese Arbeit unsere Kenntnis in unterschiedlichen Bereichen der MKS-Bekämpfung und unterstützt somit die weltweiten Bemühungen zur Ausrottung dieser Erkrankung.

1. Introduction

1.1 Taxonomy and classification of foot-and-mouth disease virus (FMDV)

FMDV belongs to the order *Picornavirales*, family *Picornaviridae* (Grubman and Baxt, 2004; Zell et al., 2017). Next to the species *Bovine rhinitis A virus* (BRAV), *Bovine rhinitis B virus* (BRBV) and *Equine rhinitis A virus* (ERAV), FMDV belongs to the genus *Aphthovirus* (Zell et al., 2017). It is the type species, eponymous for the genus (*aphtho*: from the Greek word "*aphthae*", which can be translated as "vesicles in the mouth") (Zell et al., 2017). FMDV is classified into seven serotypes, which can be differentiated from each other by their antigenic characteristics (Jamal and Belsham, 2013; Knowles and Samuel, 2003). Serotype O ("Oise") and serotype A ("Allemagne") were named after their place of origin by Vallée and Carré, who first identified these serotypes (Vallée and Carré, 1922). In 1926 a third serotype, serotype C, was discovered (Waldmann and Trautwein, 1926). Three further serotypes, Southern African Territories 1 to 3 (SAT 1-3) (Brooksby, 1958) and the seventh serotype, Asia-1, were designated after their primary area of circulation (Dhanda et al., 1957). Based on the percentage of nucleotide differences in the virus protein 1 (VP1)-coding gene, each serotype can be additionally split into distinct genetic lineages, strains and geographically clustered topotypes (Ayelet et al., 2009; Knowles and Samuel, 2003).

1.2 Virion and genome structure

The virion is a sphere-shaped icosahedron without a lipid envelope, approximately 25 to 30 nm in diameter with a smooth surface that distinguishes it from other picornaviruses (Acharya et al., 1989; Han et al., 2015). The capsid is composed of four proteins, named virus protein (VP) 1, VP2, VP3 and VP4 (Carrillo et al., 2005; Han et al., 2015). While VP1, VP2 and VP3 are exposed at the outer surface, VP4 is located inside the protein shell (Acharya et al., 1989; Domingo et al., 2002). Each VP composes of an eight-stranded beta-sandwich with the single strands labeled B, I, D, G, C, H, E and F (Mateu, 1995). The connecting loops are termed after the beta-strand they are connecting (Mateu, 1995). While the antigenic sites vary among serotypes, there is one major antigenic site located within the G-H loop of VP1 that is common to all serotypes (Grubman and Baxt, 2004; Mateu, 1995). It consists of three amino acids: arginine, glycine and aspartic acid (RGD) (Domingo et al., 2002; Grubman and Baxt, 2004) and fulfills a dual purpose: the RGD motif functions not only in antibody binding; it also recognizes integrins as cellular receptors for FMDV (Alcala et al., 2001; Berinstein et al., 1995; Domingo et al., 2002). The assembly of the viral capsid is made of one copy of each

structural protein that forms a protomer. In the following, five protomers build a pentamer and the whole capsid comprises 12 pentamers or 60 protomers (Domingo et al., 2002; Han et al., 2015) (Figure 1).

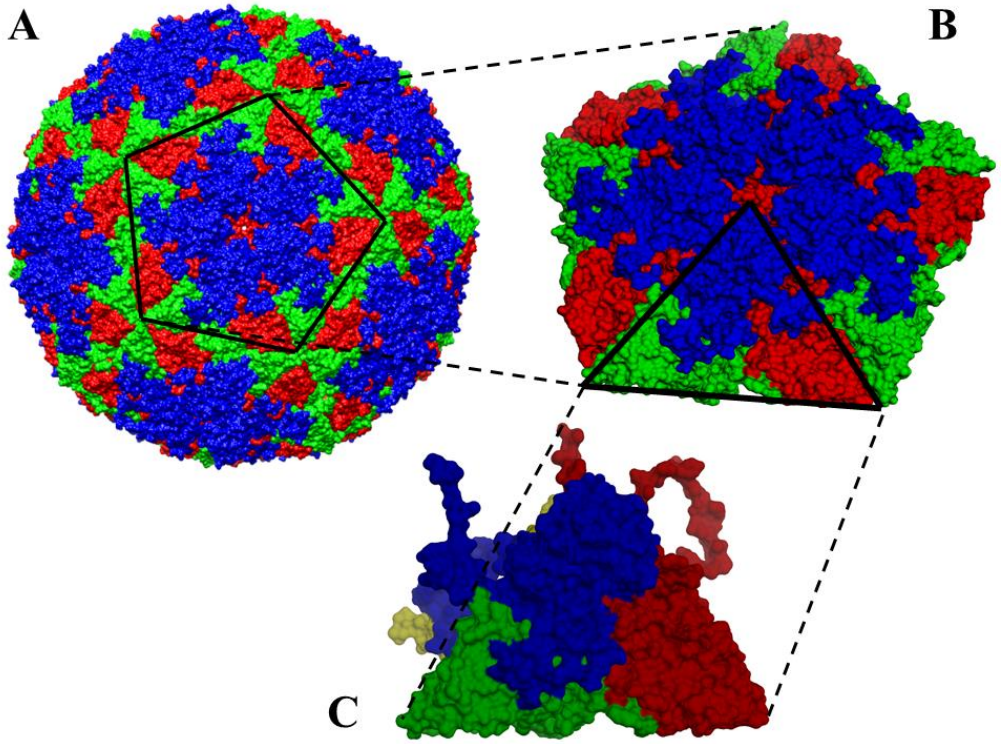

Figure 1: Icosahedrally structured FMDV particle. The assembled virus particle (A) is built of 12 identical pentameric subunits (B). Each pentamer contains five identical protomers (C) that are made of the outer capsid proteins VP1 (blue), VP2 (green) and VP3 (red), while VP4 (yellow) is located inside.
The figure was made using the X-ray crystal structures of O_1 Manisa (Logan et al., 1993) (1FOD) as template, edited with the UCSF Chimera package (Pettersen et al., 2004) and the embedded software from the MSMS package (Sanner et al., 1996). Chimera was developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco, USA (supported by NIGMS P41-GM103311).

A hole at the fivefold axis enables the entry of small molecules like caesium ions, resulting in the highest buoyant density among the picornaviruses (Acharya et al., 1989; Grubman and Baxt, 2004). When sedimenting a FMDV preparation through a sucrose density gradient, three different products can be observed: 12S particles that result from capsid dissociation into pentamers (Harmsen et al., 2011; Sangar et al., 1976), intact virions including RNA, referred to as 146S particles and empty 75S particles (Doel and Chong, 1982; Harmsen et al., 2011).

The virus genome consists of a single-stranded, positive sense RNA ((+) ssRNA), approximately 8.4 kilobases in length (Jamal and Belsham, 2013) (Figure 2). The genome

starts with the 5′ untranslated region (UTR), which is linked to a viral genome-linked protein (VPg). Among other structural elements, the internal ribosome entry site (IRES) is located within the 5´ UTR. Following the IRES, there is one open reading frame (ORF) that encodes for a single polyprotein (Belsham, 2005). Viral proteases cleave the polyprotein into four different structural capsid proteins and into eleven non-structural proteins (NSP) via different precursors (Jamal and Belsham, 2013). The NSP are responsible for genome replication and protein processing. The 3′ end of the genome consists of the 3′ UTR, containing a stem-loop structure and a poly-A tract (Belsham, 2005).

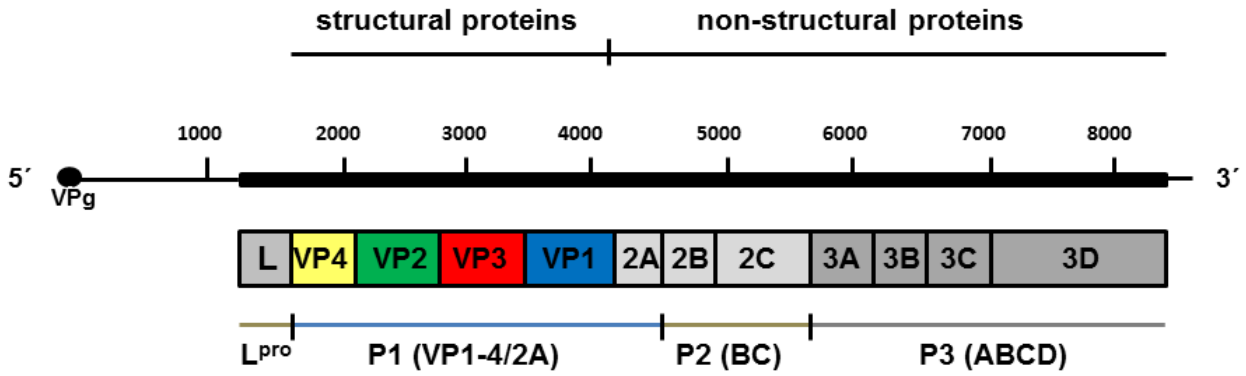

Figure 2: Genome organization of FMDV. The (+) ssRNA genome is approximately 8400 bases long and contains a single ORF, encoding structural and non-structural proteins. The Leader protease (L^{pro}) and three precursor proteins (P1, P2 and P3) are initially formed and are further cleaved by viral proteases. The figure is based on Jamal and Belsham, 2013 with modifications.

1.3 Host association and pathogenesis

FMDV is characterized by a wide range of host species, predominantly cloven-hoofed animals (order: Artiodactyla), including domesticated pigs and ruminants as well as a wide variety of wildlife species (Alexandersen et al., 2003; Schaftenaar, 2002). The severity of disease manifestation varies greatly from subclinical to fatal in wildlife species (Arzt et al., 2011). In domestic small ruminants, such as sheep and goats, infection can often go unnoticed (Alexandersen et al., 2003; Stenfeldt et al., 2016), whereas cattle generally develop mild to moderate clinical disease and pigs show the most severe course of infection (Stenfeldt et al., 2016). Vesicular lesions in and around the mouth and on the feet are the key sign of foot-and-mouth disease (FMD), although the muzzle, udder, vulva and other sites of the skin can be affected, too (Alexandersen et al., 2003). The pain caused by these vesicles leads to lameness, extreme salivation, mostly in cattle, and inappetence (Alexandersen et al., 2003; Stenfeldt et al., 2016). In extreme cases, particularly in pigs, shedding of hooves is observed. In young

animals, the disease can take a deadly course due to acute myocarditis (so called “tiger heart”, characterized by white stripes on the heart), but in general the mortality is low (Alexandersen et al., 2003). Common transmission routes are by direct contact with infected animals or by indirect contact, for instance through a contaminated environment (feed trough, rubber boots, vehicles) (Alexandersen et al., 2003). Airborne transmission is possible under certain climatic conditions (Gloster et al., 1982; Sellers and Parker, 1969). Infection via the oral route through contaminated feed such as untreated food waste is possible, too (Knowles et al., 2001).

Next to an acute course of the disease, FMDV harbors the potential to initiate a persistent infection in ruminants (Alexandersen et al., 2003). Defined by the World Organization for Animal Health (OIE), a carrier animal is an animal from which infectious FMDV can be recovered for longer than 28 days after infection (OIE, 2017a). The sites of persistence are located in the bovine nasopharynx (Stenfeldt and Belsham, 2012) and regional lymph nodes (Juleff et al., 2008). Upon exposure to FMDV, an animal can become persistently infected even with prior vaccination and in the absence of obvious clinical disease (Stenfeldt et al., 2016).

1.4 Cellular receptors for FMDV

The natural receptor for FMDV in the host and the primary receptors in cell culture are surface molecules called integrins (O'Donnell et al., 2009). Integrins are integral membrane proteins that consist of an alpha (α) and a beta (β) subunit, which can be assembled in a broad variety of heterodimers (Wang et al., 2015). They play an important role in cell signaling through conformational changes that facilitate outside-in and inside-out signaling (Stewart and Nemerow, 2007). In addition, integrins bind to extracellular matrix (ECM) proteins by recognizing RGD motifs and mediate cell adhesion (Ruiz-Saenz et al., 2009), and these motifs are also used by FMDV to bind the cell and induce internalization (Stewart and Nemerow, 2007) (Figure 3). In total, four different integrin heterodimers (αvβ1, αvβ3, αvβ6 and αvβ8) are utilized as cellular receptors by FMDV (Jackson et al., 2004; Jackson et al., 2002; Jackson et al., 2000; Neff et al., 1998).

Although cells used for FMDV culture offer at least one of the above named integrins in varying extents (King et al., 2011; Lawrence et al., 2013), subsequent passaging of FMDV in culture leads to acquisition of heparan sulfate proteoglycans (HSPG) as secondary receptors (Jackson et al., 1996) (Figure 3). Similar to integrins, HSPG belongs to the type I transmembrane proteins and is also responsible for ligand binding, especially binding of ECM components to epithelial cells, internalization events and intracellular signaling (Bernfield et

al., 1999; Wang et al., 2015). Adaption of FMDV to heparan sulfate (HS) often leads to attenuation of the field strain in the natural host (Sa-Carvalho et al., 1997), indicating that the mutations that promote growth in culture are leading to a disadvantage for pathogenesis in the natural host.

Nevertheless, virus mutants with extended receptor tropism independent of integrin and HS can arise in cell culture, initiating infection via an unknown third receptor (Berryman et al., 2013; Mohapatra et al., 2015). Recently, Jumonji C-domain containing protein 6 (JMJD6) has been proposed as an additional candidate receptor (Lawrence et al., 2016; Lawrence and Rieder, 2017) (Figure 3).

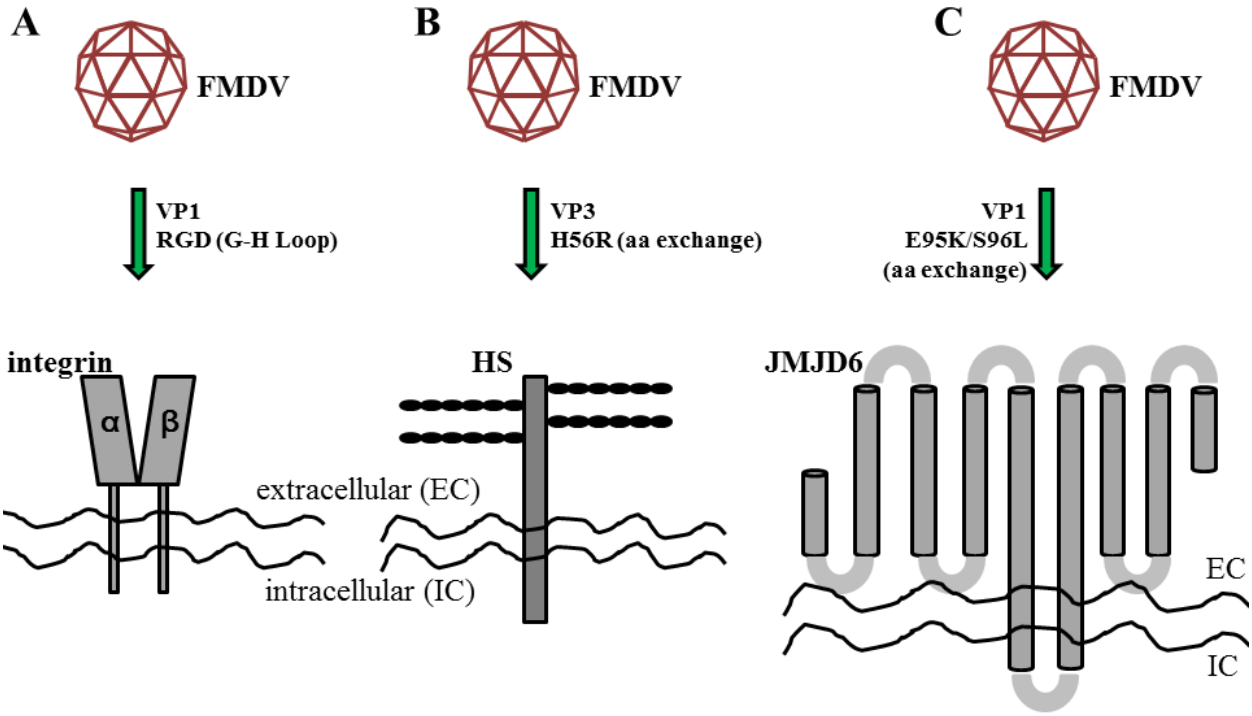

Figure 3: Cellular receptors of FMDV. Integrins are the primary receptors of FMDV, used by field isolates (A). In cell culture, the virus adapts to utilize heparan sulfate (HS) as secondary receptor by a single amino acid (aa) exchange in VP3 (B). Sometimes the usage of a tertiary receptor occurs by distinct aa exchanges in VP1, likely Jumonji C-domain containing protein 6 (JMJD6) (C). The figure is based on Lawrence and Rieder, 2017, with modifications.

1.5 Global distribution and economic impact of FMD

While North America, large parts of South America, Europe and Australia are free of FMD, the disease still occurs endemically in South and Southeast Asia, Africa and the Middle East, and sporadic outbreaks are reported from South America and Russia (Figure 4).

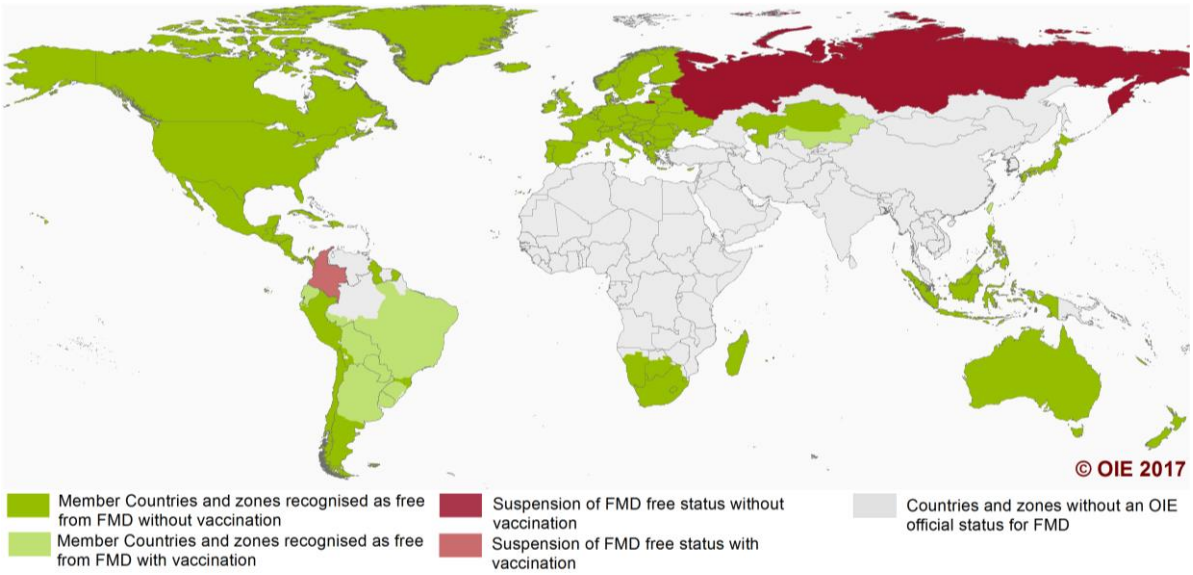

Figure 4: Official FMD status map for OIE member countries. Highlighted in green are countries that are free of FMD, either with (light green) or without (dark green) vaccination. Recent outbreaks of FMD in Mauritius, Colombia and Russia lead to the suspension of their FMD-free status (light and dark red). Grey areas do not have an official FMD status, which indicates endemic occurrence of the disease or a lack of data. Data last updated in October 2017 and kindly provided from the OIE: http://www.oie.int/en/animal-health-in-the-world/official-disease-status/fmd/en-fmd-carte/

FMDV is an OIE-listed pathogen (OIE, 2017b) and therefore outbreaks come along with a huge impact and high economic costs for the affected countries. The losses due to an outbreak can be divided into direct and indirect losses, independent of a country's FMD status (James and Rushton, 2002). Direct losses occur as limited livestock productivity and malnutrition in FMD endemic areas, while FMD free countries have to face giant costs to regain a FMD free status after an outbreak (Knight-Jones and Rushton, 2013). Restrictions to national and international trade markets as well as significant costs for disease control and management programs, including vaccination, cause high indirect losses to the country's economy (Knight-Jones and Rushton, 2013).

1.6 Obstacles to FMD control and eradication

To control an outbreak in formerly FMD-free countries, sanitary measures such as culling of infected and in-contact animals, disinfection and trade restrictions in combination with vaccination to prevent rapid spread of the disease have been applied (Saeed et al., 2015). Molecular epidemiological tracing is necessary to reveal the origin of the outbreak, to reconstruct transmission events and to retrace the viral spread (Cottam et al., 2008b).

In countries with endemic occurrence of FMD, regular mass vaccination is the control strategy of choice (Saeed et al., 2015). Unfortunately, inactivated vaccines have several limitations due to the nature of FMDV. Because of the antigenically distinct serotypes and many intratypic variants of FMDV, vaccines offer no or only little cross-protection and must closely match the circulating field strain to be effective (Rodriguez and Gay, 2011). A lack of knowledge about circulating subtypes can lead to poor outcomes of vaccination campaigns (Saeed et al., 2015). Nucleotide sequence data give not only information about the identity of the field strain, but also provide insight into geographical and temporal movement patterns of the virus (Abdul-Hamid et al., 2011).

Even with a matching vaccine, vaccine quantity and quality can be limiting factors (Saeed et al., 2015). The potency of a vaccine is mainly determined by its content of intact 146S particles (Doel and Chong, 1982) but their stability differs among serotypes (Parida, 2009). Baby hamster kidney (BHK) cells, clone 13, are mainly used to produce FMDV vaccines (Capstick et al., 1962), necessitating an adaption of the virus to cell culture, which leads to altered receptor tropism and antigenicity (Amadori et al., 1994; Jackson et al., 1996). In addition, the occurrence of BHK cells resistant to infection with FMDV harbors serious problems for the efficient cultivation of the virus for vaccine production (Clarke and Spier, 1980).

2. Objectives

FMDV is still a scourge of livestock in many parts of the world, contributing to poverty and malnutrition in endemic areas and causing high economic burdens in case of an outbreak, especially for FMD-free countries (Knight-Jones and Rushton, 2013; Thompson et al., 2002). The difficulties in eradicating this highly contagious disease demonstrate the need for a simple tool to support control measures, applicable even for emerging nations and developing countries. A better understanding of the changes to the virus that take place during cell culture adaption will improve the development of efficient vaccines. Thus, the main objectives of the study were:

I) To develop a quick and simple sequencing strategy to easily detect changes in the viral genome and support molecular diagnostics and epidemiology in an outbreak.

II) To investigate the causes of resistance of certain BHK21 cells towards infection with FMDV and how to overcome this for efficient vaccine production.

III) To examine changes in the viral genome during adaption to cell culture and their implications for the stability and immunogenicity of the virus particle.

3. Publications

I. Simple, quick and cost-efficient: A universal RT-PCR and sequencing strategy for genomic characterisation of foot-and-mouth disease viruses

Veronika Dill, Martin Beer, Bernd Hoffmann

Journal of Virological Methods 246 (2017) 58–64

doi: 10.1016/j.jviromet.2017.04.007.

Journal of Virological Methods 246 (2017) 58–64

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Simple, quick and cost-efficient: A universal RT-PCR and sequencing strategy for genomic characterisation of foot-and-mouth disease viruses

V. Dill, M. Beer, B. Hoffmann*

Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, Südufer 10, 17493 Greifswald-Insel Riems, Germany

ARTICLE INFO

Keywords:
Foot-and-mouth disease virus
Genome sequencing
RT-PCR
Sanger sequencing

ABSTRACT

Foot-and-mouth disease (FMD) is a major contributor to poverty and food insecurity in Africa and Asia, and it is one of the biggest threats to agriculture in highly developed countries. As FMD is extremely contagious, strategies for its prevention, early detection, and the immediate characterisation of outbreak strains are of great importance. The generation of whole-genome sequences enables phylogenetic characterisation, the epidemiological tracing of virus transmission pathways and is supportive in disease control strategies. This study describes the development and validation of a rapid, universal and cost-efficient RT-PCR system to generate genome sequences of FMDV, reaching from the IRES to the end of the open reading frame. The method was evaluated using twelve different virus strains covering all seven serotypes of FMDV. Additionally, samples from experimentally infected animals were tested to mimic diagnostic field samples. All primer pairs showed a robust amplification with a high sensitivity for all serotypes.

In summary, the described assay is suitable for the generation of FMDV sequences from all serotypes to allow immediate phylogenetic analysis, detailed genotyping and molecular epidemiology.

1. Introduction

Whole-genome sequencing is becoming more and more important in the field of virology, a fact that is also reflected in the fast growth of online nucleotide databases. Sequencing studies can reveal epidemiological aspects such as transmission pathways, deliver new insights into the biology and evolution of a virus and uncover genetic differences that influence host–virus interactions (Mullan et al., 2004; Radford et al., 2012).

In the case of foot-and-mouth disease virus (FMDV), molecular epidemiology, in particular for the tracing of virus transmission pathways, is a central aspect of control strategies and contingency plans (Abdul-Hamid et al., 2011; Cottam et al., 2008). This is of very high importance because FMDV outbreaks very often result in significant economic losses and drastic trade restrictions (Brito et al., 2015).

FMDV is a non-enveloped, positive-sense single-stranded RNA virus, the type species of the genus *Aphthovirus* within the family *Picornaviridae*. Its genome is approximately 8400 nucleotides (nt) long and contains one large open reading frame (ORF). The ORF consists of the leader protease (L^{pro}), four structural proteins (VP1-4), encoded by the genes 1A, 1B, 1C and 1D, as well as seven non-structural proteins (2A-C, 3A-D) (Jackson et al., 2007; Jamal and Belsham, 2013). Furthermore, there is a 5′ untranslated region (UTR) of approximately 1300 nucleotides that contains the internal ribosome entry site (IRES) and other functional elements (Belsham, 2005), as well as a 3′ UTR that folds into a stem-loop structure and contains the poly-A (Dorsch-Hasler et al., 1975).

There are seven antigenically distinct serotypes: O, A, C, Asia-1, SAT1, SAT2 and SAT3. Serotype C occurred in Kenya and Brazil for the last time in 2004, but the other six serotypes still circulate in mixed pools in Africa, the Middle East and Asia (Brito et al., 2015; Rweyemamu et al., 2008). The serotypes can be further divided into different topotypes, genetic lineages and strains. A nucleotide difference of up to 15% in the VP1-coding sequence is necessary to define a topotype within the serotypes A, O, C and Asia-1. This value is raised to 20% for SAT serotypes due to the higher variability within these serotypes (Samuel and Knowles, 2001b). Serotype O is divided into eleven topotypes (WRLFMD, 2017) and serotype A into three major geographically restricted genotypes (Knowles and Samuel, 2003; Reid et al., 2001; Samuel and Knowles, 2001a). Serotype SAT1 and SAT2 are very diverse separated into thirteen and fourteen topotypes, respectively, while SAT 3 comprises five different topotypes (WRLFMD, 2017). No distinct topotypes have been described for Asia 1 (Brito et al., 2015; Reid et al., 2001). Historically, there were three topotypes for serotype C (Knowles and Samuel, 2003; WRLFMD, 2017) but it is unknown if this serotype still exists in the wild.

* Corresponding author.
E-mail address: bernd.hoffmann@fli.bund.de (B. Hoffmann).

http://dx.doi.org/10.1016/j.jviromet.2017.04.007
Received 11 October 2016; Received in revised form 7 April 2017; Accepted 18 April 2017
Available online 23 April 2017

Traditionally, FMDV molecular epidemiology is based on analyses of the partial or full-length sequence of the genome region encoding the capsid protein VP1 (Knowles and Samuel, 2003; Xu et al., 2013). However, the VP1 coding sequence alone does not reflect all important phenotypic traits of FMDV. The full genome sequence is useful for high resolution phylogenetic analysis, to track virus movements within an outbreak and to reveal additional genomic features of a virus isolate (Cottam et al., 2006; Xu et al., 2013).

Today, several possibilities for whole-genome sequencing are available (Pareek et al., 2011). Next-generation sequencing (NGS) is independent of prior knowledge of target sequences for primer design (Logan et al., 2014), but is still expensive and requires elaborate library preparation and data analysis (Shendure et al., 2008; Wright et al., 2011).

The generation of sequences using RT-PCR and subsequent Sanger sequencing is often labour-and time-intensive because it requires rigorous primer design and testing (Logan et al., 2014). There are several approaches of covering the whole genome of FMDV by generating overlapping PCR fragments (Abdul-Hamid et al., 2011; Cottam et al., 2006, 2008). These studies either include different primer pairs for different lineages (Abdul-Hamid et al., 2011) or a multitude of primer mixes with unknown applicability for other serotypes (Cottam et al., 2008).

In order to develop a simple, quick and convenient tool to obtain the full coding sequence of FMDV, a universal primer panel for all seven serotypes of FMDV was designed. The genome was amplified and sequenced in overlapping DNA fragments reaching from the IRES to the end of the open reading frame.

2. Materials and methods

2.1. Primer design and selection

The complete genomes of 387 FMDV strains representing all seven serotypes were downloaded from the National Center for Biotechnology Information GenBank database (https://www.ncbi.nlm.nih.gov/nucleotide/). Multiple sequence alignments were performed using the MAFFT algorithm implemented in the Geneious software, Version 8 (Biomatters Limited). Primer design was performed according to standard rules (Chuang et al., 2013; Thornton and Basu, 2011) and primers were analysed for annealing temperature, dimer formation, hairpins and palindromes with OligoCalc, an online oligonucleotide properties calculator (http://biotools.nubic.northwestern.edu/OligoCalc.html) (Kibbe, 2007). Overall, a panel of 52 primer combinations was evaluated with a set of 12 FMDV cell culture isolates representing all seven serotypes, resulting in a final set of 15 genome-spanning primer combinations (Table 1).

2.2. FMDV isolates used for validation

A panel of 12 FMDV strains representing all seven serotypes and containing historic and more recent isolates was used for primer pair validation. The FMDV isolates were selected from archival stocks of the Friedrich-Loeffler-Institut (FLI). Fresh cell culture supernatants of all isolates were grown in monolayers of BHK-21 cells (RIE 164, Collection of Cell Lines in Veterinary Medicine, FLI). A serial dilution of RNA extracted from each supernatant was tested with two diagnostic real-time RT-PCR assays targeting the highly conserved IRES and 3D regions of the FMDV genome, respectively (Callahan et al., 2002; Oem et al., 2005). For each isolate, an RNA dilution that was still detectable in both assays was selected. In general, it was attempted to adjust the C_q value to 20, but there was some variation between isolates (Table 2).

Four samples from FMDV animal trials were used to demonstrate the applicability of the assay for diagnostic samples: bovine and ovine saliva, caprine serum and a porcine podal vesicle. Detailed information about the used isolates and tested samples can be found in the supplemental material (Table S1).

2.3. Selection of RT-PCR chemistry for genome amplification

Four different PCR-kits, namely the Superscript III One-Step RT-PCR System with Platinum Taq (Life Technologies GmbH, Darmstadt Germany), the SensiFAST SYBR No-ROX Kit, the MyTaq™ One-Step RT-PCR Kit (both Bioline GmbH, Luckenwalde, Germany), and the qScript™ XLT One-Step RT-PCR Kit (Quanta Biosciences, Gaithersburg, MD, USA), were tested for the best performance in relation to costs. The FMDV type A isolate EGY 1/12 was used to compare the performance of the kits with regard to amplification of products of the right size only, clear and strong bands after gel electrophoresis, and costs per reaction (data not shown). Finally, the qScript™ XLT One-Step RT-PCR Kit was selected for use in the protocol.

2.4. RNA extraction and RT-PCR

FMDV RNA was extracted using TRIzol® LS Reagent (Life Technologies), combined with the RNeasy® Mini Kit (Qiagen, Hilden, Germany) according to the manufacturers' instructions. Additionally, 10 µL of in vitro-transcribed internal control-RNA (Hoffmann et al., 2006) was added to the lysis buffer of each sample. The RNA was eluted in 100 µL, and the qScript™ XLT One-Step RT-PCR Kit was used for the PCR. One reaction consisted of 0.2 µl RNase free water, 5.0 µL 2× One-step ToughMix, 0.4 µL qScript XLT OneSTep RT, 0.4 µL SYBR® Green I (Sigma-Aldrich Chemie GmbH, Munich, Germany), and 2.0 µL of RNA template in a final reaction volume of 8.0 µL. SYBR® Green I was added to the master mix to assess the amplification performance in general. For fast optimization, a 96-well plate with the primer mixes was prepared. For the PCR reaction, 1.0 µL of each primer (10 µM) was transferred into a 96-well PCR plate, and 8.0 µL of master mix were added. The PCR conditions consisted of 20 min at 48 °C for reverse transcription, followed by 3 min at 94 °C to activate the polymerase, followed by 39 cycles of 15 s at 94 °C (denaturation), 30 s at 60 °C (annealing), 60 s at 68 °C (elongation and fluorescence data recording) and a final elongation step at 68 °C for 5 min. The PCR products were visualized on a 1.5% agarose gel according to standard procedures.

2.5. DNA sequencing and sequence analysis

PCR products of the expected length (see Table 1) were purified using the QIAquick® PCR Purification kit (Qiagen) according to the manufacturer's instructions. Purified amplicons were sequenced in 10 µL reactions containing 1 µL of BigDye® Terminator v1.1 Cycle Ready Reaction Mix (Applied Biosystems, Foster City, CA, USA), 1 µL of BigDye® Terminator 5× sequencing buffer (Applied Biosystems), 5 pmol of each primer in a 96-well PCR plate, and 5 µL of purified RT-PCR product. The cycling conditions consisted of 1 min of denaturation at 96 °C, followed by 26 cycles of 15 s at 96 °C, 10 s at 53 °C, and 4 min at 60 °C. Sequencing products were purified using the NucleoSEQ kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) according to the manufacturer's instructions. Nucleotide sequences were determined using a 3130xl Genetic Analyzer (Applied Biosystems). Sequence contigs for each isolate were assembled using CodonCode Aligner (CodonCode Corporation, Centerville, MA, USA). Extracted consensus sequences were mapped against the complete published sequence of the respective isolate using Geneious. For isolates with no available full-length sequence, an arbitrarily selected FMDV type O sequence (GenBank accession no. NC_004004, isolated from vesicular fluid of swine inoculated with material collected in Yunlin Prefecture, Taiwan, in April 1997) was used. Newly generated sequences were analysed by BLAST (http://blast.ncbi.nlm.nih.gov/) to verify the identity of every sample.

Table 1
Primer mixes.

Mix No	Primer	Primer sequence 5′–3′	Locations[a] 5′–3′	Amplicon size
1	FMD-809-F	AYG GGA CGT CWG CGC ACG	593–611	406 bp
	FMD-1214-R	TTA CCT CRG GGT ACC TRA AGG	936–915	
2	FMD-813-F	GAC GTC WGC GCA CGA AAC G	597–619	402 bp
	FMD-1214-R	TTA CCT CRG GGT ACC TRA AGG	936–915	
3	FMD-1174-F	TGG WGA CAG GCT AAG GAT GCC	895–916	890 bp
	FMD-2063-R	ATG GAG TTY TGG TAC TGY TGC AT	1762–1740	
4	FMD-1144-F	AAG GTG ACA YTG ADA CTG GTA C	865–887	940 bp
	FMD-2083-R	TGT CAC CAA GYT GYG TGT CCA T	1782–1760	
5	FMD-1842-F	GAG GAC TTY TAC CCY TGG AC	1551–1570	1382 bp
	FMD-3223-R	TGG GHC CWG TGA ACA TGA ART G	2913–2892	
6	FMD-1889-F	TTC TGG TRT TTG TYC CGT ACG A	1588–1609	1335 bp
	FMD-3223-R	TGG GHC CWG TGA ACA TGA ART G	2913–2892	
7	FMD-3161-F	TCG CVC AGT ACT ACR CAC AGT A	2851–2872	1143 bp
	FMD-4303-R	TGA CGT CRG AGA AGA AGA ARG G	3939–3918	
8	FMD-4249-F	GCA GGR GAC GTB GAG TCC AA	3911–3931	943 bp
	FMD-5191-R	CGT CRA AGT GGT CRG GGT C	4827–4809	
9	FMD-4249-F	GCA GGR GAC GTB GAG TCC AA	3911–3931	955 bp
	FMD-5203-R	GTT GGT TGT ARC CGT CRA AGT G	4839–4818	
10	FMD-5143-F	AGA ACY GAY TCA GTT TGG TAC TG	4735–4758	1205 bp
	FMD-6347-R	GCT TTC ACT TTC AAA GCG ACA GG	5953–5931	
11	FMD-5143-F	AGA ACY GAY TCA GTT TGG TAC TG	4735–4758	1238 bp
	FMD-6380-R	GCA CCA CTC TCA GTG ACR AT	5983–5964	
12	FMD-6143-F	AAC CRC AAG CTG AAG GAC CCT	5749–5769	481 bp
	FMD-6623-R	TCT GAG AGC ATG TCC TGT CC	6226–6207	
13	FMD-6567-F	TGA CTW CAG AGT GTT TGA GTT TGA	6170–6193	1042 bp
	FMD-7608-R	TCT GCC AAT CAT CAT YCT RGT GTA	7208–7185	
14	FMD-6567-F	TGA CTW CAG AGT GTT TGA GTT TGA	6170–6193	1050 bp
	FMD-7616-R	GCA CAR AAT CTG CCA ATC ATC AT	7216–7194	
15	FMD-7491-F	CCA RAC CTT CCT GAA GGA CGA	7091–7111	795 bp
	FMD-8285-R	AAC TTC TCC TGK ATG GTC CCA	7885–7865	
16[b]	EGFP-15-F	GAG CAA GGG CGA GGA GC		712 bp
	EGFP-10-R	CTT GTA CAG CTC GTC CAT GC		

[a] Serotype O isolate NC_004004 was used as reference sequence. Note: location numbers are estimated for some primers.
[b] Published in Hoffmann et al. (2006).

Table 2
Product analysis using gel electrophoresis for the selected virus isolates.

Mix	A EGY 9/2011	A22 IRQ 24/64	Asia HKN 5/2005	Asia1 Shamir ISR 89	O MOG 7/2010	O1 Manisa TUR 69	SAT1 SAR 3/2000	SAT1 ZIM 25/89	SAT2 EGY 24/2014	SAT2 ZIM 11/91	SAT3 ZIM 4/81	C1 Noville SWI 65
Mix-1	+ +	+ +	+ +	+ +	+ +	+ + +	+ +	+ +	+ + +	+ +	+ +	+
Mix-2	+ +	+ +	+	+ +	+ +	+ + +	+ +	+ +	+ + +	+ +	+ +	+
Mix-3	+ + + +	+ + + +	+ + +	+ + +	+ + +	+ + + +	+ + +	+ + + +	+ + + +	+ + +	+ + + +	+ +
Mix-4	+ + + +	+	+ + +	+ + +	+ + +	+ + + +	+ +	+ + +	+ + + +	+ +	+ + + +	+ +
Mix-5	+ +	+	+ +	+ +	+ + +	+ + + +	–	–	+ + + +	+ +	–	+ +
Mix-6	+	+	+ +	+ +	+ + +	+ +	+	+	+ + +	+	+	+ +
Mix-7	+ + + +	+ + + +	+ +	+ + +	+ + +	+ + + +	+ +	+ + + +	+ +	+ +	+ + +	+ +
Mix-8	+ + + +	+ + +	+ + +	+ + +	+ + +	+ + + +	+ + +	+ + +	+ + + +	+ +	+ + + +	+ +
Mix-9	+ + + +	+ + +	+ + +	+ +	+ + +	+ + + +	+ + + +	+ + +	+ + + +	+ +	+ + + +	+ +
Mix-10	+ + + +	+ + + +	+ + +	+ + +	+ + + +	+ + + +	+ + +	+ + +	+ + + +	+ + +	+ + +	+ +
Mix-11	+ + + +	+ + + +	+ + +	+ + +	+ + + +	+ + + +	+ +	+ +	+ + +	+ + +	+	+ +
Mix-12	+ + + +	+ + +	+ + +	+ +	+ + + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ +
Mix-13	+ + +	+ + +	+ + +	+ +	+ +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + + +	+ +
Mix-14	+ + + +	+ + +	+ + +	+ + +	+	+ + + +	+ + + +	+ +	+ + + +	+ +	+ + + +	+ +
Mix-15	+ + +	+ + + +	+ + +	+ + +	+ + + +	+ + + +	+ + + +	+ + +	+ + +	+ + +	+ + + +	+ +
Mix-16	+ + +	+	+	+	+	+ +	+ +	+	+	+	+ +	+ +
Cq 3D-OIE[a]	22.2	16.1	22.0	17.0	20.6	16.8	25.2	19.7	20.9	18.4	23.0	23.7
Cq IRES[b]	25.7	21.6	24.4	17.8	21.1	17.5	32.0	26.4	23.4	22.2	27.2	26.3

– no band; +hardly visible band; + + weak visible band; + + + thin bright band; + + + + thick bright band.
[a] Callahan et al. (2002).
[b] Oem et al. (2005).

2.6. In silico analysis

The complete genomes of available FMDV strains representing different topotypes within the seven serotypes were downloaded from GenBank. The topotype was determined by using the VP1 oracle from the FMDV-Tools suite of the Pirbright Institute (Kim et al., 2016). It was intended to include at least one strain of every topotype of the different serotypes in the analysis but that was not possible for all serotypes. A detailed listing of all strains, including accession numbers, topotypes and lineages where applicable, can be found in the supplemental material (Table S2). Multiple sequence alignments for all serotypes were performed using the MUSCLE algorithm as implemented in Geneious, the primers were mapped to the sequences in the alignment and the nucleotides at the primer binding sites were tabulated.

2.7. Cost estimation for genome sequencing

Only the direct costs for materials based on catalogue prices without institutional discounts were used for the calculation. In general, the listed prices per kit were divided through the number of reactions in the kit. Deviations from the manufacturer's instructions for RT-PCR and sequencing reactions are described in the relevant sections. The calculations are based on current prices as of August 2016.

3. Results

3.1. RT-PCR, sequencing and genotyping

A panel of 52 different primer combinations was evaluated with a set of 12 FMDV isolates representing all seven serotypes. In total, the 15 most suitable genome-spanning primer combinations were selected (Table 1). This allowed robust amplification of the entire ORF with a double-amplicon strategy. Most regions of the ORF were covered by more than one primer pair to improve the overall reliability of the amplification (Fig. 1).

All amplicons were sequenced, and consensus sequences of about seven kilobases (kb) from the internal ribosome entry site (IRES) to the end of the 3D protein or the 3′ untranslated region (UTR) were produced. The size of the generated consensus sequences did not vary significantly between the tested samples. All sequences were of high quality and matched the predicted genotypes. Sequences have been uploaded to Genbank (KY825717–KY825732).

In order to exclude false-negative results, the 15 genome-spanning primers in the final panel were complemented with an internal control (Hoffmann et al., 2006). Therefore, the final RT-PCR amplification protocol uses 16 primer mixes, which can pre-dispensed into a 96-well PCR plate. This primer plate was stable at −20 °C for the duration of the experiment (at least nine months; data not shown).

The analytical sensitivity of the mixes in the protocol was determined with a 10-fold dilution series of FMDV O/MOG/7/2010 RNA. PCR products were obtained with all mixes at all tested dilutions of the template, down to a maximum dilution of 10^{-5} (Table 3). For the mixes 1, 2, 5, 11 and 12, the effect of the dilution is clearly visible in the decreasing intensity of the gel bands. Other mixes, for example mix 3 or 15, produced strong bands even with a very dilute template (Fig. S1). To illustrate the decreasing amount of template, the dilution series was tested with standard diagnostic real-time RT-PCRs targeting the 3D and IRES genome regions (Callahan et al., 2002; Oem et al., 2005) (Table 3).

3.2. Evaluation with animal samples

Samples from animal trials were used to demonstrate the applicability of the protocol for diagnostic material. Representative samples were selected to cover the most important host animals and sample types: bovine and ovine saliva, caprine serum and a porcine podal vesicle. The animals had been infected with viruses of serotypes O, A, Asia-1 and SAT2.

Sample handling was the same as described above. The genome load of the samples ranged from a Cq of 25 (serum, 3 dpi, SAT2 EGY 24/14, 3D-OIE assay) to 29 (fluid from porcine podal vesicle, A22 Iraq, IRES assay) (Table 4).

The obtained viral RNAs were processed using the final primer panel, and all targeted genome regions were amplified correctly, resulting in sequences covering the genome from the IRES to the 3′ end of the 3D protein (Table 4, Fig. S2).

3.3. In silico analysis

To show that the primers have broad sensitivity for diverse viral strains, an in silico analysis with sequence alignments was performed.

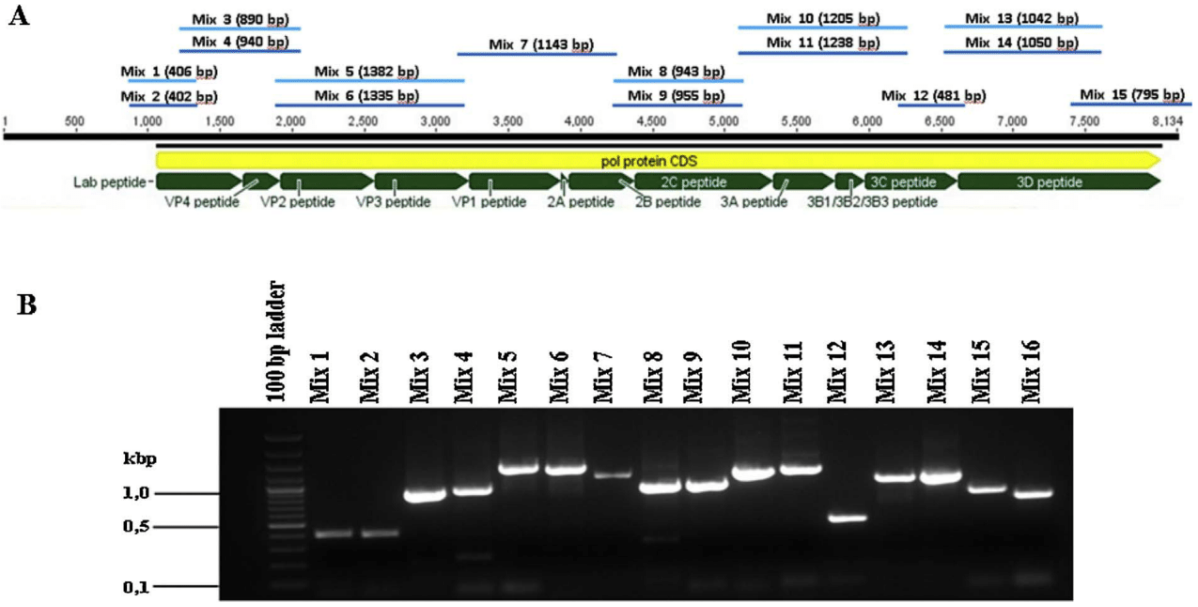

Fig. 1. Amplification of the open reading frame of FMDV SAT 2 EGY 24/2014 (B), isolated from serum with primer mixes 1–16 (A). (A) Modified from Geneious version 9.0 created by Biomatters.

Table 3
Analytical sensitivity of the primer mixes on FMDV O MOG 7/2010.

Mix	Original	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Mix-1	+ + +	+ + +	+ +	+ +	+/+ +	+
Mix-2	+ + +	+ + +	+ +	+ +	+/+ +	+
Mix-3	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + +
Mix-4	+ + + +	+ + +	+ + +	+ + +	+ + +	+ + +
Mix-5	+ + + +	+ + + +	+ + +	+ + +	+ +	+ +
Mix-6	+ + +	+ +	+ +	+ +	+ +	+ +
Mix-7	+ + + +	+ + + +	+ + +	+ + +	+ + +	+ + +
Mix-8	+ + + +	+ + + +	+ + +	+ + +	+ + +	+ + +
Mix-9	+ + + +	+ + + +	+ + +	+	+ + +	+ +
Mix-10	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + +
Mix-11	+ + + +	+ + + +	+ + +	+ + +	+ + +	+ +
Mix-12	+ + +	+ + +	+ + +	+ + +	+ + +	+ +
Mix-13	+ + +	+ + +	+ + +	+ + +	+ + +	+ +
Mix-14	+ + + +	+ + + +	+ + +	+ + +	+ + +	+ + +
Mix-15	+ + + +	+ + + +	+ + + +	+ + + +	+ + +	+ + +
Cq 3D-OIE[a]	16.7	20.4	24.3	28.5	32.5	37.2
Cq IRES[b]	17.3	20.4	23.9	27.4	30.9	34.9

+ hardly visible band; + + weak visible band; + + + thin bright band; + + + + thick bright band.
[a] Callahan et al., 2002.
[b] Oem et al., 2005.

The detailed results of the analysis can be found in the supplemental material (Table S2) and an overview of the nucleotide mismatches is shown in Table 5. Most primers had a maximum mismatch with any sequence in the alignment of less than or equal to 5% (FMD-809-F, FMD-813-F, FMD-1174-F, FMD-1214-R, FMD-2063-R, FMD-5191-R, FMD-5203-R, FMD-6143-F, FMD-6347-R, FMD-6567-F, FMD-6623-R, FMD-7608-R, FMD-7616-R, FMD-8285-R) or slightly higher (FMD-7491-F: 5.7%,), which corresponds to one or two mismatched nucleotides. Ten primers (FMDV-1144-F, FMD-1842-F, FMD-1889-F, FMD-2083-R, FMD-3161-F, FMD-3223-R, FMD-4249-F, FMD-4303-R, FMD-5143-F, FMD-6380-R) showed sequence variations of more than 5%, mostly due to variation within SAT serotypes. One primer, FMD-1889-F, had a maximum mismatch of 30.7%, caused by large discrepancies within the sequences of the SAT serotypes. The genomic region covered by this primer is also covered by the forward primer of another pair.

Table 4
Product analysis of the full-ORFRT-PCR assay with animal samples.

Sample	Asia1 Shamir ISR 89 Bovine saliva 5 dpi	A22 IRQ 24/64 Porcine podal vesicle 7 dpi	O1 Manisa TUR 69 Ovine saliva 5 dpi	SAT2 EGY 24/ 2014 Caprine serum 3 dpi, 24 dpv
Mix 1	+ +	+ +	+ +	+ +
Mix 2	+ +	+ +	+ +	+ +
Mix 3	+ + + +	+ + + +	+ + + +	+ + + +
Mix 4	+ + +	+ +	+ + +	+ + +
Mix 5	+	+	+ + +	+ + +
Mix 6	+ +	+	+ + +	+ + +
Mix 7	+ + +	+ + + +	+ + +	+
Mix 8	+ + +	+ + + +	+ + +	+ + +
Mix 9	+ + +	+ + +	+ +	+ + +
Mix 10	+ + + +	+ + + +	+ + + +	+ + + +
Mix 11	+ + + +	+ + +	+ + +	+ + +
Mix 12	+ + +	+ + +	+ + +	+ + +
Mix 13	+ + +	+ + +	+ + +	+ + +
Mix 14	+ + + +	+ + + +	+ + + +	+ + + +
Mix 15	+ + + +	+ + + +	+ + +	+ + +
Mix 16	+ +	+ +	+ + +	+ + +
Cq 3D-OIE[a]	28.2	24.8	26.8	25.3
Cq IRES[b]	26.2	29.4	28.1	28.1

dpi: days post infection; dpv: days post vaccination; − no band; + hardly visible band; + + weak visible band; + + + thin bright band; + + + + thick bright band.
[a] Callahan et al. (2002).
[b] Oem et al. (2005).

Table 5
Nucleotide mismatches of the particular primer sequences in percent.

Primer	A	O	Asia1	C	SAT1	SAT2	SAT3	Max mismatch
FMD-809-F	1.2	0.0	0.0	0.0	2.5	2.2	2.1	2.5
FMD-813-F	1.5	0.0	0.0	0.0	2.3	3.2	2.0	3.2
FMD-1144-F	5.1	0.3	0.6	4.5	4.1	5.3	6.3	6.3
FMD-1174-F	0.4	0.7	0.6	2.4	1.4	0.8	0.0	2.4
FMD-1214-R	1.7	0.3	0.0	2.4	1.0	2.6	1.8	2.6
FMD-1842-F	3.6	3.6	2.0	5.0	13.5	10.0	13.1	13.5
FMD-1889-F	9.7	4.9	5.8	11.4	27.3	24.5	30.7	30.7
FMD-2063-R	3.2	1.9	2.6	0.0	3.9	5.0	3.8	5.0
FMD-2083-R	2.7	2.9	4.5	4.5	8.6	5.2	8.0	8.6
FMD-3161-F	5.6	2.3	4.8	4.5	6.4	14.0	9.7	14.0
FMD-3223-R	14.3	1.3	6.4	6.8	12.7	8.4	11.4	14.3
FMD-4249-F	5.9	2.5	3.3	35.0	6.0	4.6	6.3	35.0
FMD-4303-R	5.8	2.3	4.5	2.3	7.3	6.3	8.0	8.0
FMD-5143-F	6.1	2.8	1.2	8.7	15.2	15.7	16.3	16.3
FMD-5191-R	0.2	0.4	0.4	2.6	4.2	2.8	2.0	4.2
FMD-5203-R	2.7	3.9	1.2	2.3	2.7	3.5	1.1	3.9
FMD-6143-F	0.9	1.0	0.6	0.0	0.5	3.5	0.0	3.5
FMD-6347-R	1.4	0.6	1.2	0.0	0.5	2.3	2.2	2.3
FMD-6380-R	2.0	1.1	0.7	0.0	22.0	17.3	22.5	22.5
FMD-6567-F	1.3	0.6	0.6	0.0	0.8	3.2	1.0	3.2
FMD-6623-R	1.4	0.0	0.7	0.0	0.0	1.9	5.0	5.0
FMD-7491-F	3.9	3.4	4.4	0.0	5.7	4.4	4.8	5.7
FMD-7608-R	0.2	1.5	0.8	2.1	2.5	2.2	0.0	2.5
FMD-7616-R	1.0	1.2	0.6	0.0	1.7	2.0	0.0	2.0
FMD-8285-R	0.9	0.0	0.3	0.0	1.4	1.8	1.8	1.8

3.4. Cost efficiency and time scale

The protocol generates sequences covering the full FMDV ORF in a short time and with high sensitivity even for samples with low FMDV RNA load. For maximum versatility, all primer mixes can be predispensed into 96-well-plates for both the FMDV-specific RT-PCRs and the cycle sequencing reactions. The amplification and sequencing procedure for FMDV described here can be used in all standard molecular diagnostic laboratories without expensive equipment or reagents. RNA extraction, RT-PCR genome amplification, gel electrophoresis, and amplicon purification can be completed in one day. On the second day, the sequencing PCR, the purification of the sequencing reaction, and the electrophoretic analysis can be performed if a Genetic Analyzer is available. Alternatively, the purified products can be submitted to a commercial service for off-site sequencing. Finally, the obtained sequence data must be analysed and evaluated. In summary, generation of a nearly complete FMDV genome sequence can be done in less than 2 days with costs of approximately €200 per FMDV genome (based on catalogue prices of August 2016). The costs can only be estimated because the actual costs for materials etc. vary between countries and even from laboratory to laboratory.

4. Discussion

Whole genome sequences or at least sequences covering the whole open reading frame of FMDV are of increasing importance for the molecular epidemiology of FMDV. In the past, the VP1 coding region has been the primary target for epidemiological studies. However, it has been shown that most inter- and intra-serotypic recombination events occur in the non-structural genes of FMDV (Carrillo et al., 2005; Klein, 2009; Lee et al., 2009) and that important evolutionary events can occur outside of VP1 (Knowles et al., 2001; Nobiron et al., 2005). Sequences of the L gene or the whole P1 coding region can supplement VP1 phylogenies (Xu et al., 2013), but a solid phylogenetic analysis requires whole genome or at least full ORF sequences. Today, these genomes are often generated by next-generation sequencing (NGS)

technologies (Pareek et al., 2011; Radford et al., 2012). However, NGS is still complex, expensive and is not available in all diagnostic laboratories. Furthermore, direct sequencing of viruses from diagnostic samples requires high genome loads (Marston et al., 2013; Pareek et al., 2011).

This study presents a new panel of 15 primer combinations for the rapid, easy and cheap amplification of FMDV genomes for subsequent Sanger sequencing. The introduced sequencing strategy allows amplification of genome fragments, reaching from the IRES to the end of the open reading frame. Untranslated regions upstream of the ORF, including the S-fragment, and downstream regions of the 3′ UTR are not covered. While the whole genome of FMDV cannot be attained by using this sequencing strategy, nevertheless, it is possible to achieve a broad genomic characterisation, including the sequence of the L-protein as well as the structural and non-structural proteins of FMDV.

The panel was evaluated for all seven FMDV serotypes, and the performance was similar across all serotypes. Keeping in mind that SAT viruses have a higher genetic diversity than the serotypes A, O and Asia-1 (Bastos et al., 2001; Vosloo et al., 1995), this result underlines the stability and universality of the chosen primer panel.

Nevertheless, only a limited number of FMDV strains were used for the experimental validation of the protocol. To supplement the laboratory results, an in silico analysis has been performed to evaluate the primers against a broader range of viral strains. The analysis predicted good performance for all serotypes and for the different topotypes within the serotypes. Overall, there were more predicted mismatches for SAT viruses than for the Eurasian serotypes O, A, C and Asia-1. Nevertheless, it cannot be ruled out that some of the 15 PCR fragments may not be obtained for every virus isolate or that sequencing of further FMDV strains will show that optimization of the method is required. To mitigate this, the “double-check design” provides redundant primer pairs for about two thirds of the ORF. In the case of a strain that is still not amplified completely, missing genome regions can be filled in by designing additional primers based on sequence data from adjacent genomic regions. Although the need for primer walking is one of the known disadvantages of full-genome sequencing by Sanger technology, this will be most likely a rare exception when using the presented primer panel. And even NGS-based approaches can have genome gaps that must be closed by conventional RT-PCRs and Sanger sequencing (Radford et al., 2012). For field samples, it has to be taken into account that amplification of long targets may become a problem. The PCR products range from 400 to 1400 bp, which can be difficult to amplify if the sample RNA is degraded. In this case, it might also be necessary to design additional primers based on the sequencing data obtained from the first runs of the sample.

To reduce the time required for the whole process from sample preparation to the final sequence, the 16 primer pairs in the final protocol were pre-dispensed in paired columns on a 96-well plate. In this way, six different samples can be processed at once. While NGS is often time-consuming due to extensive pre- and post-processing pipelines (Radford et al., 2012; Shendure and Ji, 2008), the generation of an almost full-genome sequence using our primer panel can be done in less than two days.

The presented primer panel showed a very high analytical sensitivity. PCR products for sequencing were obtained even with very dilute template. For most mixes, the intensities of the gel bands decreased with the increasing dilution of the template, but some remained the same. This can be explained by the different analytical sensitivities of the primer mixes, e.g. due to sequence conservation or secondary structures and binding site accessibility.

The final dilution of the template was only weakly positive in the diagnostic real-time RT-PCRs, indicating that the introduced method would also be applicable for diagnostic samples with small sample quantities or a low viral genome load. The robustness of the assay was confirmed with animal samples from infection experiments. All materials were processed with standard methods and allowed amplification of the respective FMDV genome.

Redundancies within the presented primer panel, i.e. coverage of the same genomic region by more than one primer pair (“double-check strategy”), increase the likelihood of obtaining a full ORF sequence and improve the resolution of the sequence data. At best, four sequence fragments for one genomic region can be obtained by bidirectional sequencing of two overlapping PCR products.

Routine Sanger sequencing cannot reliably collect information on the quasispecies distribution in a sample. For many research questions, however, such as transmission pathways or other analyses of between-host viral evolution, mapping and comparing the consensus sequences is sufficient. Where the occurrence of ambiguous bases indicates the presence of more than one genotype this could be confirmed by additional methods like cloning and sequencing or specific RT-qPCR assays.

The protocol does not require the use of a real-time PCR cycler. If one is available, however, successful amplification of genome fragments can be monitored in real time by the addition of a fluorescent dye like SYBR® Green I to the PCR reaction mix. The advantage of the real-time RT-PCR is that a failed amplification can already be detected by the absent signal of the fluorescent dye, and negative reactions do not need to be run on a gel. For all positive samples, however, a further evaluation of the PCR products by gel electrophoresis is inevitable. Amplification products of the desired size must be purified for sequencing.

The costs of the protocol are manageable due to the universal primers, small PCR reaction volumes as well as the application of inexpensive standard kits and equipment. No complex bioinformatics are necessary to obtain the Sanger sequences, unlike NGS data that requires extensive post-processing.

Both systems for whole-genome analysis of FMDV, NGS or Sanger sequencing, have advantages and disadvantages regarding costs, time, and quality of the obtained sequences. This should be taken into consideration for the selection of a sequencing strategy. Currently, Sanger sequencing still represents the “gold standard” of DNA sequencing. With high costs per base and a low throughput (Liu et al., 2012), however, Sanger sequencing is no longer an attractive option for large genomes. For the analysis of the quasi-species character of FMDV, NGS also provides clear advantages with its possibility to obtain an ultra-deep coverage of the entire genome (Wright et al., 2011). But for the generation of consensus sequences of small viral genomes like FMDV, Sanger sequencing based on genome-specific primers is still a quick and easy alternative. In addition, the primer panel from this study can be adapted in the future to become part of a NGS pipeline by using barcodes or other tags appended to the primer sequences (Clarke et al., 2014; Moser et al., 2016).

In conclusion, this RT-PCR amplification protocol represents a new efficient method to generate full-ORF sequences for all seven serotypes of FMDV. In comparison to other recent methods, the primer panel presented here can identify the genotype in samples of an unknown FMDV strain without the need of any prior knowledge for the selection of suitable primers. The method can genotype FMDV without the need of NGS or other complex techniques and analyses. With a single multi-well PCR run followed by sequencing, the full coding sequence is obtained rapidly and independently of the FMDV serotype present in the sample. This quick turnaround time is particularly crucial in the event of an outbreak.

Funding

This research was funded by Merck Life Science, Darmstadt, Germany. There is no involvement of the funder in collection, analysis and interpretation of data, in the writing of the report and in the decision to submit the article for publication.

Acknowledgements

We thank Dr Michael Eschbaumer for his support and for editing the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jviromet.2017.04.007.

References

Abdul-Hamid, N.F., Firat-Sarac, M., Radford, A.D., Knowles, N.J., King, D.P., 2011. Comparative sequence analysis of representative foot-and-mouth disease virus genomes from Southeast Asia. Virus Genes 43, 41–45.

Bastos, A.D., Haydon, D.T., Forsberg, R., Knowles, N.J., Anderson, E.C., Bengis, R.G., Nel, L.H., Thomson, G.R., 2001. Genetic heterogeneity of SAT-1 type foot-and-mouth disease viruses in southern Africa. Arch. Virol. 146, 1537–1551.

Belsham, G.J., 2005. Translation and replication of FMDV RNA. Curr. Top. Microbiol. Immunol. 288, 43–70.

Brito, B.P., Rodriguez, L.L., Hammond, J.M., Pinto, J., Perez, A.M., 2015. Review of the global distribution of foot-and-mouth disease virus from 2007 to 2014. Transbound. Emerg. Dis.

Callahan, J.D., Brown, F., Osorio, F.A., Sur, J.H., Kramer, E., Long, G.W., Lubroth, J., Ellis, S.J., Shoulars, K.S., Gaffney, K.L., Rock, D.L., Nelson, W.M., 2002. Use of a portable real-time reverse transcriptase-polymerase chain reaction assay for rapid detection of foot-and-mouth disease virus. J. Am. Vet. Med. Assoc. 220, 1636–1642.

Carrillo, C., Tulman, E.R., Delhon, G., Lu, Z., Carreno, A., Vagnozzi, A., Kutish, G.F., Rock, D.L., 2005. Comparative genomics of foot-and-mouth disease virus. J. Virol. 79, 6487–6504.

Chuang, L.Y., Cheng, Y.H., Yang, C.H., 2013. Specific primer design for the polymerase chain reaction. Biotechnol. Lett. 35, 1541–1549.

Clarke, L.J., Czechowski, P., Soubrier, J., Stevens, M.I., Cooper, A., 2014. Modular tagging of amplicons using a single PCR for high-throughput sequencing. Mol. Ecol. Resour. 14, 117–121.

Cottam, E.M., Haydon, D.T., Paton, D.J., Gloster, J., Wilesmith, J.W., Ferris, N.P., Hutchings, G.H., King, D.P., 2006. Molecular epidemiology of the foot-and-mouth disease virus outbreak in the United Kingdom in 2001. J. Virol. 80, 11274–11282.

Cottam, E.M., Wadsworth, J., Shaw, A.E., Rowlands, R.J., Goatley, L., Maan, S., Maan, N.S., Mertens, P.P., Ebert, K., Li, Y., Ryan, E.D., Juleff, N., Ferris, N.P., Wilesmith, J.W., Haydon, D.T., King, D.P., Paton, D.J., Knowles, N.J., 2008. Transmission pathways of foot-and-mouth disease virus in the United Kingdom in 2007. PLoS Pathog. 4, e1000050.

Dorsch-Hasler, K., Yogo, Y., Wimmer, E., 1975. Replication of picornaviruses. I. Evidence from in vitro RNA synthesis that poly(A) of the poliovirus genome is genetically coded. J. Virol. 16, 1512–1517.

Hoffmann, B., Depner, K., Schirrmeier, H., Beer, M., 2006. A universal heterologous internal control system for duplex real-time RT-PCR assays used in a detection system for pestiviruses. J. Virol. Methods 136, 200–209.

Jackson, A.L., O'Neill, H., Maree, F., Blignaut, B., Carrillo, C., Rodriguez, L., Haydon, D.T., 2007. Mosaic structure of foot-and-mouth disease virus genomes. J. Gen. Virol. 88, 487–492.

Jamal, S.M., Belsham, G.J., 2013. Foot-and-mouth disease: past, present and future. Vet. Res. 44.

Kibbe, W.A., 2007. OligoCalc: an online oligonucleotide properties calculator. Nucleic Acids Res. 35, W43–W46.

Kim, J., Ferretti, L., Bachanek-Bankowska, K., Knowles, N.J., King, D.P., Ribeca, P., 2016. VIBAsys and FMDV-Tools: practical resources for foot-and-mouth disease sequence analysis. Open Session of the Standing Technical and Research Committees of the European Commission for the Control of Foot-and-Mouth Disease. EuFMD Meeting.

Klein, J., 2009. Understanding the molecular epidemiology of foot-and-mouth-disease virus. Infect. Genet. Evol. 9, 153–161.

Knowles, N.J., Davies, P.R., Henry, T., O'Donnell, V., Pacheco, J.M., Mason, P.W., 2001. Emergence in Asia of foot-and-mouth disease viruses with altered host range: characterization of alterations in the 3A protein. J. Virol. 75, 1551–1556.

Knowles, N.J., Samuel, A.R., 2003. Molecular epidemiology of foot-and-mouth disease virus. Virus Res. 91, 65–80.

Lee, K.N., Oem, J.K., Park, J.H., Kim, S.M., Lee, S.Y., Tserendorj, S., Sodnomdarjaa, R., Joo, Y.S., Kim, H., 2009. Evidence of recombination in a new isolate of foot-and-mouth disease virus serotype Asia 1. Virus Res. 139, 117–121.

Liu, L., Li, Y., Li, S., Hu, N., He, Y., Pong, R., Lin, D., Lu, L., Law, M., 2012. Comparison of next-generation sequencing systems. J. Biomed. Biotechnol. 2012, 251364.

Logan, G., Freimanis, G.L., King, D.J., Valdazo-Gonzalez, B., Bachanek-Bankowska, K., Sanderson, N.D., Knowles, N.J., King, D.P., Cottam, E.M., 2014. A universal protocol to generate consensus level genome sequences for foot-and-mouth disease virus and other positive-sense polyadenylated RNA viruses using the Illumina MiSeq. BMC Genomics 15, 828.

Marston, D.A., McElhinney, L.M., Ellis, R.J., Horton, D.L., Wise, E.L., Leech, S.L., David, D., de Lamballerie, X., Fooks, A.R., 2013. Next generation sequencing of viral RNA genomes. BMC Genomics 14, 444.

Moser, L.A., Ramirez-Carvajal, L., Puri, V., Pauszek, S.J., Matthews, K., Dilley, K.A., Mullan, C., McGraw, J., Khayat, M., Beeri, K., Yee, A., Dugan, V., Heise, M.T., Frieman, M.B., Rodriguez, L.L., Bernard, K.A., Wentworth, D.E., Stockwell, T.B., Shabman, R.S., 2016. A universal next-generation sequencing protocol to generate noninfectious barcoded cDNA libraries from high-containment RNA viruses. mSystems 1.

Mullan, B., Sheehy, P., Shanahan, F., Fanning, L., 2004. Do Taq-generated RT-PCR products from RNA viruses accurately reflect viral genetic heterogeneity? J. Viral Hepat. 11, 108–114.

Nobiron, I., Rémond, M., Kaiser, C., Lebreton, F., Zientara, S., Delmas, B., 2005. The nucleotide sequence of foot-and-mouth disease virus O/FRA/1/2001 and comparison with its British parental strain O/UKG/35/2001. Virus Res. 108, 225–229.

Oem, J.K., Kye, S.J., Lee, K.N., Kim, Y.J., Park, J.Y., Park, J.H., Joo, Y.S., Song, H.J., 2005. Development of a Lightcycler-based reverse transcription polymerase chain reaction for the detection of foot-and-mouth disease virus. J. Vet. Sci. 6, 207–212.

Pareek, C.S., Smoczynski, R., Tretyn, A., 2011. Sequencing technologies and genome sequencing. J. Appl. Genet. 52, 413–435.

Radford, A.D., Chapman, D., Dixon, L., Chantrey, J., Darby, A.C., Hall, N., 2012. Application of next-generation sequencing technologies in virology. J. Gen. Virol. 93, 1853–1868.

Reid, S.M., Ferris, N.P., Hutchings, G.H., De Clercq, K., Newman, B.J., Knowle, N.J., Samuel, A.R., 2001. Diagnosis of foot-and-mouth disease by RT-PCR: use of phylogenetic data to evaluate primers for the typing of viral RNA in clinical samples. Arch. Virol. 146, 2421–2434.

Rweyemamu, M., Roeder, P., Mackay, D., Sumption, K., Brownlie, J., Leforban, Y., Valarcher, J.F., Knowles, N.J., Saraiva, V., 2008. Epidemiological patterns of foot-and-mouth disease worldwide. Transbound. Emerg. Dis. 55, 57–72.

Samuel, A.R., Knowles, N.J., 2001a. Foot-and-mouth disease type O viruses exhibit genetically and geographically distinct evolutionary lineages (topotypes). J. Gen. Virol. 82, 609–621.

Samuel, A.R., Knowles, N.J., 2001b. Foot-and-mouth disease virus: cause of the recent crisis for the UK livestock industry. Trends Genet. 17, 421–424.

Shendure, J., Ji, H., 2008. Next-generation DNA sequencing. Nat. Biotechnol. 26, 1135–1145.

Shendure, J.A., Porreca, G.J., Church, G.M., 2008. Overview of DNA sequencing strategies. Curr. Protoc. Mol. Biol. Unit 7.1.

Thornton, B., Basu, C., 2011. Real-time PCR (qPCR) primer design using free online software. Biochem. Mol. Biol. Educ. 39, 145–154.

Vosloo, W., Kirkbride, E., Bengis, R.G., Keet, D.F., Thomson, G.R., 1995. Genome variation in the SAT types of foot-and-mouth disease viruses prevalent in buffalo (*Syncerus caffer*) in the Kruger National Park and other regions of southern Africa, 1986–93. Epidemiol. Infect. 114, 203–218.

Wright, C.F., Morelli, M.J., Thebaud, G., Knowles, N.J., Herzyk, P., Paton, D.J., Haydon, D.T., King, D.P., 2011. Beyond the consensus: dissecting within-host viral population diversity of foot-and-mouth disease virus by using next-generation genome sequencing. J. Virol. 85, 2266–2275.

WRLFMD, 2017. Prototypes. World Reference Laboratory for Foot-and-Mouth Disease, Pirbright, UK. (accessed on 2017/03/09). http://www.wrlfmd.org/fmd_genotyping/prototypes.htm.

Xu, L., Hurtle, W., Rowland, J.M., Casteran, K.A., Bucko, S.M., Grau, F.R., Valdazo-Gonzalez, B., Knowles, N.J., King, D.P., Beckham, T.R., McIntosh, M.T., 2013. Development of a universal RT-PCR for amplifying and sequencing the leader and capsid-coding region of foot-and-mouth disease virus. J. Virol. Methods 189, 70–76.

Supplementary Materials: Simple, quick and cost-efficient: A universal RT-PCR and sequencing strategy for genomic characterisation of foot-and-mouth disease viruses.

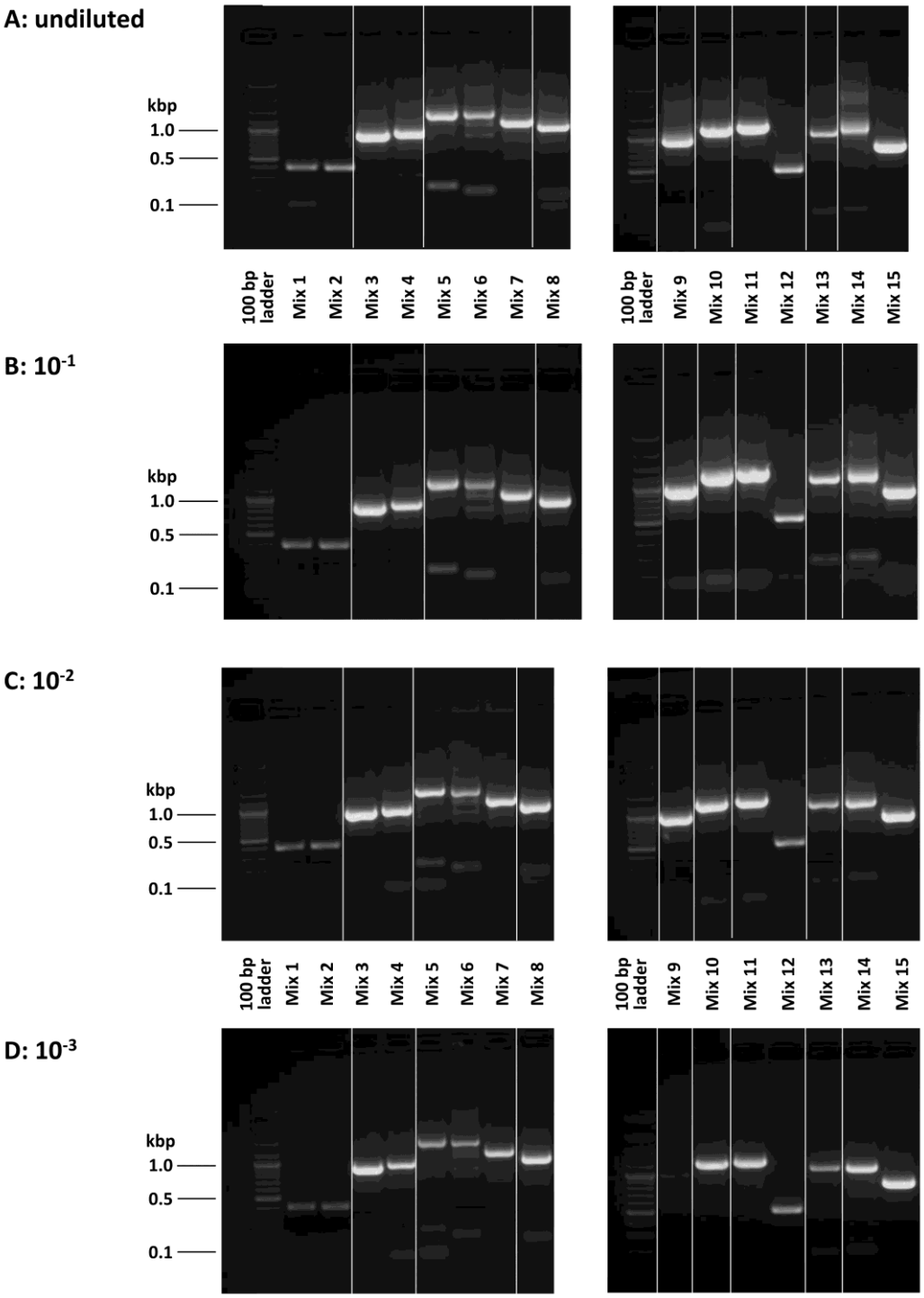

Figure S1: Dilution series of FMDV O MOG 7/10 to examine analytical sensitivity of primer mixes 1-15.
A: undiluted RNA. B: 10^{-1}. C: 10^{-2}. D: 10^{-3}. E: 10^{-4}. F: 10^{-5}.
Additional gel lanes that did not belong to primer mixes 1-15 were removed from the images. Relevant lanes were spliced together, which is indicated by thin white lines between the gel pieces that have been juxtaposed.

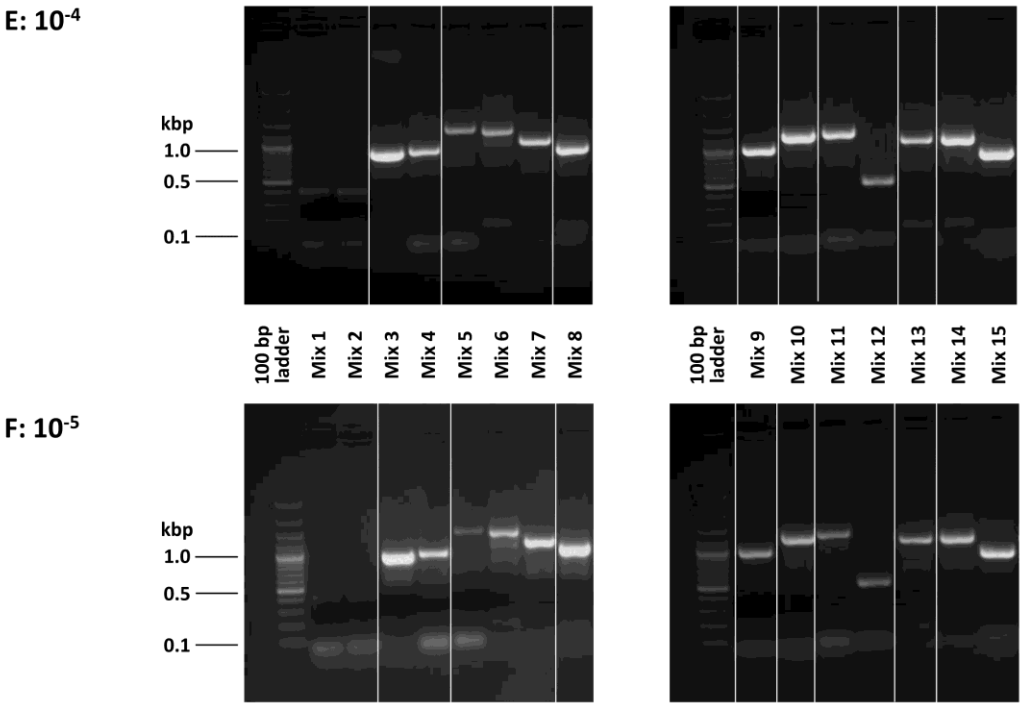

Figure S1 (continued)

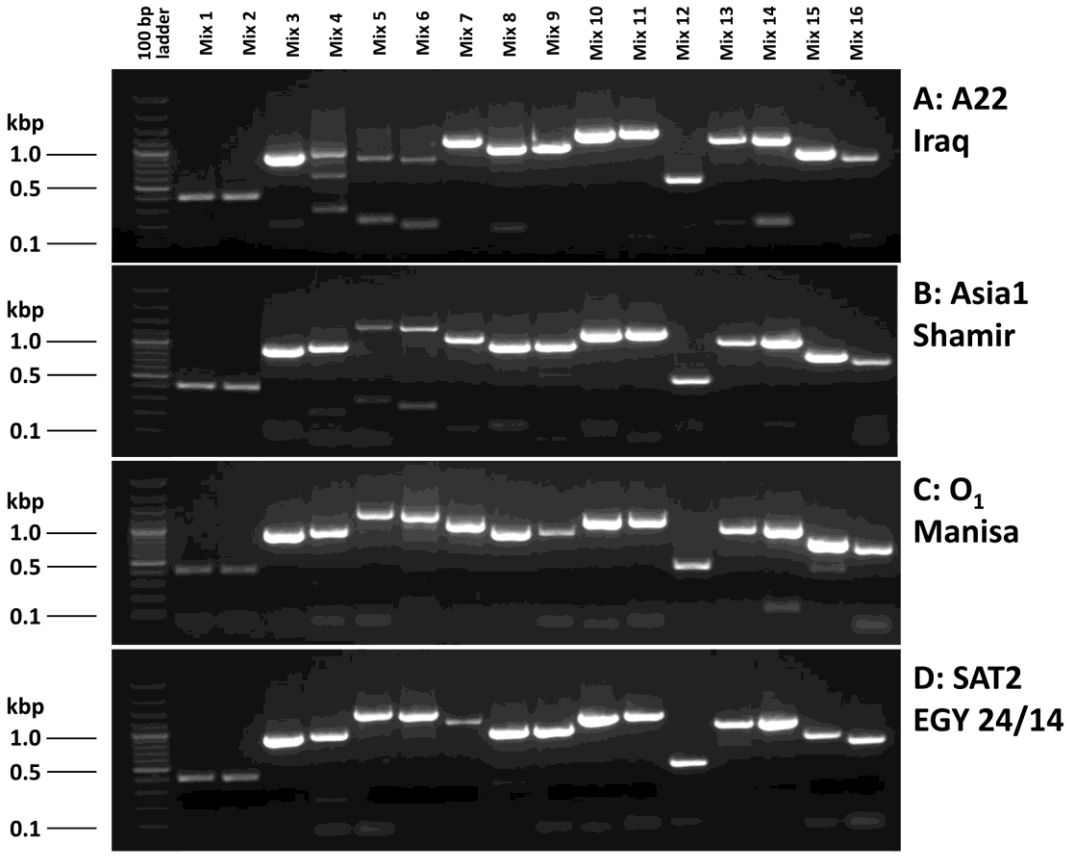

Figure S2: Gel images of the whole-ORF RT-PCR assay used with animal samples.
A: A_{22} Iraq, vesicular fluid. B: Asia1 Shamir, saliva. C: O_1 Manisa, saliva. D: SAT2 EGY 24/14, serum.

Table S1: Overview about the samples used in this study, their origin and passage history.

virus isolate	origin	material	passage history*
A EGY 9/2011	cell culture	supernatant	BTY3 WRL, BHK1 FLI
A22 IRQ 24/64	cell culture	supernatant	CP2 BTY2 BHK2 BTY1 WRL, BHK2 FLI
Asia1 HKN 5/2005	cell culture	supernatant	WRL, BHK1 FLI
Asia1 Shamir ISR 89	cell culture	supernatant	BHK6 FLI
O1 MOG 7/2010	cell culture	supernatant	BTY1 WRL, BHK1 FLI
O1 Manisa TUR 69	cell culture	supernatant	BHK8 WRL, BHK6 FLI
SAT1 SAR 3/2000	cell culture	supernatant	PK1 RS2 WRL, BHK1 FLI
SAT1 ZIM 22/89	cell culture	supernatant	BTY2 WRL, BHK1 FLI
SAT2 EGY 24/2014	cell culture	supernatant	BTY1 WRL, BHK1 FLI
SAT2 ZIM 11/91	cell culture	supernatant	BTY WRL, BHK4 FLI
SAT3 ZIM 4/81	cell culture	supernatant	RS2 BHK2 WRL, BHK4 FLI
C1 Noville SWI 65	cell culture	supernatant	C filt, BTY3 BHK2 WRL, BHK3 FLI
Asia1 Shamir ISR 89	animal trial	saliva; 5 dpi from cattle	vesicle, BHK1 FLI
A22 IRQ 24/64	animal trial	vesicle on a pig's trotter; 7dpi	vesicle, BHK1 FLI
O1 Manisa TUR 69	animal trial	saliva; 5 dpi from sheep	vesicle, BHK1 FLI
SAT2 EGY 24/2014	animal trial	serum; 3 dpi, 24 dpv from goat	BTY1 WRL, BHK1 GP1 FLI

* passage host/passage number/location

cell lines: BTY: primary bovine (calf) thyroid, BHK: baby hamster kidney, PK: porcine kidney, RS: renal swine cells

CP: cattle passage, GP: goat passage, C filt: cattle epithelium homogenate

WRL: world reference laboratory, Pirbright, UK; FLI: Friedrich-Loeffler-Institut, Greifswald, Germany

Table S2: In-silico analysis covering most topotypes within the different serotypes of FMDV. Primer sequences aligned with a panel of selected virus strains for all seven serotypes. All primer sequences are given in 5´ to 3´ notation. Reverse primers are shaded in grey.

Table S2: In-silico analysis covering most topotypes within the different serotypes of FMDV. Primer sequences aligned with a panel of selected virus strains for all seven serotyp All primer sequences are given in 5' to 3' notation. Reverse primers are shaded in grey

				FMD-809-F (593-611)	FMD-813-F (597-619)	FMD-1144-F (865-887)	FMD-1174-F (895-916)	FMD-1214-R (936-915)
				AYG GGA CGT CWG CGC ACG	GAC GTC WGC GCA CGA AAC G	AAG GTG ACA YTG ADA CTG GTA C	TGG WGA CAG GCT AAG GAT GCC	TTA CCT CRG GGT ACC TRA AGG
SeroType	TopoType	Lineage	Accession No					
A	Asia	G-VII	AY593751	... ...	... ...	... ...	... ..A	... ...
A	Asia	G-VII	AY593768	... ...	... ...	..A	... ...	... ...
A	Asia	Sea-97	AY593760	... ...	... ...	..A	... ...	..C ... ...
A	Asia	Sea-97	AY593753	... ...	... ...	..A	... ...	... ...
A	Asia	Iran-05 (SIS-10)	KT968663	... ...	... ...	..A	... ...	... ...
A	Asia	Iran-05	AY593771	... ... T..	... ..T	..A ... ..G	... ...	... ..C ... ...
A	Asia	Iran-96	AY593780	... ...	... ...	..A	... ...	..G ... ...
A	EURO-SA	A3	AY593794	... ...	... ...	..A ... ..G	... ...	... ...G ...
A	EURO-SA	A5	AY593778	T..	... ...	..A	... ...	... ...
A	EURO-SA	A10	KJ754939	... ...	... ...	... ...	... ...	... ...
A	EURO-SA	A12	AY593793	... ...	... ...T ...	... ... .C.	... ... .A.	... ...
A	EURO-SA	A16	KC440882	missing sequence data	missing sequence data	missing sequence data	... ...	... ...
A	EURO-SA	A17	KC440881	missing sequence data	missing sequence data	missing sequence data	... ...	... ...G ...
A	EURO-SA	A24	AY593777	... ... .C.	... ... C..	... ...	... ...	... ...
A	EURO-SA	A27	EF117837	missing sequence data	... ...	... .AA	... ...	... ...
A	EURO-SA	A79	AY593785	... ...	... ...	... A..	... ...	... ...
A	EURO-SA	A81	AY593789	... ...	... ...T ...	... ...	... ...	..G ... ...
A	Africa	G-III	AY593761	... ... T..	... ..T	... .CA ..G	... ...	... ... T.. ... ...
A	Africa	G-IV	AY593757	... ...	... ...	..A	... ...	... ...
A	Africa	G-IV	JF749843	... ...	... ...	..A ... ..G	... ...	... ...
A	Africa	G-VII	KP940474	missing sequence data	missing sequence data	missing sequence data	... ...	... ...G ...
A	Africa	G-VIII	HM854025	... ...	... ...	... ...	... ...	... ...
O	Cathay		EF175732	... ...	... ...	... ...	... ... A..	... ... G..
O	ME-SA	PanAsia	AF506822	... ...	... ...	... ...	... ...	... ...
O	ME-SA	PanAsia	AJ539140	... ...	... ...	... ...	... ...	... ...
O	ME-SA	PanAsia	KR265075	... ...	... ...	... ...	... ...	... ...
O	ME-SA	PanAsia2	FJ175666	... ...	... ...	... ... .T.	... ...	... ...
O	Me-SA	PanAsia2	GU384683	... ...	... ...	... ...	... ...	... ...
O	ME-SA	PanAsia2	JX040500	... ...	... ...	... ...	... ...	... ...
O	ME-SA	PanAsia2	KC440883	missing sequence data	missing sequence data	missing sequence data	... ...	... ...
O	ME-SA	Ind-2001	KJ825809	... ...	... ...	... ...	... ...	... ...
O	EURO-SA	O1	JX869188	... ...	... ...	... ...	... ...	... ...
O	EURO-SA	O2	AY593826	... ...	... ...	... ...	... ...	... ...
O	SEA	Mya-98	HM229661	... ...	... ...	... ...	... ...	... ...
O	SEA	Mya-98	LC036265	... ...	... ...	... ...	... ...	... ...
O	EA-2		KU821591	... ...	... ...	... ...	... ... A..	... ...
Asia-1	Asia		AY593796	... ...	... ...	... ...	... ...	... ...
Asia-1	Asia		AY593797	... ...	... ...	... ...	... ...	... ...
Asia-1	Asia		AY593800.1	... ...	... ...	... ...	... ...	... ...
Asia-1	Asia		DQ989309	... ...	... ...	... ...	... ...	... ...
Asia-1	Asia		DQ989321	... ...	... ...	... ...	... ...	... ...
Asia-1	Asia		DQ989323	... ...	... ...	... ...	... ...	... ...
Asia-1	Asia		EF614458	... ...	... ...	... ...	... ...	... ...
Asia-1	Asia		GT125645	... ...	... ...	... ...	... ..G	... ...
Asia-1	Asia		GT125646	... ...	... ...	... ...	... ...	... ...
Asia-1	Asia		HQ113233	missing sequence data	missing sequence data	... ...	... ...	... ...
Asia-1	Asia		KT360085	... ...	... ...	... ...	... ...	... ...
Asia-1	Asia		HQ631363	... ...	... ...	... ...	... ..G	... ...
Asia-1	Asia	Sindh-08	HQ632774	... ...	... ...	... .G	... ...	... ...
Asia-1	Asia	Sindh-08	JN006719	missing sequence data	missing sequence data	... ...	... ...	... ...
Asia-1	Asia	Sindh-08	JN006720	missing sequence data	missing sequence data	... ... .A.	... ...	... ...
C	EURO-SA	C1	AF274010.1	... ...	... ...	..A	... ...	... ...
C	Africa		KM268897.1	... ...	... ...	..A	... ... T..	..G ... ...
SAT 1	I		JF749860	... ... .C.	... ... C..	... ..A	... ... .T.	... ...
SAT 1	I		KM268899	... ...	... ...	... ...	... ... .T.	... ...
SAT 1	II		AY593839	... ... .C.	... ... C..	... ..A	.A.	... ...
SAT 1	II		AY593846	... ...	... ...	... ..A	... ...	... ...
SAT 1	III		KT821590	... ...A	... ... .A.	... ..A	... ...	... .A ... ...
SAT 1	III		AY593840	... ...	... ...	... ..A	... ...	... ...
SAT 1	III		AY593841	... ... .C.	... ... C..	... ..A	... ...	... ...
SAT 1	III		AY593842	... ...	... ...	... ..A	... ...	... ...
SAT 1	IV		HM067706	missing sequence data	missing sequence data	... ..A	... ...	... ... .T. ... ...
SAT 1	VI		AY593844	... ...	... ...	... ..A	... ...	... ...
SAT 2	I		AY593848	... ... .C.	... ... C..	..A ..A	... ...	... ...
SAT 2	I		JF749864	... ... .C.	... ... C..	... ..A	... ...	... ...
SAT 2	II		AF540910	... ...	... ...	... ..A	... ...	... ...
SAT 2	III		AY593847	... ...	... ...	... ..A	... ...	... ...
SAT 2	IV		JF749861	... ... .C.	... ... C..	... ...	... ... .T.	... ...
SAT 2	IV		KM268900	... ...	... ...	... ...	... ...	... ...G ...
SAT 2	VII	Ghb-12	JX014255	... ...	... ...C ...	..A ..A	... ...	... ...G ...
SAT 2	VII	Ghb-12	JX014256	... ...	... ...C ...	..A ..A	... ...	... ...G ...
SAT 2	VII	Ghb-12	KC440884	missing sequence data	missing sequence data	missing sequence data	missing sequence data	... ...G ...
SAT 2	IX		AY593849	... ... .A. ...	... ... A..	... ..A	... ...	... ...
SAT 2	X		HM067704	missing sequence data	missing sequence data	... ..A	... ...	... ... .C. ... ...
SAT 2	X		HM067705	missing sequence data	missing sequence data	... ..A ... ... T..	... ..G	... ... .C. ... ...
SAT 2	X		JF749862	... ...	... ...	... ..A	... ...	... ... .C. ... ...
SAT 3	I		AY593850	... ... .A. ...	... ... A..	... ..A ... ... G..	... ...	... ...
SAT 3	I		KM268901	... ...	... ...	... ..A	... ...	... ...
SAT 3	I		KR108950	... ...	... ...	... ..A	... ...	..G ... ...
SAT 3	I		KX375417	... ... T..	... ..T	... ..A ... ... G.. ... .A. ..	... ...	... ...
SAT 3	II		AY593851	... ...	... ...	... ..A	... ...	... ...G ...
SAT 3	II		AY593852	... ...	... ...	... ..A	... ...	... ...
SAT 3	II		AY593853	... ... G..	... ..G	... ..A	... ...	... ...
SAT 3	V		KJ820999	... ...	... ...	... ..A	... ...	... ... .C. ... ...

Table S2 (continued)

SeroType	TopoType	Lineage	Accession No	FMD-1842-F (1551-1570) GAG GAC TTY TAC CCY TGG AC	FMD-1889-F (1588-1609) TTC TGG TRT TTG TYC CGT ACG A	FMD-2063-R (1762-1740) ATG GAG TTY TGG TAC TGY TGC AT	FMD-2083-R (1782-1760) TGT CAC CAA GYT GYG TGT CCA T	FMD-3161-F (2851-2872) TCG CVC AGT ACT ACR CAC AGT A
A	Asia	G-VII	AY593751	... ...G	.CT ... ... .C.	... ...T	... ...	.T. ... ...
A	Asia	G-VII	AY593768	... ...A	..T	... ...T	... .T. .C. A..	.T. .T. ... ...
A	Asia	Sea-97	AY593760	... ..G	... ...	... ...	... ...	.T. ... ...
A	Asia	Sea-97	AY593753	... ...A	.CT	... ...	... .G.	.T. ... ... T.. ... ...
A	Asia	Iran-05 (SIS-10)	KT968663	... ...	.C.	... ...	... .T. ... ... .A.	.A. ... ...
A	Asia	Iran-05	AY593771	... ..A	.CT	... ...	... ...	.T. ... ...
A	Asia	Iran-96	AY593780	... ..A	.C.	... ...A	... ...	.T. ... ...
A	EURO-SA	A3	AY593794	... ...	.C.	...A ..A	... ...	.T. .T. ... ...
A	EURO-SA	A5	AY593778	... ..A	.C.	... ...	... ...	.T. ... ...
A	EURO-SA	A10	KJ754939	... ...	... ...	... ...	... .T.	.A. ... ...
A	EURO-SA	A12	AY593793	..A ... ...G	... .T .C. .C.	... ...	.A.	.T. ... ...
A	EURO-SA	A16	KC440882	... ...T ..G	.C.	... ..A	.A. .G.	.A. ... ...
A	EURO-SA	A17	KC440881	... ...A	.CT ... GT. ... .C. .A. ...	...T ..A	... ...	.T. ... ...
A	EURO-SA	A24	AY593777	... ...	.C. ... ... .A.	... ..A ... .T	... ...	.T. ... ...
A	EURO-SA	A27	EF117837	... ...G	.C.	... ...	... .G.	.A. ... ... .G. ...
A	EURO-SA	A79	AY593785	... ...	.C.	... ...	... ...	.T. ... ...
A	EURO-SA	A81	AY593789	... ...	.C. ... ... .C. .C.	... ...	... ...	.T. ... ...
A	Africa	G-III	AY593761	... ..T	.CT	...T	... ... .G.	.T. ... ...
A	Africa	G-IV	AY593757	... ...A	.CT	... ...	... ...	.T. ... ... T.. ... ...
A	Africa	G-IV	JF749843	... ...	.C. .A. .CT C.. ... .C. ...	... ...	... ...	.T. ... ...
A	Africa	G-VII	KP940474	... ...A	.CT ... GT. ... .C. .A. ...	...T ..A	... ...	.T. ... ...
A	Africa	G-VIII	HM854025	... ...	... .T ... .C.	...A ..A ... .T	... .C.	.A. ... ...
O	Cathay		EF175732	... ...	.CT ... .T. ... ... .A. ...	..A	... .G. ... ... .G.	... ...
O	ME-SA	PanAsia	AF506822	..A ..A	... ...	... ...	.A.	... ...
O	ME-SA	PanAsia	AJ539140	... ...	... ...	... ...	... ...	... ...
O	ME-SA	PanAsia	KR265075	... ...G	... ...	... ...	... ...	... ...
O	ME-SA	PanAsia2	FJ175666	... ...	... ... .T.	... ...	.A.	... ...
O	Me-SA	PanAsia2	GU384683	... ...	... ...	... ...	... ...	... ...
O	ME-SA	PanAsia2	JX040500	..A ..A	... ...	... ...	... ...	... ...
O	ME-SA	PanAsia2	KC440883	..A	... ... .T.	... ...	.A.	... ...
O	ME-SA	Ind-2001	KJ825809	..A ..T ... .G	... ...	...A	... .T.	... ...
O	EURO-SA	O1	JX869188	... ...	... ...	... ...A	... ...	.T. ... ...
O	EURO-SA	O2	AY593826	... ...	.CT	... ..A	... .G.	... ...
O	SEA	Mya-98	HM229661	... ..T	.G. ... .C.	... ...	... ... .G.	.T. ... .A. ... ... .G. ...
O	SEA	Mya-98	LC036265	... ...	.G. ... .C.	... ...	... ... .G.	.T. ... ... .G. ...
O	EA-2		KU821591	... ...	..T ... .T. ... ... .T. ...	... ..A ... .T	... ...	... ... .A. ... ...
Asia-1	Asia		AY593796	... ...	.C.	... ..A	... .G.	.G. ... ... .G. ...
Asia-1	Asia		AY593797	..A	.C. ... ... .G.	... ..A ... .T	... ...	.G. ... ...
Asia-1	Asia		AY593800.1	... ...	... ...	... ..A	... ...	.G. ... ...
Asia-1	Asia		DQ989309	... ...	... ... .T.	.T.	... ...	.G. ... ...
Asia-1	Asia		DQ989321	..A	..T	... ...T	... ...	.G. ... ...
Asia-1	Asia		DQ989323	..A	..T	... ...T	... ...	.G. ... ...
Asia-1	Asia		EF614458	... ...	.C.	... ...	.A. .T.	.G. ... ...
Asia-1	Asia		GT125645	... ...	.CT	... ...	... .T.	.G. ... ...
Asia-1	Asia		GT125646	... ..A	.C. ... ... .T.	... ...	... .T.	.G. ... ...
Asia-1	Asia		HQ113233	... ...	... ...	... ...T	... .T.	.G. ... ...
Asia-1	Asia		KT360085	... ...	.CT ... ... .C.	... ...	.A. .T.	.G. ... ...
Asia-1	Asia		HQ631363	... ...	.CT	... ...	.A. .T.	.G. ... ...
Asia-1	Asia	Sindh-08	HQ632774	..A	.C.	... ...T	... ...A. ...	.G. ... ...
Asia-1	Asia	Sindh-08	JN006719	... ...	... ...	... ...	... .T. .G. ... .G.	.G. ... ...
Asia-1	Asia	Sindh-08	JN006720	... ...T	... ... .T.	... ..A	... .G.	.G. ... ...
C	EURO-SA	C1	AF274010.1	... ...	.C. ... ... .A. ...	... ...	... .G. .G.	.G. ... ...
C	Africa		KM268897.1	.G. ... ...A	.CT ... ... .T.	... ...	... ...	.G. ... ...
SAT 1	I		JF749860	... .TG ... .A	.A. .C. C.. AC. .A. .C. ...	... ...	... .G. ... ... .G.	.T. ... ...
SAT 1	I		KM268899	... .TG ... .A	.A. .C. C.. AC. .G.	...T ..A	... .G. ... ... .G.	... ...
SAT 1	II		AY593839	..A .TG ... .A	.A. .C. C.. AC. .G. .C. ...	... ...	... .T. .G. ... .G.	... ...
SAT 1	II		AY593846	... .TG ... ..T ..G	.G. .C. C.. AC. ... .C. ...	... ...T	... .T. ... ... .G.	... ...
SAT 1	III		KT821590	... .TG ... .A	.G. .C. C.. AC. ... .C. ...	...T	... .G. ... ... .G.	.G. ... ... T.. ... ...
SAT 1	III		AY593840	... .GT ... .A	.A. .C. C.. AC. ... .C. ...	... ...	... .G. ... ... .G.	.G. ... ... TT. ... .A.
SAT 1	III		AY593841	... .TG	.A. .T. C.. AC. ... .C. ...	... ..A	... .G. ... ... .G.	.G. ... ... TT. ... ...
SAT 1	III		AY593842	... ATA ... .A	.G. .C. C.. AC. ... .C. ...	...T	... .G. ... ... .G.	.G. ... ... T.. ... ...
SAT 1	IV		HM067706	... ...	.C. .CT GT.	...T	... ... .G.	.T. ... ...
SAT 1	VI		AY593844	... ...G	.C. ..T GT. ... ... .A. ...	...T ..A	... ... .G.	.G. ... ...
SAT 2	I		AY593848	... .TG .A. ... ..G	.A. .C. C.. AC. .G. .C. ...	...T	... ... .G. ... .G.	... .TG .C. ... ...
SAT 2	I		JF749864	... .TG ... .G	.A. ..G C.. AC. ... .C. ...	...T	... ... .G.	.T. ..G .C. ... ...
SAT 2	II		AF540910	... .TG	.A. .C. C.. ... ... .C. ...	...T ..A	... .G. ... ... .G.	.A. ..G .T. ... ... .C. ...
SAT 2	III		AY593847	... .TG ... .G	.G. .C. C.. AC. ... .C. ...	...A	... ... .G.	... .TG .T. ... ... .T. ...
SAT 2	IV		JF749861	... .TT ... .A	.G. .T. C.. AC. .A.	...A ... .A	... .G. .C.	.T. ..G .C. ... ... .C. ...
SAT 2	IV		KM268900	..A .T. ... .A	.A. .C. C.. AC. .A. ... .T.	... ...	... .G. ... ... .G.	.T. ..G .C. ... ... .C. ...
SAT 2	VII	Ghb-12	JX014255	... ...A	.C. ..T GT. ... ... .T.	...T	... ...	.G. ..G .C. ... ...
SAT 2	VII	Ghb-12	JX014256	... ...A	.C. ..T GT. ... ... .T.	...T	... ...	.G. ..G .C. ... ...
SAT 2	VII	Ghb-12	KC440884	... ...A	.C. ..T GT. ... ... .T.	...T	... ...	.G. ..G .C. ... ...
SAT 2	IX		AY593849	... ...A	.C. ..T GT. .C. ... .A. ...	...C	... .G. .G.	.T. ... ...
SAT 2	X		HM067704	... .GC	.C. .TT GT.	...T	... ...	.G. ..G .C. ... ...
SAT 2	X		HM067705	... ...A	.C. ..T GT.	...T ... ...T	... .G. .G. ... .G.	.T. ..G .C. ... ...
SAT 2	X		JF749862	... ...A	.C. ..T GT.	...T	... ...	... ..G .C. ... ...
SAT 3	I		AY593850	... .TG ... .G	.G. .T. C.. AC. .G. .C. ...	...T ..A	... .G. ... ... .G.	... ... .C.
SAT 3	I		KM268901	... .TG ... ..T ..A	.A. .C. C.. AC. .G. .A. ...	...C	... .G.	... ... .C.
SAT 3	I		KR108950	... .TG ... ..T ..A	.A. .C. C.. AC. .A. .C. ...	... ..A	... .T. .G. ... .G.	... ... .C.
SAT 3	I		KX375417	... .TG ... ..T ..A	.G. .C. C.. AC. ... .C. ...	... ...	... .G. ... ... .G.	... ... .C.
SAT 3	II		AY593851	... .TG	.G. .C. C.. AC. .A. .A. ...	... ...	... ... .G.	.G. .T. .A. ... ... .C. ...
SAT 3	II		AY593852	... .TG	.G. .C. C.. AC. .A. .A. ...	... ...	... ... .G.	.G. .T. .A. ... ... .C. ...
SAT 3	II		AY593853	... .TG	.A. .T. C.. AC. ... .C. ...	...A ..A	... .G. ... ... .G.	.T. ... .A. ... ... .C. ...
SAT 3	V		KJ820999	... ...	.C. .TT GT. ... ... .C. .T.	...C	... .G. ... ... .G.	... ... TT. ... ...

Table S2 (continued)

SeroType	TopoType	Lineage	Accession No	FMD-3223-R (2913-2892) TGG GHC CWG TGA ACA TGA ART	FMD-4249-F (3911-3931) GCA GGR GAC GTB GAG TCC AA	FMD-4303-R (3939-3918) TGA CGT CRG AGA AGA AGA ARG G	FMD-5143-F (4735-4758) AGA ACY GAY TCA GTT TGG TAC TG	FMD-5191-R (4827-4809) CGT CRA AGT GGT CRG GGT C
A	Asia	G-VII	AY593751	... AG.	..G ..T	.A. ... ... C..	... ...G	... ...
A	Asia	G-VII	AY593768	... AA.	..C	.A.	..G	... ...
A	Asia	Sea-97	AY593760	.T. AG.	..T ..T	.A. .A. ... .A.	.A. ... ...T ..	... ...
A	Asia	Sea-97	AY593753	... AG.	..G ..C	.A.	..G	... ...
A	Asia	Iran-05 (SIS-10)	KT968663	... AA. ... .A.	..G	.A.	... ...	... ...
A	Asia	Iran-05	AY593771	.T. AG.	..T	.A.	C.G	.A. ... ...
A	Asia	Iran-96	AY593780	.T. AG.	..T	.A.	C.G ... ...C	... ...
A	EURO-SA	A3	AY593794	... AG.	..T	... .A.	..G ... .G	... ...
A	EURO-SA	A5	AY593778	.T. AG.	..T	.A.	C.G ... ...C	... ...
A	EURO-SA	A10	KJ754939	.A. AA. ... .A.	..G	.C. ... ... C..	... ...	... ...
A	EURO-SA	A12	AY593793	.A. AA.	..T ... ...A	.A. ... ... .A.	... ...T	... ...
A	EURO-SA	A16	KC440882	.T. AG.	... ...	.A.	... ...	... ...
A	EURO-SA	A17	KC440881	CA. AG. ... .A. ... .A. ...	... ...	.A.	..G ..A ... .C	... ...
A	EURO-SA	A24	AY593777	... AG.	..G ..T	.A. ... ... CA.	..G	... ...
A	EURO-SA	A27	EF117837	.A. AG.	..G ... ...T ..	... ...	... ...	... ...
A	EURO-SA	A79	AY593785	.T. AA.	..G	... ...	... ...G	... ...
A	EURO-SA	A81	AY593789	.A. AG. ... ... .A. ...	..G ..T	.A.	... ...G	... ...
A	Africa	G-III	AY593761	CA. AG.	... ...	... .A.	... ...G	... ...
A	Africa	G-IV	AY593757	... AG.	..G ..C	.A.	..G	... ...
A	Africa	G-IV	JF749843	.A. AA.	..T	.A.	..G ..A ... ..G ..C	... ...
A	Africa	G-VII	KP940474	CA. AG. ... .A. ... .A. ...	... ...	.A.	..G ..A ... .C	... ...
A	Africa	G-VIII	HM854025	... AG. ... .A.	..G	.A. ... ... .A.	... ...	... ...
O	Cathay		EF175732	... ... .C.	. G ... ..T	... ...	..G ... ...C	... ...
O	ME-SA	PanAsia	AF506822	... ...	... ...	... ...	... .	... ...
O	ME-SA	PanAsia	AJ539140	... ...	... ...	... ...	... ...	... ...
O	ME-SA	PanAsia	KR265075	... ...	... ...	... ...	... ...	... ...
O	ME-SA	PanAsia2	FJ175666	... ...	... ... A..	.C.	... ...	... ...
O	Me-SA	PanAsia2	GU384683	... ...	... ...	.C.	... ...	... ...
O	ME-SA	PanAsia2	JX040500	... ...	..G	... ...	... ...	... ...
O	ME-SA	PanAsia2	KC440883	... ... .C.	... ...	.C. ... ... G.. .	.A.	... ...
O	ME-SA	Ind-2001	KJ825809	... ...	..G	.A.	... ...	... ...
O	EURO-SA	O1	JX869188	... ...	... ...	.A.	... ...G	... ...
O	EURO-SA	O2	AY593826	... ... .A.	..T	... ...	... ...G	... ...
O	SEA	Mya-98	HM229661	... ...	... ...	... ...	..G	... ...
O	SEA	Mya-98	LC036265	... ... C..	... ...	... ...	..G	... ...
O	EA-2		KU821591	... ...	... .T	... .A.	... .A ... .C	.A. ... ...
Asia-1	Asia		AY593796	.A. ... .G.	..G	.A.	.A.	... ... .A. .
Asia-1	Asia		AY593797	.A. ... .G. ... ... .A. ...	..G	.A.	.A.	... ...
Asia-1	Asia		AY593800.1	... ... .G.	... ...	.A.	... ...	... ...
Asia-1	Asia		DQ989309	... ... .G.	..G	.A.	... ...T	... ...
Asia-1	Asia		DQ989321	.A.	... ...	.A.	... ...	... ...
Asia-1	Asia		DQ989323	.A.	... ...	.C.	... ...	... ...
Asia-1	Asia		EF614458	... ...	... ...	... .A.	... ...	... ...
Asia-1	Asia		GT125645	... ... .G.	... ...	... ...	... ...	... ...
Asia-1	Asia		GT125646	... .G. .G. .A.	... ...	.A. ... ... .A.	... ...	... ...
Asia-1	Asia		HQ113233	... ... .G.	..G ... ..T	... ...	... ...	... ...
Asia-1	Asia		KT360085	... ... .G.	... ... C.. ..	... .A.	... ...	... ...
Asia-1	Asia		HQ631363	... ... .G.	... ...	... .A.	... ...	... ...
Asia-1	Asia	Sindh-08	HQ632774	.A. ... .G. .A.	... .T	... ...	... ...T ..	... ...
Asia-1	Asia	Sindh-08	JN006719	... ... .G.	..T	.A. ... ...T	... ...	... ...
Asia-1	Asia	Sindh-08	JN006720	... ... .G.	..T ... ...A	.A. ... ...T	... ...	... ...
C	EURO-SA	C1	AF274010.1	.C.	.GC T.. ... ... GTG .C	.A.	... .T. ... .G	... ...
C	Africa		KM268897.1	.C. ... .G.	.GC T.. ... ... GTG .C	... ...	..G ... ... .T	... ... .A. ... ...
SAT 1	I		JF749860	.T. ... .T	..C ... .T	... ... C..	C.G GTG ... .C	... ...
SAT 1	I		KM268899	.T. .G. ... .AT	..C ... ... .T. ..	... ... C..	C.G GTG ... .C	... ...
SAT 1	II		AY593839	.T. .G. ... ..T	..T	... .A. ... C..	C.C GTG ... .C	... ...
SAT 1	II		AY593846	.T. .G. ... ..T	..T	... .A. ... C..	C.T GTG ... .C	... ...
SAT 1	III		KT821590	.A. .G. ... ..T ... .A. ...	..C	... .A. ... C..	... ...T ..C	.A. ... ...
SAT 1	III		AY593840	... .G. ... ..T	..C	... ... C..	... ...C	.A. ... ...
SAT 1	III		AY593841	... .G. ... ..T	..C	... .A. ... C..	... ...T	.A. ... ... .A. .
SAT 1	III		AY593842	... .G. ... ..T	..T	... ... C.. ... ... G.. .	... ...T ..C	.A. ... ...
SAT 1	IV		HM067706	... ...T ... .A. ...	..C	... ... C..	C.T ... .G ... .T ..	... ... .A. .
SAT 1	VI		AY593844	.T. .G. ... A.T	... .T	... ... CA.	... ...T	.A. ... ... .A. ... ...
SAT 2	I		AY593848	... ...T	..C	... ... C..	C.C GTG ... .C	... ...
SAT 2	I		JF749864	... ... .AT	..C	... .A. ... C..	... ..A ... ..C ..C	.A. ... ...
SAT 2	II		AF540910	.A. ... .T	..T	... ...	... ...T ..C	.A. ... ...
SAT 2	III		AY593847	.T. ... .T	..C	... ... C..	CGT GTG ... .C	.A. ... ... .A. ... ...
SAT 2	IV		JF749861	.A. ... .T	..C	... .A. ... C..	C.G GTG ... .C	... ...
SAT 2	IV		KM268900	... ...T	..T	... T.. ... C..	..G GTG ... .C	... ...
SAT 2	VII	Ghb-12	JX014255	... ...T	... ...	.A. T..	... ..A ... .C	... ...
SAT 2	VII	Ghb-12	JX014256	... ...T	... ...	.A. T..	... ..A ... .C	... ...
SAT 2	VII	Ghb-12	KC440884	... ...T	... ...	.A. T..	... ..A ... .C	... ...
SAT 2	IX		AY593849	.T. .G. .C. ..T	..G ... ...T ..	... ...	... ...T ..G	... ...
SAT 2	X		HM067704	... .G. .C. ... ... .A. ...	..C	... .A. ... C..	C.T ... .G ..C	... ...
SAT 2	X		HM067705	.A. ... .C.	..C ... ..T	... ... C..	C.T ... ...C	.A. ... .A. .A. ... ...
SAT 2	X		JF749862	... ... .C. ..T	..C	... ... C..	C.T ... ...T ..	... ...
SAT 3	I		AY593850	... ... .G. ..T	..T ... ..T	... ... C..	C.T GTG ... .C	... ...
SAT 3	I		KM268901	... ...T	..T ... ..T	... .A. ... C..	C.C GTG ... .C	... ...
SAT 3	I		KR108950	... ... .G. ..T	..C	... ... .T. C..	C.C GTG	... ...
SAT 3	I		KX375417	.T. ... .G. ..T	..C	... ... CG. ... ... .TT .	C.C GTG ... .C	... ...
SAT 3	II		AY593851	.T. ... .G. ..T	..C	... ... C..	... ...T	.A. ... ...
SAT 3	II		AY593852	.T. ... .G. ..T	..C	... ... C..	... ...T	.A. ... ...
SAT 3	II		AY593853	.T. ... .G. ..T	..C	... .A. ... C..	.A. ... .T	... ... .A. ... ...
SAT 3	V		KJ820999	.C. .G. ... ..T	..C	... ... C..	C.T ... .G	... ...

Table S2 (continued)

SeroType	TopoType	Lineage	Accession No	FMD-5203-R (4839-4818) GTT GGT TGT ARC CGT CRA AGT	FMD-6143-F (5749-5769) AAC CRC AAG CTG AAG GAC CCT	FMD-6347-R (5953-5931) GCT TTC ACT TTC AAA GCG ACA GG	FMD-6380-R (5983-5964) GCA CCA CTC TCA GTG ACR AT	FMD-6567-F (6170-6193) TGA CTW CAG AGT GTT TGA GTT TGA
A	Asia	G-VII	AY593751	... ...	... ...	... ...	... ...	... T.. ... ...
A	Asia	G-VII	AY593768	... ...	... ...	... ...A	... ...	... ...
A	Asia	Sea-97	AY593760	.C.	... ... .A.	... ...A	... ...	... ...
A	Asia	Sea-97	AY593753	... ... .A.	... ... G..	... ...	... ...	... ...
A	Asia	Iran-05 (SIS-10)	KT968663	.C.	... ...	... ..A	... ...	... ...
A	Asia	Iran-05	AY593771	... ... .A.	... ...	... ...G	... ...	... ...
A	Asia	Iran-96	AY593780	... ...	... ...	... ...	... ...	... ... C..
A	EURO-SA	A3	AY593794	.C.	... ...	T..	... ...	... ...
A	EURO-SA	A5	AY593778	... ...	... ...	... ...	... ...	... ... G.. ... ... C..
A	EURO-SA	A10	KJ754939	.C.	... ...	... ...	... ...	... ...
A	EURO-SA	A12	AY593793	... ...	... ...	... ...	... ...	... ...
A	EURO-SA	A16	KC440882	... ...	... ...	... ... G.. ..	... ...	... ...
A	EURO-SA	A17	KC440881	... ... .C.	... ...	... ...	..T ... .C ... .T. ..	... T.. ... ...
A	EURO-SA	A24	AY593777	.C. ... .A.	... ... .C.	... ...	... ...	... T.. ... ...
A	EURO-SA	A27	EF117837	.C.	... ...	... ...	... ...	... ...
A	EURO-SA	A79	AY593785	... ...	... ...	... ...	... ...	... ...
A	EURO-SA	A81	AY593789	... ...	... ...	... ...	... ...T ..	... ...
A	Africa	G-III	AY593761	.C.	... ...	... ...	... ...	... ...
A	Africa	G-IV	AY593757	... ...	... ... G..	... ...	... ...	... ...
A	Africa	G-IV	JF749843	... ...	... ...	... ...	..T	... ...
A	Africa	G-VII	KP940474	... ... .C.	... ...	... ... A..	..T ... .C ... .T. ..	... T.. ... ...
A	Africa	G-VIII	HM854025	.C.	... ...	... ...	..T	... ...
O	Cathay		EF175732	.C.	... ... G..	... ...	... ...T ..	C.. ... ... C.. ...
O	ME-SA	PanAsia	AF506822	.C.	... ...	... ...	... ...	... ...
O	ME-SA	PanAsia	AJ539140	.C.	... ...	... ...	... ...	... ...
O	ME-SA	PanAsia	KR265075	.C.	... ...	... ...	... ...	... ...
O	ME-SA	PanAsia2	FJ175666	.C.	... ...	... ...	... ...	... ...
O	Me-SA	PanAsia2	GU384683	... ...	... ...	... ...A	... ...	... ...
O	ME-SA	PanAsia2	JX040500	.C.	.G.	... ...A	... ...	... ...
O	ME-SA	PanAsia2	KC440883	.C.	... ...	... ...	... ...	... ...
O	ME-SA	Ind-2001	KJ825809	... ...	... ...	... ...	... ...	... ...
O	EURO-SA	O1	JX869188	.C.	... ...	... ...	... ...	... ...
O	EURO-SA	O2	AY593826	.C.	... ...	... ...	... ...	... ...
O	SEA	Mya-98	HM229661	.C.	... ... .G.	... ...	... ...	... ...
O	SEA	Mya-98	LC036265	.C.	... ...	... ...	... ...G	... ...
O	EA-2		KU821591	... ... .A.	... ...	... ...	..G	... ...
Asia-1	Asia		AY593796	... ...	... ...	... ...	... ...	C.. ... ...
Asia-1	Asia		AY593797	... ...	... ...	... ... .G.	... ...	... ... C.. ... ...
Asia-1	Asia		AY593800.1	.C.	... ... .G.	... ...	... ...	... ...
Asia-1	Asia		DQ989309	... ...	... ...	... ... .G.	..G	... ...
Asia-1	Asia		DQ989321	.C.	... ...	... ...A	..G	... ...
Asia-1	Asia		DQ989323	.C.	... ...	... ...A	... ...	... ...
Asia-1	Asia		EF614458	... ...	... ...	... ...	... ...	... ...
Asia-1	Asia		GT125645	... ...	... ...	... ...	... ...	... ...
Asia-1	Asia		GT125646	... ...	... ... .T.	... ...	... ...	... ...
Asia-1	Asia		HQ113233	... ...	... ...	... ...	... ...	... ...
Asia-1	Asia		KT360085	... ...	... ...	... ...	... ...	... ...
Asia-1	Asia		HQ631363	... ...	... ...	... ...	... ...	... ...
Asia-1	Asia	Sindh-08	HQ632774	... ...	... ...	... ...	... ...	... ...
Asia-1	Asia	Sindh-08	JN006719	.C.	... ...	... ...	... ...	... ...
Asia-1	Asia	Sindh-08	JN006720	... ...	... ...	... ...	... ...	... ...
C	EURO-SA	C1	AF274010.1	.C.	... ...	... ...	... ...	... ...
C	Africa		KM268897.1	... ...	... ...	... ...	... ...	... ...
SAT 1	I		JF749860	... ...	... ...	... ...G	CAT ..G ..T	... ... C.. ... ...
SAT 1	I		KM268899	... ...	... ...	... ...G	CAC ..G ..T	... ...
SAT 1	II		AY593839	... ...	... ... .C.	... ...	CAT ..G ..T	... ...
SAT 1	II		AY593846	... ...	... ...	... ...	CAT ..G ..T	... ...
SAT 1	III		KT821590	... ... .A. ... .A.	... ...	... ...	CAT ..G ..T	... ...
SAT 1	III		AY593840	... ... .A.	... ...	... ...	CAT ..G ..T	... ...
SAT 1	III		AY593841	... ... .A.	... ...	... ...	CAT ..G ..T	... ...
SAT 1	III		AY593842	... ... .A.	... ...	... ...	CAT ..G ..T	... ...
SAT 1	IV		HM067706	... ...	... ...	... ...G	... ..G ... ...T ..	... ...
SAT 1	VI		AY593844	... ... .A.	... ...	... ...	..T ... .C	... T.. ... ...
SAT 2	I		AY593848	... ...	... ...	... ...	CAT ..G ..T	... ... T.. ... ...
SAT 2	I		JF749864	... ... .A.	... ...	... ...	CAT ..G ..T	... ...
SAT 2	II		AF540910	... ... .A. ... .A.	... ...	... ...	CAT ..G ..T	... ... C..
SAT 2	III		AY593847	... ... .A.	G..	... ...	CAA ..G G..	... ... G.. ... ... C..
SAT 2	IV		JF749861	... ... .T.	... ...	... ... .GG ... ..G ..	CAC ..G ..T	... ...
SAT 2	IV		KM268900	... .A.	... ...	... ...G	CAT ..G ..T	... ...
SAT 2	VII	Ghb-12	JX014255	... ...	... ...	... ...	..T ... .C	... T.. ... ...
SAT 2	VII	Ghb-12	JX014256	... ...	... ...	... ...	..T ... .C	... T.. ... ...
SAT 2	VII	Ghb-12	KC440884	... ...	... ...	... ...	..T ... .C	... T.. ... ...
SAT 2	IX		AY593849	... ... .A.	... .A .G.	... ...	..T ... .C ... ..T ..	... T.. ... ...
SAT 2	X		HM067704	... ...	... ...	... ...G	... ..G ... ...T ..	... ... T.. ... ...
SAT 2	X		HM067705	... ... .A. ... .A.	... ...	... ...G	... ..G ... ...T ..	... ... G.. ... ...
SAT 2	X		JF749862	... ... .C.	... ...	... ...G	... ..G ... ...T ..	... ...
SAT 3	I		AY593850	... ...	... ...	... ...	CAT ..G ..T	... ...
SAT 3	I		KM268901	... ...	... ...	... ...	CAT ..G	... ... T.. ... ...
SAT 3	I		KR108950	... ...	... ...	... ...	CAT ..G ..T	... ...
SAT 3	I		KX375417	... ...	... ...	... ...	CAT ..G ..T	... ...
SAT 3	II		AY593851	... ... .A.	... ...	.T.	CAT ..G ..T	... ...
SAT 3	II		AY593852	... ... .A.	... ...	.T.	CAT ..G ..T	... ...
SAT 3	II		AY593853	... ...	... ...	... ...A	CAT ..G ..T	... ... C..
SAT 3	V		KJ820999	... ...	... ...	... ...G	... ..G ... ...T ..	... ...

Table S2 (continued)

SeroType	TopoType	Lineage	Accession No	FMD-6623-R (6226-6207) TCT GAG AGC ATG TCC TGT CC	FMD-7491-F (7091-7111) CCA RAC CTT CCT GAA GGA CGA	FMD-7608-R (7208-7185) TCT GCC AAT CAT CAT YCT RGT GTA	FMD-7616-R (7216-7194) GCA CAR AAT CTG CCA ATC ATC AT	FMD-8285-R (7885-7865) AAC TTC TCC TGK ATG GTC CCA
A	Asia	G-VII	AY593751					
A	Asia	G-VII	AY593768					
A	Asia	Sea-97	AY593760					
A	Asia	Sea-97	AY593753					
A	Asia	Iran-05 (SIS-10)	KT968663					
A	Asia	Iran-05	AY593771					
A	Asia	Iran-96	AY593780					
A	EURO-SA	A3	AY593794					
A	EURO-SA	A5	AY593778					
A	EURO-SA	A10	KJ754939					
A	EURO-SA	A12	AY593793					
A	EURO-SA	A16	KC440882					
A	EURO-SA	A17	KC440881					
A	EURO-SA	A24	AY593777					
A	EURO-SA	A27	EF117837					
A	EURO-SA	A79	AY593785					
A	EURO-SA	A81	AY593789					
A	Africa	G-III	AY593761					
A	Africa	G-IV	AY593757					
A	Africa	G-IV	JF749843					
A	Africa	G-VII	KP940474					
A	Africa	G-VIII	HM854025					
O	Cathay		EF175732					
O	ME-SA	PanAsia	AF506822					
O	ME-SA	PanAsia	AJ539140					
O	ME-SA	PanAsia	KR265075					
O	ME-SA	PanAsia2	FJ175666					
O	Me-SA	PanAsia2	GU384683					
O	ME-SA	PanAsia2	JX040500					
O	ME-SA	PanAsia2	KC440883					
O	ME-SA	Ind-2001	KJ825809					
O	EURO-SA	O1	JX869188					
O	EURO-SA	O2	AY593826					
O	SEA	Mya-98	HM229661					
O	SEA	Mya-98	LC036265					
O	EA-2		KU821591					
Asia-1	Asia		AY593796					
Asia-1	Asia		AY593797					
Asia-1	Asia		AY593800.1					
Asia-1	Asia		DQ989309					
Asia-1	Asia		DQ989321					
Asia-1	Asia		DQ989323					
Asia-1	Asia		EF614458					
Asia-1	Asia		GT125645					
Asia-1	Asia		GT125646					
Asia-1	Asia		HQ113233					
Asia-1	Asia		KT360085					
Asia-1	Asia		HQ631363					
Asia-1	Asia	Sindh-08	HQ632774					
Asia-1	Asia	Sindh-08	JN006719					
Asia-1	Asia	Sindh-08	JN006720					
C	EURO-SA	C1	AF274010.1					
C	Africa		KM268897.1					
SAT 1	I		JF749860					
SAT 1	I		KM268899					
SAT 1	II		AY593839					
SAT 1	II		AY593846					
SAT 1	III		KT821590					
SAT 1	III		AY593840					
SAT 1	III		AY593841					
SAT 1	III		AY593842					
SAT 1	IV		HM067706					
SAT 1	VI		AY593844					
SAT 2	I		AY593848					
SAT 2	I		JF749864					
SAT 2	II		AF540910					
SAT 2	III		AY593847					
SAT 2	IV		JF749861					
SAT 2	IV		KM268900					
SAT 2	VII	Ghb-12	JX014255					
SAT 2	VII	Ghb-12	JX014256					
SAT 2	VII	Ghb-12	KC440884					
SAT 2	IX		AY593849					
SAT 2	X		HM067704					
SAT 2	X		HM067705					
SAT 2	X		JF749862					
SAT 3	I		AY593850					
SAT 3	I		KM268901					
SAT 3	I		KR108950					
SAT 3	I		KX375417					
SAT 3	II		AY593851					
SAT 3	II		AY593852					
SAT 3	II		AY593853					
SAT 3	V		KJ820999					

II. Adaption of FMDV Asia-1 to Suspension Culture: Cell Resistance Is Overcome by Virus Capsid Alterations

Veronika Dill, Bernd Hoffmann, Aline Zimmer, Martin Beer, Michael Eschbaumer

Viruses 2017, 9, 231

doi:10.3390/v9080231

viruses

MDPI

Article

Adaption of FMDV Asia-1 to Suspension Culture: Cell Resistance Is Overcome by Virus Capsid Alterations

Veronika Dill [1], Bernd Hoffmann [1], Aline Zimmer [2], Martin Beer [1] and Michael Eschbaumer [1,*]

1 Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, Südufer 10, 17493 Greifswald-Insel Riems, Germany; veronika.dill@gmx.de (V.D.); bernd.hoffmann@fli.de (B.H.); martin.beer@fli.de (M.B.)

2 Merck KGaA, Merck Life Sciences, Upstream R&D, Frankfurter Straße 250, 64293 Darmstadt, Germany; aline.zimmer@merckgroup.com

* Correspondence: michael.eschbaumer@fli.de; Tel.: +49-38351-71211

Academic Editor: Andrew Mehle
Received: 2 June 2017; Accepted: 14 August 2017; Published: 18 August 2017

Abstract: Foot-and-mouth disease virus (FMDV) causes a highly contagious disease with catastrophic economic impact for affected countries. BHK21 suspension cells are preferred for the industrial production of FMDV vaccine antigen, but not all virus strains can be successfully propagated in these cells. Serotype Asia-1 is often affected by this phenomenon. In this study, the Asia-1 strain Shamir was used to examine viral, cellular and environmental factors that contribute to resistance to cell culture infection. Cell media composition, pH and ammonium chloride concentration did not affect Asia-1 differently than other serotypes. Virus replication after transfection of viral genome was not impaired, but the adhesion to the cells was markedly reduced for Asia-1 in comparison to serotype A. The Asia-1 Shamir virus was successfully adapted to grow in the resistant cells by using a closely related but susceptible cell line. Sequence analysis of the adapted virus revealed two distinct mutations in the capsid protein VP1 that might mediate cell attachment and entry.

Keywords: foot-and-mouth disease virus; BHK21; susceptibility; resistance; serotype Asia-1; adaption; suspension cells; adhesion; receptor; antigen production

1. Introduction

In spite of international control efforts, foot-and-mouth disease virus (FMDV) is still widespread in the Middle East, Asia and Africa, where it causes severe disruptions of livestock production and trade [1]. FMDV comprises seven distinct serotypes, namely serotype A, O, C, Southern African Territories (SAT) 1–3 and Asia-1, and a previous infection with one serotype does not protect against an infection with any of the other six [2]. To fight this highly contagious disease, inactivated vaccines are produced at a large scale. The dominant cell line for industrial production of FMDV vaccine antigen is baby hamster kidney-21, clone 13 (BHK21C13) by MacPherson and Stoker [3], adapted to grow in suspension by Capstick et al. [4], and used in large fermenters as first described by Telling and Elsworth [5]. Unfortunately, BHK21 cells have some phenotypic features that are detrimental for production of FMDV antigen. Along with alterations in cell ploidy and a down-regulated surface expression of particular integrins correlated with the loss of actin stress fibers (especially in suspension) [6–8], BHK cells vary in their susceptibility for different FMDV strains [9,10]. Furthermore, the ability for FMDV infection can get lost on repeated subculturing [9,11].

To test the susceptibility for FMDV of a cell line, serotype Asia-1 viruses may serve as an indicator because this serotype has the most difficulties to grow in different BHK cell lines. A cell line that supports growth of Asia-1 usually is susceptible to all other serotypes of FMDV [9,10].

While some studies assumed that some of the deficits of BHK cells are due to a generally reduced capacity for virus attachment [12], Clarke and Spier proposed that the susceptibility of a cell population is determined by the proportion of susceptible and resistant cells within the population [9].

However, the mechanisms that determine susceptibility are still not fully solved. This study investigates why FMDV serotype Asia-1 in particular is disadvantaged in infecting BHK cells in the context of the underlying mechanisms that form the basis of cell susceptibility to infection with FMDV.

2. Materials and Methods

2.1. Cells

The suspension cell line BHK21C13-2P (BHK-2P) provided by the European Collection of Authenticated Cell Cultures (ECACC 84111301), was adapted to grow in the serum-free Cellvento^{TM} BHK200 medium (Merck KGaA, Darmstadt, Germany) in TubeSpin® bioreactors (TPP Techno Plastic Products AG, Trasadingen, Switzerland), cultured in a shaker incubator with 320 revolutions per minute (rpm) at 37 °C, 5% carbon dioxide and 80% relative humidity.

An adherent BHK21 cell line (clone 13, derived from American Type Culture Collection [ATCC] CCL-10™, held as CCLV-RIE 179 in the Collection of Cell Lines in Veterinary Medicine, Friedrich-Loeffler-Institut, Greifswald, Germany; in short: BHK179) served as a positive control for virus susceptibility. It was cultured in Minimum Essential Medium Eagle (MEM) supplemented with Hanks' and Earle's salts (Sigma-Aldrich, St. Louis, MO, USA) with 10% fetal bovine serum (FBS) during maintenance and passaging, and with 5% FBS during infection experiments. Cells were incubated in sealed flasks at 37 °C.

The assessment of cell death and viability is fundamentally different between adherent and suspension cells. In adherent cells, cell death can be visually evaluated and is reported as the percentage of cells in a field of view that exhibit cytopathic effect (CPE). Cell death in suspension culture cannot as easily be evaluated under a microscope. Cell viability is quantified by a dye exclusion method. In addition, to account for the rapid growth of healthy suspension cells, the cell density in an infected culture is compared to an identically seeded negative culture. CPE and cell viability are related concepts, but are not identical.

The Chinese hamster ovary (CHO) cell lines CHO-K1 (ATCC CCL-61, held as CCLV-RIE 134) and CHO677 (CRL 2244, held as CCLV-RIE 1524) were maintained in Ham's MEM mixed 1:2 with Iscove's Modified Dulbecco's Medium (Thermo Fisher Scientific, Darmstadt, Germany) and with 10% FBS at 37 °C in sealed flasks.

2.2. Susceptibility of BHK-2P for FMDV Stock Viruses

Different Asia-1 isolates (Asia TUR 6/2014, Asia TUR 6/2014-PT, Asia HKN 5/2005, Asia PAK 5/2012) were selected from archival stocks of the Friedrich-Loeffler-Institut (FLI) and were tested for their ability to successfully infect BHK-2P cells. Virus was first grown on adherent BHK164 cells and the gained supernatant was used to infect the BHK-2P, followed by repeated passaging in BHK-2P. Successful virus replication was defined as a decrease in cell viability and growth. Cell viability was determined using trypan blue (Bio-Rad, Hercules, CA, USA) and an automatic cell counter (Bio-Rad, model TC20™, Munich, Germany). To check the supernatant for remaining infectivity, it was passaged on adherent cells (infection control).

For further experiments, a representative Asia-1 strain, the Shamir/ISR/1989 isolate, was used. As comparison, a second strain, FMDV A_{24} Cruzeiro, which can infect the adherent and the suspension cell lines, was used in selected experiments. A third strain, FMDV O_1 Manisa, was used in experiments

testing the pH sensitivity. All virus strains were used as cell culture supernatant. For a detailed listing of the used isolates and passage histories see the Supplementary Materials (Table S1).

2.3. Virus Titrations

Viral titers were estimated by endpoint titration. Titers are expressed as 50% tissue culture infectious doses ($TCID_{50}$) per 100 µL calculated with the Spearman-Kärber method [13,14]. Titrating BHK179-passaged virus on BHK179 cells could bias the results, therefore all titrations were performed on adherent BHK21 clone "Tübingen" cells (CCLV-RIE 164; referred to as BHK164).

2.4. Storage Conditions

To rule out that the difficulties of Asia-1 Shamir to infect BHK-2P are due to the media in which the cells are maintained, different storage conditions were tested. Equal amounts of virus (Asia-1 Shamir or A_{24} Cruzeiro) were added in a final dilution of 1:100 to 1.5 mL of serum-free Cellvento™ BHK200, Cellvento™ BHK200 with 5% FBS, or MEM with 5% FBS. The samples were incubated at 37 °C with agitation (350 rpm) for 0, 4, 8, 12, or 24 h and the remaining infectivity was determined as described above. All experiments were independently performed three times.

2.5. pH Sensitivity

A protocol already published by Martín-Acebes et al. [15] was used with some modifications. In short, equal amounts of virus (Asia-1 Shamir, O_1 Manisa or A_{24} Cruzeiro) were mixed with 1 mL phosphate-buffered saline (PBS) solutions of different pH (7.5, 7.0, 6.8, 6.5, 5.5) in a final dilution of 1:100 and incubated for 30 min at room temperature. Afterwards, the samples were neutralized with 1M Tris-HCl (pH 8.0). The remaining infectivity in each sample was determined by titration as described above. All experiments were independently performed three times.

2.6. Blockage of Endosomal Acidification with Ammonium Chloride

The assay was performed as published [15] with some modifications. BHK179 monolayers as well as aliquots of BHK-2P suspension cells were treated with culture medium supplemented with 25 mM or 50 mM NH_4Cl (Sigma-Aldrich) one hour prior to infection. The same concentration of NH_4Cl was maintained throughout the rest of the infection. Cells in culture medium without supplementation served as positive controls. Adherent cells were infected with Asia-1 Shamir or A_{24} Cruzeiro, whereas suspension cells were only infected with A_{24} Cruzeiro adapted to BHK-2P. The cells were incubated for six hours post infection and virus was harvested by freezing at −70 °C and thawing. Viral yields were estimated by titration as described above. All experiments were performed independently in duplicates three times.

2.7. RNA Transfection and Immunofluorescence

2.7.1. RNA Transfection

Aliquots of Asia-1 and A_{24} Cruzeiro grown in BHK179 were used as source of FMDV RNA. Nucleic acid was isolated manually with the QIAamp® Viral RNA Mini Kit (Qiagen GmbH, Hilden, Germany) as described by the manufacturer. The RNA content of the eluates was quantified with a NanoDrop™ 2000c spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA), and FMDV genome load was assessed using a real-time reverse-transcription polymerase chain reaction (RT-PCR) assay targeting the 3D-coding region of the viral genome [16].

BHK-2P cells grown in suspension were transfected with the RNA using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. In short, 10 µL of RNA and 0.75 µL or 1.5 µL of Lipofectamine diluted in Cellvento™ BHK200 were mixed and incubated at room temperature for 20 min to allow complex formation. Afterwards, these mixtures were transferred to 78 µL of BHK-2P cells (approximately 1×10^5 cells/mL) in 24-well plates and medium was added

up to 200 µL. The cells were incubated for 4 h at 37 °C and 700 µL medium was then added, followed by further incubation at 37 °C for 24 h. After 24 h, the entire contents of the wells were harvested and frozen at −70 °C. The thawed cell lysate was clarified by centrifugation and used to treat adherent BHK164 cells.

2.7.2. Immunofluorescent Staining

Confluent monolayers of BHK164 cells grown in 96-well plates were treated with 50 µL of clarified cell lysate harvested directly from the transfected cells (see Section 2.7.1), as well as with stock virus (positive control) and with 10 µL of the extracted RNA itself (negative control). Cells were then incubated at 37 °C for 4h. After the incubation period, the supernatant was discarded, cells were washed with PBS once and fixed with 4% (*w*/*v*) paraformaldehyde (Sigma) dissolved in PBS for 20 min at room temperature (RT). Cells were washed again once with PBS and permeabilized with PBS with 0.1% (*v*/*v*) Triton X-100 for 5 min at RT. The permeabilized cells were then incubated with the primary antibody (polyclonal serum from cattle experimentally infected with O_1 Manisa) diluted 1:200 in PBS with 0.05% (*v*/*v*) polysorbate 20 (Tween; in short: PBS/T), for 20 minutes at RT. Next, cells were washed two times with PBS/T and incubated with the secondary antibody, rabbit anti-bovine immunoglobulin G (IgG) conjugated with fluorescein (FITC) (Thermo Fisher Scientific, Darmstadt, Germany) (diluted 1:500 in PBS/T) for 20 min at RT. Afterwards, cells were washed two times with PBS/T, nuclei were counterstained with DAPI (4′,6-diamidino-2′-phenylindole, dihydrochloride, Thermo Fisher Scientific; diluted 1:1000 in PBS/T) for 5 min at RT, followed by three washes with PBS/T. Cells were examined with a fluorescence microscope (Axio Vert.A1; Carl Zeiss, Oberkochen, Germany) and data were collected utilizing the prepared controls of the anti-FMDV antibodies with uninfected cells to give the negative background levels. The captured images were processed with ImageJ to globally adjust contrast and brightness and merge the green and blue color channels [17].

2.8. Susceptibility of Other Cell Lines and Cross-Infection Experiments

2.8.1. Susceptibility of Other Cell Lines

To determine if FMDV Asia-1 Shamir generally cannot infect suspension cells or if that failure is more specifically caused by changes in each cell line during the adaption to suspension culture, several other lines of BHK suspension cells were inoculated with the virus: The adherent original BHK21, clone 13 (BHK21C13; derived from ATCC CCL-10™), maintained in GMEM with 8.5% FBS (referred to as cell line #1), the same cells adapted to grow in suspension by sequential withdrawal of serum (#3), the previously introduced BHK-2P (Section 2.1) maintained either in GMEM with 10% FBS (#2) or adapted to BHK200 in two different processes (lines #4 and #5), plus four additional suspension cell lines, all maintained in BHK200 (BHK21-C, #6; BHK21-Hektor, #7; BHK21-InVitrus, #8 (Sigma); production BHK, #9). All cell lines except #8 were provided by Merck KGaA, Darmstadt, Germany. Successful virus replication in suspension cells was defined as a decrease in cell viability in comparison to the uninfected cells (negative control, NC), together with a decrease in the required infection volume. In adherent cell lines, the occurrence of cytopathic effect (CPE) was defined as successful virus replication.

2.8.2. Infection and Virus Adaption Experiments

All susceptible cell lines were used to repeatedly passage Asia-1 Shamir. The virus grown in these cell lines was then put back on BHK-2P to determine if it could now infect them.

Infection experiments and adaption studies were performed with the following virus isolates, obtained by passaging Asia-1 Shamir in the cell lines described in the previous section: #3 Asia-1, after four passages on BHK21C13; #8 Asia-1, after seven passages on BHK21-InVitrus and #9 Asia-1, after five passages on production BHK (see also Table S1). BHK-2P cells were seeded with a cell density of 1×10^6 cells/mL and infected with 5 mL cell culture supernatant, corresponding to 1/6 of the

total culture volume of 30 mL. After an incubation period of 20–24 h, cell viability was determined. Once the cell viability dropped below 15%, the infection volume for the next passage was reduced. The adaption of the virus was considered successful when an infection volume of 5% of the total culture volume (i.e., 1.5 mL) was sufficient to kill at least 90% of the cells after 20–24 h. To prove successful virus replication, one aliquot taken at the time of infection (0 h post infection, hpi) and one aliquot taken at the end of the incubation period (24 hpi) were titrated as described above (Section 2.3). A mock-infected negative control was passaged under the same conditions as the virus.

2.8.3. Attachment Test

BHK179 cell monolayers were washed with medium and then incubated with Asia-1 Shamir ($10^{6.1}$ $TCID_{50}$), Asia-#3 ($10^{5.1}$ $TCID_{50}$), Asia-#8 ($10^{5.5}$ $TCID_{50}$), Asia-#9 ($10^{4.7}$ $TCID_{50}$) or A_{24} Cruzeiro ($10^{5.4}$ $TCID_{50}$) in 1 mL of MEM with 5% FBS. BHK-2P were adjusted to 1×10^6 cells per mL, centrifuged at $300\times$ *g* for 5 min and resuspended in 1 mL fresh Cellvento^{TM} BHK200 with the same viruses and doses as used for the adherent cells.

The cells were kept on ice to prevent the internalization of the virus [18]. After 15 min, the supernatant was discarded and the cells were washed two times with medium to remove unbound virus. Monolayers were detached with 0.05% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA). The cell pellet was collected in 1 mL MEM with 5% FBS and titrated. All experiments were performed in duplicate and repeated for a total of three times.

2.8.4. Sequence and Structure Analysis

FMDV RNA was extracted from the original virus stock of Asia-1 Shamir and the final passages of #3 Asia-1, #8 Asia-1 and #9 Asia-1 using TRIzol® LS Reagent (Invitrogen, Karlsruhe, Germany) and the RNeasy® Mini Kit (Qiagen, Hilden, Germany) according to the manufacturers' instructions.

Reverse transcription and PCR was done using a method previously described [19]. Three additional primer pairs were used to fill in gaps (VP1-3165F, VP1-3632R, VP3-2835F, VP3-3217R, 3D-7320F and 3D-8097R, see Table S3). The nucleotide sequences were assembled and mapped with Geneious (Biomatters Limited, Auckland, New Zealand) against the complete published sequence for Asia-1 Shamir (Genbank accession no. JF739177). Sequences of the initial Asia-1 Shamir strain and the #3-, #8- and #9-Asia-1 isolates have been uploaded to Genbank (MF063053-MF063056).

The capsid map was created with the Virus Particle Explorer (VIPER, http://viperdb.scripps.edu/) [20] using FMDV O1/BFS/1860 and A10/Argentina/61 as templates (Protein Data Bank accessions 1BBT [21] and 1ZBE [22]). The crystallographic structure of the mutations located in the capsid pentamer was analyzed with the UCSF Chimera package [23], using 1ZBE as template. Chimera is developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco, CA, USA (supported by NIGMS P41-GM103311).

2.9. Infectivity Assay on Receptor-Deficient Cells

Infectivity assays on CHO K1 and CHO677 cells were performed as described by Jackson et al. [24] with one modification: harvested virus-infected CHO cells were titrated on BHK164. All experiments were performed in duplicate and repeated for a total of three times.

2.10. Virus Neutralization Test

The virus neutralization test (VNT) was performed with Asia-1 Shamir, Asia-#3, -#8 and -#9 and BHK164 cells as prescribed by the World Organisation for Animal Health (OIE) [25]. Neutralization titers are expressed as the $\log_{10}$ of the reciprocal of the final dilution of serum where 50% of wells are protected, i.e., show no CPE. Two different sera of bovine origin were used for the VNT. Serum "P2/99" had been collected 21 days after vaccination (dpv) with a commercial Asia-1 vaccine (Bayer AG, lot W4829). Serum "RD460" was taken 19 days after infection with Asia-1 stock virus (second passage on BHK164). The experiments were conducted in duplicates, three times independently. R_1 values

Viruses **2017**, *9*, 231

were calculated by dividing the mean neutralization titer of each serum against the adapted virus by the mean neutralization titer of the serum against the original isolate.

2.11. Statistical Analysis

In all experiments, the differences between treatment groups were evaluated with linear mixed-effects models using R (http://www.r-project.org) and lme4 [26]. Wald chi-square tests for fixed effects and their interactions were calculated with the car and phia packages. p-Values of <0.01 were considered significant.

3. Results

3.1. FMDV Serotype Asia-1 Cannot Infect BHK-2P Cells

Three independent attempts have been performed to infect the suspension cell line BHK-2P with the Asia-1 Shamir isolate as well as single attempts with other Asia-1 isolates. Supernatant to inoculate the first passage on BHK-2P was obtained from adherent BHK cells that also served as positive controls for successful virus replication. After each passage, BHK-2P were lysed by freezing and thawing, and the clarified lysate was used to inoculate a fresh culture of BHK-2P cells. After repeated passaging without any decrease in cell viability, lysate of the last BHK-2P passage was transferred to adherent BHK or LFBKαvβ6 to check for residual infectivity (infection control). No live virus was detected in any of these experiments (see Table S4).

3.2. The Ability of FMDV to Infect BHK-2P is not Dependent on the Culture Environment

Different culture conditions such as the media in which cells and virus are cultured as well as its pH were examined for their influence on virus particle stability or cell susceptibility.

First, equal amounts of FMDV A_{24} Cruzeiro and Asia-1 were incubated over a period of 24 h in three different culture media. Every four hours, one aliquot was titrated to determine the remaining infectivity. Because BHK-2P are maintained in media without serum or any other animal-derived components, the experiments were designed to investigate whether the medium alone has detrimental effects on virus particle stability.

Viral titers declined over time for both serotypes. Statistical analysis did not show any significant differences between A_{24} Cruzeiro and Asia-1 Shamir in any of the three media (Figure 1). Therefore, the media does not directly influence the potential of the virus to infect the BHK-2P cells.

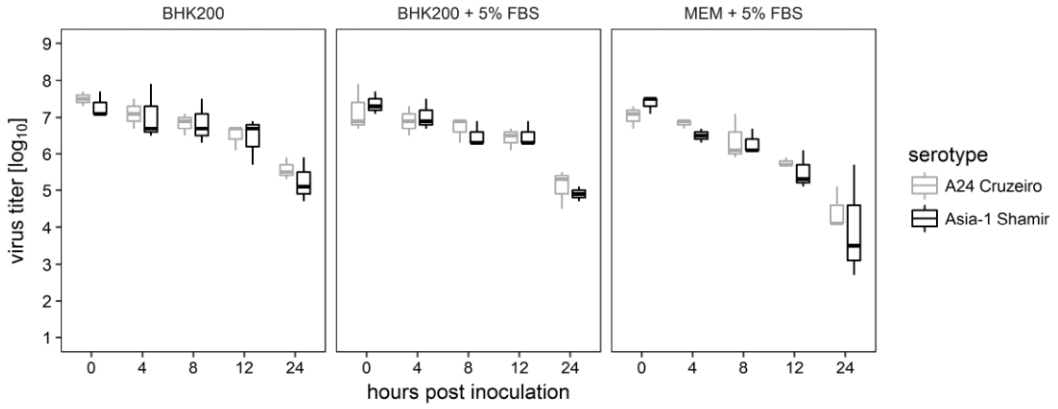

Figure 1. Storage of FMDV A_{24} Cruzeiro and Asia-1 Shamir in different culture media for 24 h. Equal amounts of virus were incubated in serum-free Cellvento™ BHK200 media, Cellvento™ BHK200 supplemented with 5% fetal bovine serum (FBS), and Minimum Essential Medium Eagle (MEM) supplemented with 5% FBS. Every four hours, one aliquot was titrated to determine remaining infectivity. All experiments were independently performed three times.

Viruses **2017**, *9*, 231

Next, the pH-dependent disintegration of the serotype Asia-1 isolate was compared to serotypes A or O. Equal amounts of each virus were incubated in PBS solutions of different pH for 30 min and remaining infectivity was determined by titration. Results indicate a wide spread in infectivity for the solution with pH 6.5 but overall show no difference between serotypes for the different pH conditions (Figure 2).

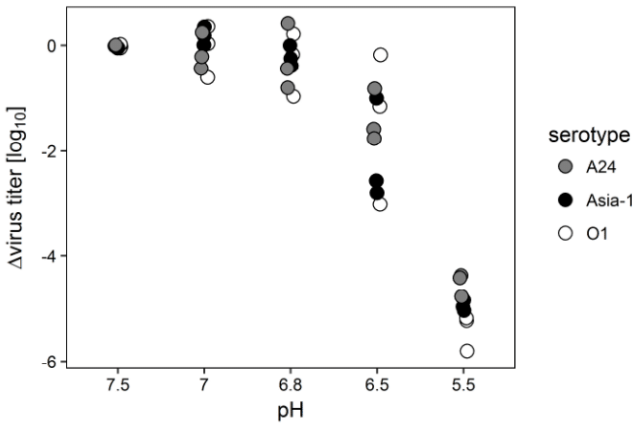

Figure 2. Incubation of FMDV A_{24} Cruzeiro, O_1 Manisa and Asia-1 Shamir in PBS solutions of different pH for 30 min. Equal amounts of virus were incubated at pH 7.5, 7.0, 6.8, 6.5 and 5.5 for 30 min. The solutions were neutralized and remaining infectivity was determined by titration. Values on the y-axis represent the reduction in titer compared to virus incubated at pH 7.5. All experiments were independently performed three times.

Finally, BHK179 cell monolayers as well as BHK-2P suspension cells were treated with 25 mM or 50 mM ammonium chloride. Untreated cells served as controls. Virus yields for all strains were decreased by the ammonium chloride treatment. The original A_{24} virus strain in BHK179 cells was significantly more affected by the ammonium chloride treatment than the other strains (Figure 3), but there was no significant difference between Asia-1 in adherent BHK179 and the 2P-adapted strain of A_{24} in suspension culture. Therefore, even though adaption to suspension cells increased the ammonium chloride resistance of A_{24}, Asia-1 Shamir does not display a degree of susceptibility that could explain its inability to infect BHK-2P cells.

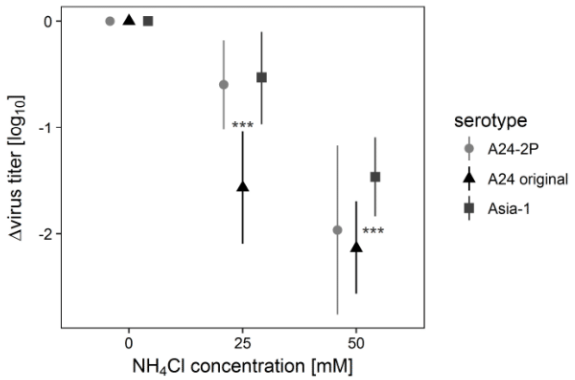

Figure 3. Different NH_4Cl sensitivity of FMDV serotypes A and Asia-1. BHK monolayers as well as suspension cells were treated with 50 mM or 25 mM ammonium chloride or left untreated. BHK179 were infected with FMDV Asia-1 Shamir and A_{24} Cruzeiro (original), and BHK-2P were infected with the adapted virus strain A24-2P. After 24 h of incubation, the remaining infectivity was determined by titration. Values on the y-axis represent the reduction in virus yield compared to untreated cells. Experiments were performed in duplicates three times independently. Significance code: *** $p < 0.001$.

Viruses **2017**, *9*, 231

In summary, environmental conditions such as cell culture media and pH, as well as endosome acidification, do not explain the inability of FMDV Asia-1 Shamir to infect certain cell lines.

3.3. BHK-2P Cells Can Produce Infectious Asia-1 FMDV

Viral RNA of A_{24} Cruzeiro and Asia-1 Shamir was extracted und transfected into BHK-2P. When the supernatant of the transfected cells was added to BHK164 monolayers, they showed strong CPE and stained positive for FMDV antigen after 24 h of incubation (Figure 4). However, virus production in the BHK-2P cells occurred only in a single cycle, i.e., while the transfected cells did produce virus, the virus that was released was not amplified by a passage in BHK-2P cells. These results indicate that the reduced susceptibility of BHK-2P cells is related to a blocked virus entry or an inefficient virus adhesion at the cell surface, but not caused by a defect in the replication of the virus and the production of infectious progeny. The cells are resistant but permissive.

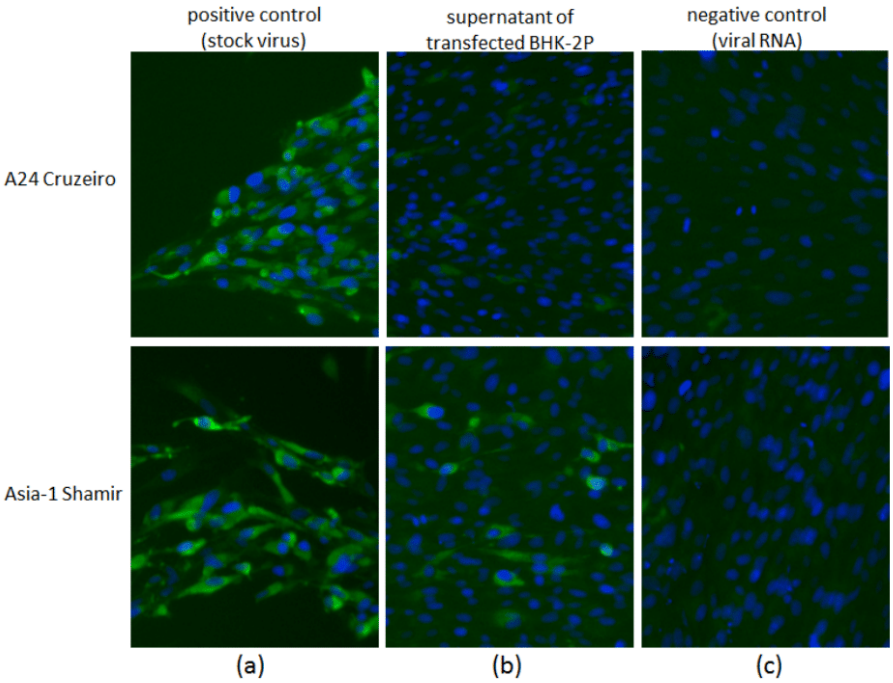

Figure 4. Detection of FMDV antigen in adherent BHK164 after treatment with supernatant from transfected BHK-2P. Adherent BHK164 were incubated with stock virus, clarified supernatant from transfected BHK-2P, or viral RNA for 4 h at 37 °C, then fixed and stained with serum from FMDV-infected cattle (green staining). Cell nuclei are shown in blue (**a**) Cells infected with A_{24} Cruzeiro and Asia-1 Shamir stock virus as positive controls (PC); (**b**) Cells incubated with supernatant from transfected BHK-2P; (**c**) Cells incubated with viral RNA alone (no transfection reagent) as negative controls (NC).

3.4. Cellular Resistance Can be Overcome by Virus Adaption to a "Wet Nurse" Cell Line

Several different suspension cell lines as well as the parental adherent line were inoculated with Asia-1 Shamir to test their susceptibility for this virus. Results show that the BHK-2P line was not susceptible to the original Asia-1 Shamir isolate under any culture conditions (#2, #4, #5). Two of the other suspension cell lines tested (#6, #7) also did not support the replication of Asia-1 Shamir.

Three cell lines proved susceptible to FMDV Asia-1 Shamir: an adherent BHK21 clone 13 adapted to grow in suspension in serum-free medium after sequential withdrawal of serum (short: BHK21C13, "#3"), the suspension cell line BHK21-InVitrus ("#8"), and another production BHK-derived suspension cell line (short: production BHK, "#9") (Table S2).

Viruses **2017**, *9*, 231

After several passages on these susceptible cells, the recovered Asia-1 viruses were tested for their capacity to infect BHK-2P cells. With these viruses, productive infection in BHK-2P could be achieved after serial passaging (Table 1). Adaptation was considered complete when inoculation of 0.5 mL of supernatant from the previous passage led to a 90% decrease in cell viability within 20 h. The fully BHK-2P-adapted virus isolates (#3 Asia-1, #8 Asia-1 and #9 Asia-1) were then sequenced and compared with the genome of the original Asia-1 Shamir virus strain (Table S6). The quickest adaption onto BHK-2P was achieved for #9 Asia-1 after only four passages. The virus strains #3 Asia-1 and #8 Asia-1 required nearly twice as many passages for complete adaption as #9 Asia-1.

Table 1. Decrease of cell viability and the required infection volume during serial passaging of Asia-1 isolates with different original cell adaption background on BHK-2P.

Passage No.	#3 Virus from BHK21C13 in Cellvento™ BHK200					NC
	Cell Viability 0 hpi	Cell Viability 20 hpi	Viral Titer 0 hpi *	Viral Titer 20 hpi *	Infection Volume	Cell Viability 20 hpi
1	98%	63%	4.9	5.9	5 mL	99%
2	99%	73%	5.5	5.7	5 mL	98%
3	99%	79%	5.7	5.9	5 mL	96%
4	99%	60%	5.3	6.1	5 mL	99%
5	99%	13%	5.5	5.9	5 mL	98%
6	97%	6%	5.5	6.1	4 mL	97%
7	97%	16%	4.9	6.1	3 mL	99%
8	98%	5%	5.3	5.9	1.5 mL	97%
9	98%	8%	4.3	6.1	0.5 mL	96%
Passage No.	**#8 Virus from BHK21-InVitrus Cells in Cellvento™ BHK200**					**NC**
	Cell Viability 0 hpi	**Cell Viability 20 hpi**	**Viral Titer 0 hpi ***	**VIRAL titer 20 hpi ***	**Infection Volume**	**Cell Viability 20 hpi**
1	98%	60%	4.7	5.7	5mL	99%
2	99%	72%	5.5	5.9	5mL	98%
3	99%	86%	5.9	6.1	5 mL	96%
4	99%	53%	5.5	5.9	5 mL	99%
5	99%	16%	5.1	6.3	5 mL	98%
6	97%	10%	5.5	5.9	4 mL	97%
7	97%	12%	5.1	6.3	3 mL	99%
8	98%	6%	5.3	5.9	1.5 mL	97%
9	98%	9%	4.1	6.5	0.5 mL	96%
Passage No.	**#9 Virus from Production BHK Cells in Cellvento™ BHK200**					**NC**
	Cell Viability 0 hpi	**Cell Viability 20 hpi**	**Viral Titer 0 hpi ***	**Viral Titer 20 hpi ***	**Infection Volume**	**Cell Viability 20 hpi**
1	98%	61%	5.9	6.3	5 mL	99%
2	99%	8%	5.5	5.9	5mL	98%
3	99%	14%	5.1	6.1	3 mL	96%
4	99%	2%	4.9	6.1	1.5 mL	99%
5	99%	3%	3.9	5.7	0.5 mL	98%

* Titers are expressed in $\log_{10}$ $TCID_{50}$ per 100 µL. 3.5 Attachment of FMDV to BHK-2P cells differs between virus isolates. hpi: Hours post-infection; NC: Negative control.

FMDV A_{24} Cruzeiro, Asia-1 Shamir and the adapted Asia-1 isolates #3, #8 and #9 were incubated with adherent BHK179 cells or BHK-2P for 15 min. Cells and virus were incubated on ice to avoid virus uptake into the cells. Afterwards, the cells were thoroughly washed and the amount of bound virus was determined by titration. The comparison between the two cell lines revealed no significant difference for A_{24} Cruzeiro ($\log_{10}$ $titer_{179}$ with standard deviation = 4.6 ± 0.2; $titer_{-2P}$ = 4.9 ± 0.3) but a highly significant loss of titer for Asia-1 Shamir ($titer_{179}$ = 4.5 ± 0.6; $titer_{-2P}$ = 3.1 ± 0.5). In contrast, Asia-1 isolates #3, #8 and #9 showed a significantly higher titer on BHK-2P ($\log_{10}$ titers = 5.5 ± 0.3; 5.7 ± 0.3; 4.3 ± 0.3, respectively) in comparison to the adherent BHK179 ($\log_{10}$ titers = 4.3 ± 0.2; 4.5 ± 0.4; 3.0 ± 0.5, respectively) (Figure 5).

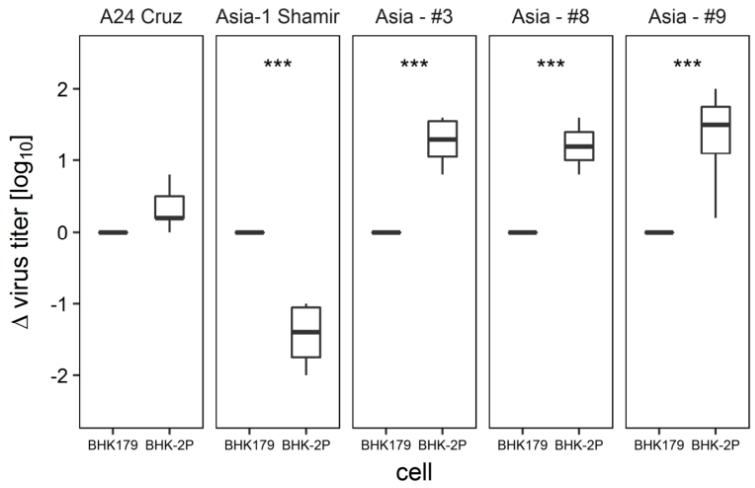

Figure 5. Infectivity after attachment of A_{24} Cruzeiro, Asia-1 Shamir and adapted Asia-1 isolates to BHK179 or BHK-2P. The viruses were incubated either with the adherent cell line BHK179 or the suspension cell line BHK-2P for 15 min on ice to prevent internalization. Cells were washed to remove unbound virus and titrated. The *y*-axis shows the difference in titer between the BHK-2P and the BHK179 cell preparation. Experiments were performed in duplicates and repeated for a total of three times. Significance code: *** $p < 0.001$.

3.5. Sequence Analysis Revealed Mutations in the Five-Fold Axis and Extended Receptor Tropism

For #9 Asia-1, five non-synonymous mutations (i.e., amino acid changes) were found in the genome of the fully adapted virus. The virus strains #3 Asia-1 and #8 Asia-1 acquired three identical non-synonymous mutations during the adaption process. All three virus strains replaced an uncharged glutamine (Q) at residue 110 of the VP1 capsid protein with either a positively charged arginine (R) (#3 Asia-1 and #8 Asia-1) or a positively charged lysine (K) (#9 Asia-1). This mutation always occurred together with a second exchange in VP1, either Q108R for #3 Asia-1 and #8 Asia-1 or T83A (threonine to alanine) for #9 Asia-1. All modified amino acids are exposed on the outer capsid surface and are located in close spatial proximity to each other with no known interactions at any protomer interfaces (Figure 6).

Besides that, virus strain #9 Asia-1 exhibited one additional mutation in the C-terminus of VP1 (E202K) and an amino acid exchange from a negatively charged glutamic acid to a positively charged lysine at position 59 (E59K) of the VP3 protein (Table 2). The mutations in the C-terminus of VP1 as well as the mutation in the VP3 protein are part of the heparin-binding site, which is formed by residues 55–58 of VP3 and the following residues 58–60 of the successional loop, the C- terminus of VP1 (residues 195–197) and residues 133–138 of the VP2 protein [22].

Table 2. Overview of amino acid changes during passaging of Asia-1 Shamir.

Protein	Structure *	Virus Isolate		
		#3 Asia-1	#8 Asia-1	#9 Asia-1
VP3	beta-B 'knob'	-	-	E59K
VP1	capsid surface	-	-	T83A
VP1	capsid surface	Q108R	Q108R	-
VP1	capsid surface	Q110R	Q110R	Q110K
VP1	C-terminus	-	-	E202K
2C	-	K285Q	K285Q	K285Q

* According to Fry et al. [22].

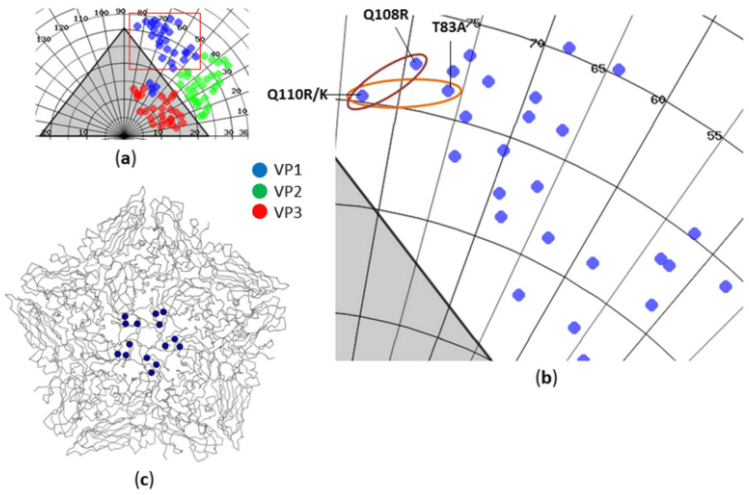

Figure 6. Capsid maps of the outer surface molecules of FMDV. The capsid map was created with VIPER [20] using FMDV O_1/BFS/1860 and A_{10}/Argentina/61 as templates: (**a**) overview of the exposed residues on the outside of the virus capsid, color-coded for VP1 (blue), VP2 (green) and VP3 (red); (**b**) zoom on the location where mutations were found in #3 Asia-1, #8 Asia-1 and #9 Asia-1; (**c**) crystallographic structure of an FMDV capsid pentamer. The VP1 residues 83, 109 and 110 are highlighted. All three mutations are in close spatial proximity to each other and to the fivefold symmetry axis. Residue 110 was mutated in all three viruses, and it appears that this exchange was only possible in combination with a second exchange: either Q108R (in #3 Asia-1 and #8 Asia-1) or T83A (in #9 Asia-1).

Moreover, all three virus strains acquired the same heterologous mutation in the non-structural 2C protein (K285Q), which is responsible for RNA replication [27].

To determine if the Asia-1 mutants adapted to use heparan sulfate (HS) or a non-integrin, non-HS receptor, the original virus isolate Asia-1 Shamir and the adapted mutants were incubated on different CHO cell lines. CHO-K1 cells express HS but none of the integrins αvβ1, αvβ3, αvβ6 or αvβ8. CHO677 cells are also deficient for these integrins, but do not express HS. All three adapted isolates were able to grow on both cell lines, while the wildtype Asia-1 Shamir was unable to infect either of them (Table 3). For growth on CHO-K1 cells, no significant differences between #3 Asia-1, #8 Asia-1 and #9 Asia-1 could be found. Interestingly, for the #9 Asia-1 isolate, there was also no significant difference between CHO-K1 or CHO677 cells, whereas titers of #3 Asia-1 and #8 Asia-1 were significantly reduced on CHO677 in comparison to CHO-K1 ($p < 0.001$). These findings suggest that all three adapted isolates extended their receptor usage and that the additional mutations in the capsid of #9 Asia-1 offer an advantage in cell culture infection compared to #3 and #8 Asia-1.

Table 3. Growth comparison of Asia-1 Shamir and its mutants on receptor-deficient cell lines.

Cell Line	Virus Isolate			
	Asia-1 Shamir	**#3 Asia-1**	**#8 Asia-1**	**#9 Asia-1**
CHO-K1	negative	3.7 ± 0.5	3.7 ± 0.6	4.2 ± 0.6
CHO677	negative	2.4 ± 0.5	2.2 ± 0.2	3.8 ± 0.7

Values represent mean virus titers and standard deviation in $\log_{10}$ $TCID_{50}$ per milliliter ($TCID_{50}$/mL).

3.6. Virus Adaptation Alters Neutralization Profile

Virus neutralization tests were conducted to determine if any antigenic changes took place during adaptation of Asia-1 Shamir to the BHK-2P cell line. Serum of bovine origin, collected after infection

Viruses **2017**, *9*, 231

with Asia-1 Shamir ("RD460"), strongly neutralized the original Asia-1 and the adapted #9 isolate (r_1 value = 0.92), but showed a highly significant loss in titer against the #3 and #8 adapted Asia-1 isolates (r_1 = 0.21 and 0.36, respectively). The neutralization titers of a second serum, taken from a vaccinated animal ("PC2/99"), generally were lower compared to the serum of the infected animal. Similar to the infected animal, the loss in neutralizing activity of this serum for the adapted isolates #3 and #8 (r_1 = 0.33 and 0.48) was highly significant. The titers obtained with the adapted isolate #9 (r_1 = 0.78) were reduced compared to the original Asia-1 isolate, but the difference was not significant (p = 0.05) (Figure 7).

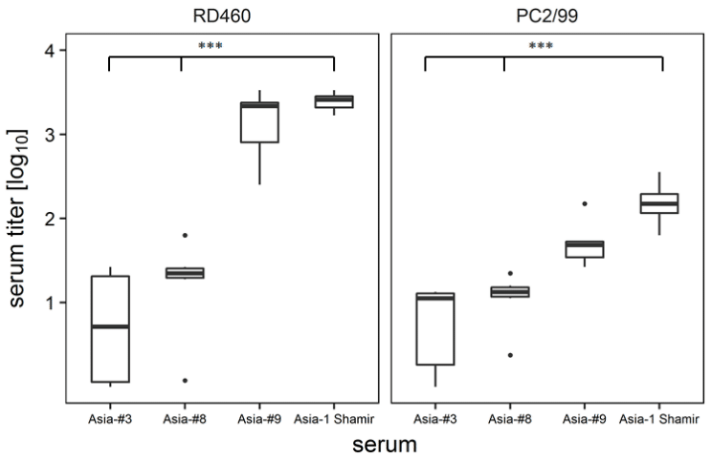

Figure 7. Serum neutralization tests revealed an altered neutralization profile of the adapted viruses. Asia-1 Shamir and the adapted Asia-1 isolates #3, #8 and #9 were incubated with serum from experimentally infected ("RD460") or vaccinated ("PC2/99") cattle. Experiments were performed in duplicates and repeated for a total of three times. Significance code: *** $p < 0.001$.

4. Discussion

Since more than fifty years ago, BHK cells are the cell line of choice for propagation of FMDV and production of FMDV vaccines [2], even though specific problems with this cell line have been known for at least 35 years [9]. In addition to alterations in cell morphology and reduced surface expression of integrins, especially in BHK suspension cells [6], the susceptibility of BHK cells for FMDV infection is also known to change [9]. While some BHK cell lines lose the ability for efficient FMDV production on repeated subculturing, other BHK cells do not seem to be susceptible to FMDV Asia-1 at all. In this context, strains of serotype Asia-1 seem to be the most sensitive [9]. Previous investigations of this topic have suggested two possible reasons for the resistance of BHK cells. Diderholme and Dinter proposed resistance due to deficiencies in virus attachment at the cell surface [12], while Clarke and Spier assumed that cell populations are heterogeneous and the overall susceptibility of a cell line depends on the relative proportions of cells with different susceptibilities [9]. Inhibition of viral replication can occur at any point of the viral growth cycle [10] and the exact underlying mechanisms remain unclear.

With serotype Asia-1 being an indicator for the general FMDV susceptibility of a cell line, Asia-1 Shamir was chosen for our investigations. At first, the study focused on the exclusion of external factors in the culture environment that might influence virus particle stability in a serotype-specific manner and thus bias the results. Serum-free media often have reduced pH buffering capacity and fewer stabilizing components compared to serum-containing media [28]. In industrial production, the pH is regulated automatically, but in our small-scale experimental setting no such automatic control was possible. Within the scope of our experiments, the virus particle stability was not dependent on the culture medium or pH and did not differ between serotypes. Similarly, the sensitivity towards ammonium chloride was the same for Asia-1 Shamir, which was not able to infect BHK-2P cells,

and for the 2P-adapted A_{24} Cruzeiro (A24-2P) virus strain. Based on this, external factors that might influence the experimental setting and can inhibit infection right from the start due to particle instability were excluded.

Clarke and Spier have argued that the resistance of a cell line to virus replication is not due to an inhibited release of newly synthesized virus particles [10]. This was confirmed by the results of the present study. Transfection experiments with viral RNA showed a single cycle of virus replication after transfection of BHK-2P cells with viral RNA of Asia-1 Shamir. The production of stable and infectious virus particles was confirmed by passaging the supernatant of the transfected cell culture on to adherent BHK cells. This leads to the conclusion that BHK-2P are indeed capable of virus production, and provides further evidence that their resistance to infection with FMDV Asia-1 is caused by a deficiency in cell binding, entry or uncoating of the virus particle.

Our experiments revealed three susceptible BHK suspension cultures (BHK21C13, BHK21-InVitrus, production BHK). Once Asia-1 Shamir had been successfully adapted to these three cell lines, the adapted strains were used in another attempt to infect BHK-2P cells. Surprisingly, all three pre-adapted strains replicated in BHK-2P and good viral growth was achieved after a few passages.

A previous study proposed a restriction at the stage of virus entry and/or uncoating as reason for the cellular resistance to FMDV, while a lack of virus attachment to the cell surface had been ruled out [10]. Therefore, the next investigations focused on the cell-virus interactions. The attachment capacity of BHK-2P for Asia-1 Shamir was significantly reduced in comparison to BHK179. On the other hand, the BHK-2P adapted Asia-1 isolates attached significantly stronger to BHK-2P than BHK179. These results provide an indication that BHK-2P cells have an altered surface structure in comparison to BHK179 and that the natural isolates of FMDV serotype Asia-1 are disadvantaged in utilizing the presented surface molecules for attachment and entry. FMDV is able to utilize four different integrin molecules for cell binding: αvβ1, αvβ3, αvβ6 and αvβ8 [24,29–31]. The utilization of these receptors varies between serotypes, for example serotype O prefers integrin αvβ6, followed by αvβ1, and serotype A prefers integrin αvβ3 and αvβ6 [32]. For Asia-1 no such preferences are known. BHK cells only possess one of the four integrins, integrin αvβ1, which is even downregulated in suspension cells or highly passaged adherent cells [7]. Another possibility for FMDV binding is the utilization of heparan sulfate proteoglycan (HSPG) as a receptor, typically as an adaption to cell culture [33]. Depending on the receptor used, the virus is either internalized via caveola-mediated endocytosis (heparan sulfate) [34] or in a clathrin-dependent manner (integrins) [35]. Many structural changes take place when cells adapt to grow in suspension, amongst others a restructuring of the cytoskeleton with disappearance of actin stress fibers [7]. For CHO cells, changes in the expression and clustering of integrins have been described after adaption to grow in suspension [36].

Sequencing of the viral genome revealed three amino acid exchanges (VP1: Q108R, Q110R; 2C: K285Q) which were shared by two (#3 Asia-1, #8 Asia-1) of the three isolates. The third isolate (#9 Asia-1) harbored the same mutation in the 2C protein and a similar mutation at position 110 of the VP1 protein (Q to K). Residue 108 was conserved in #9 Asia-1, but there was another amino acid exchange (T83A) in close spatial proximity to the mutations acquired by the other two isolates. In addition, the genome of the third isolate carried two more mutations, introducing positive charges to regions of VP1 (E202K) and VP3 (E59K) that form the HSPG-binding pocket in type O and A viruses [22,37]. The accumulation of positive charges on the capsid surface argues for the acquisition of heparan sulfate binding, particularly for #9 Asia-1. The mutations at residues 108 and 110 of VP1, however, fall outside of the canonical HSPG binding pocket. HSPG binds to the center of the protomer [38], whereas residues 108 and 110 are located at the edge of the protomer, next to the five-fold symmetry axis of the capsid pentamer. The acquisition of positively charged amino acids in this region is associated with the binding of a third, not conclusively identified FMDV receptor [37,38]. In fact, the introduction of a positive charge at residue 110 of VP1 into an FMDV Asia-1 strain enabled it to infect αvβ6- and HSPG-deficient CHO cells, provided that a second positive charge existed in close proximity—residue 109 as described by Berryman et al. [38]. In the present study, all three

isolates gained the ability to successfully infect CHO-K1 cells as well as HSPG-deficient CHO677 cells. Strikingly, #9 Asia-1 developed significantly higher titers when replicating in CHO677 cells in comparison to isolates #3 and #8. This raises the question whether the introduced HS-specific mutations also support the usage of another, unknown receptor.

It is also interesting that all three virus isolates exhibit the same mutation in the non-structural 2C protein. This protein is highly conserved among the different serotypes with >85% identity of the amino acids [39] and has important functions during virus replication [27]. The 2C protein is a membrane-binding component of the replication complex with additional functions, including RNA binding activities, ATPase and GTPase activities as well as the induction of apoptosis in BHK cells [39–41]. Recent studies revealed an interaction between 2C and cellular Beclin1, which blocks autophagosome fusion to lysosomes and prevents RNA degradation as well as enhances viral replication [39]. Possible binding sites were predicted at positions 225–280 and 288–294 within the 2C protein that consists of 318 amino acids in total [39]. The detected mutation within all three adapted Asia-1 strains is located at position 285, in between the possible binding sites for Beclin1. Another function of 2C is the interaction with cellular vimentin [42]. Vimentin is important for cell spreading in adherent cells [43], but culturing of cells in suspension leads to a decrease in vimentin biosynthesis [44]. During FMDV infection, vimentin builds cage-like structures around the non-structural 2C protein [42]. An intact vimentin network is indispensable for FMDV replication and its chemical disruption leads to a decrease in viral yield [42]. Although the observed mutation is distant from the predicted binding site for vimentin (at position 78–84), it is possible that the mutation in the 2C protein enhances viral growth within the cell, especially in the context of altered vimentin synthesis in BHK suspension cells.

Unfortunately, these studies did not fully elucidate the root cause of resistance of BHK-2P and other suspension cell lines towards native FMDV Asia-1. It appears that the infection can be impeded by the lack of suitable (or preferred) receptors on the cell surface. For instance, necessary receptors can be more accessible or available in higher quantity on the surface of susceptible cell lines than on resistant cell lines. By using a closely related but permissive cell line as a "wet nurse" for the adaption of the virus, this obstacle can be overcome. This is of high importance for the production of FMDV vaccine antigen, if the production cell line of choice shows resistance to some FMDV strains. Intriguingly, when incubating the adapted viruses with serum of infected or vaccinated animals, an altered neutralization profile compared to the original Asia-1 Shamir isolate became evident. This is an interesting finding, but does not prove that a vaccine made from these mutants would be without protective effect. BHK-2P cells do not seem to present receptor molecules on their surface for the virus to invade the cells via the "natural way", i.e., by using integrins [45–47]. Therefore, alternative routes had to be found by the virus and were found in the mutations around the five-fold axis. At the same time, the structures important for natural infection, such as the RGD motif, remained unchanged. There is currently no indication that the induction of antibodies against these structures would be affected by the capsid mutations. Thus, infection or vaccination with the adapted viruses could still lead to an immune response that protects against infection with field isolates of Asia-1. Further studies, including animal trials, are necessary to answer these questions and to prove the quality and quantity of the antigen obtained with the described mutants and/or other strains that have been adapted in this rather circuitous manner.

Supplementary Materials: The following are available online at www.mdpi.com/1999-4915/9/8/231/s1, Table S1: Overview about the virus isolates used in this study and their passage history, Table S2: Passaging of FMDV Asia-1 Shamir on different BHK cell lines, Table S3: Additional primer mixes used for sequencing, Table S4: Virus passages of Asia-1 Shamir and other serotype Asia-1 isolates on BHK-2P, Table S5: Nucleotide and amino acid changes during passaging of Asia-1 in suspension cells.

Acknowledgments: This research was funded by Merck Life Science, Darmstadt, Germany.

Author Contributions: V.D., B.H., M.B. and M.E. conceived and designed the experiments; V.D. performed the experiments; V.D. and M.E. analyzed the data; A.Z. contributed to reagents and materials; V.D. and M.E. wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest. A.Z. is an employee of Merck Life Science. Merck provided reagents and materials, but had no role in the design of the study; in the collection, analyses, or interpretation of data and in the writing of the manuscript.

References

1. Brito, B.P.; Rodriguez, L.L.; Hammond, J.M.; Pinto, J.; Perez, A.M. Review of the global distribution of foot-and-mouth disease virus from 2007 to 2014. *Transbound. Emerg. Dis.* **2017**, *64*, 316–332. [CrossRef] [PubMed]
2. Doel, T.R. FMD vaccines. *Virus Res.* **2003**, *91*, 81–99. [CrossRef]
3. MacPherson, I.; Stoker, M. Polyoma transformation of hamster cell clones—An investigation of genetic factors affecting cell competence. *Virology* **1962**, *16*, 147–151. [CrossRef]
4. Capstick, P.B.; Telling, R.C.; Chapman, W.G.; Stewart, D.L. Growth of a cloned strain of hamster kidney cells in suspended cultures and their susceptibility to the virus of foot-and-mouth disease. *Nature* **1962**, *195*, 1163–1164. [CrossRef] [PubMed]
5. Telling, R.C.; Elsworth, R. Submerged culture of hamster kidney cells in a stainless steel vessel. *Biotechnol. Bioeng.* **1965**, *7*, 417–434. [CrossRef]
6. Amadori, M.; Berneri, C.; Archetti, I.L. Immunogenicity of foot-and-mouth disease virus grown in BHK-21 suspension cells. Correlation with cell ploidy alterations and abnormal expression of the α5β1 integrin. *Vaccine* **1994**, *12*, 159–166. [CrossRef]
7. Amadori, M.; Volpe, G.; Defilippi, P.; Berneri, C. Phenotypic features of BHK-21 cells used for production of foot-and-mouth disease vaccine. *Biologicals* **1997**, *25*, 65–73. [CrossRef] [PubMed]
8. Brown, F. Problems with BHK 21 cells. *Dev. Biol. Stand.* **1998**, *93*, 85–88. [PubMed]
9. Clarke, J.B.; Spier, R.E. Variation in the susceptibility of BHK populations and cloned cell lines to three strains of foot-and-mouth disease virus. *Arch. Virol.* **1980**, *63*, 1–9. [CrossRef] [PubMed]
10. Clarke, J.B.; Spier, R.E. An investigation into causes of resistance of a cloned line of BHK cells to a strain of foot-and-mouth disease virus. *Vet. Microbiol.* **1983**, *8*, 259–270. [CrossRef]
11. Syusyukin, A.A.; Tsvetkova, N.E.; Kudryatseva, G.A.; Syusyukin, M.S.; Efimov, N.I. Cultures of foot-and-mouth disease virus in different sublines of BHK 21 cells. *Veterinariya (Moscow)* **1976**, *51*, 46–48.
12. Diderholm, H.; Dinter, Z. A Line of polyoma-transformed BHK21 Cells insusceptible to foot-and-mouth disease virus. *Virology* **1965**, *26*, 369–372. [CrossRef]
13. Spearman, C. The method of "right and wrong cases" (constant stimuli) without Gauss's formula. *Br. J. Psychol.* **1908**, *2*, 227–242.
14. Kärber, G. Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche. *Arch. Exp. Pathol. Pharmakol.* **1931**, *162*, 480–483. [CrossRef]
15. Martin-Acebes, M.A.; Rincon, V.; Armas-Portela, R.; Mateu, M.G.; Sobrino, F. A single amino acid substitution in the capsid of foot-and-mouth disease virus can increase acid lability and confer resistance to acid-dependent uncoating inhibition. *J. Virol.* **2010**, *84*, 2902–2912. [CrossRef] [PubMed]
16. Callahan, J.D.; Brown, F.; Osorio, F.A.; Sur, J.H.; Kramer, E.; Long, G.W.; Lubroth, J.; Ellis, S.J.; Shoulars, K.S.; Gaffney, K.L.; et al. Use of a portable real-time reverse transcriptase-polymerase chain reaction assay for rapid detection of foot-and-mouth disease virus. *J. Am. Vet. Med. Assoc.* **2002**, *220*, 1636–1642. [CrossRef] [PubMed]
17. Schneider, C.A.; Rasband, W.S.; Eliceiri, K.W. NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* **2012**, *9*, 671–675. [CrossRef] [PubMed]
18. Brown, F.; Cartwright, B.; Stewart, D.L. Further studies on the infection of pig-kidney cells by foot-and-mouth disease virus. *Biochim. Biophys. Acta* **1962**, *55*, 768–774. [CrossRef]
19. Dill, V.; Beer, M.; Hoffmann, B. Simple, quick and cost-efficient: A universal RT-PCR and sequencing strategy for genomic characterisation of foot-and-mouth disease viruses. *J. Virol. Methods* **2017**, *246*, 58–64. [CrossRef] [PubMed]
20. Carrillo-Tripp, M.; Montiel-Garcia, D.J.; Brooks, C.L., 3rd; Reddy, V.S. CapsidMaps: Protein-protein interaction pattern discovery platform for the structural analysis of virus capsids using Google Maps. *J. Struct. Biol.* **2015**, *190*, 47–55. [CrossRef] [PubMed]

21. Fry, E.; Acharya, R.; Stuart, D. Methods used in the structure determination of foot-and-mouth disease virus. *Acta Crystallogr. Sect. A Found. Crystallogr.* **1993**, *49*, 45–55. [CrossRef]
22. Fry, E.E.; Newman, J.W.; Curry, S.; Najjam, S.; Jackson, T.; Blakemore, W.; Lea, S.M.; Miller, L.; Burman, A.; King, A.M.; et al. Structure of foot-and-mouth disease virus serotype $A10_{61}$ alone and complexed with oligosaccharide receptor: Receptor conservation in the face of antigenic variation. *J. Gen. Virol.* **2005**, *86*, 1909–1920. [CrossRef] [PubMed]
23. Pettersen, E.F.; Goddard, T.D.; Huang, C.C.; Couch, G.S.; Greenblatt, D.M.; Meng, E.C.; Ferrin, T.E. UCSF Chimera—A visualization system for exploratory research and analysis. *J. Comput. Chem.* **2004**, *25*, 1605–1612. [CrossRef] [PubMed]
24. Jackson, T.; Sheppard, D.; Denyer, M.; Blakemore, W.; King, A.M. The epithelial integrin αvβ6 is a receptor for foot-and-mouth disease virus. *J. Virol.* **2000**, *74*, 4949–4956. [CrossRef] [PubMed]
25. World Organisation for Animal Health (OIE). Foot and Mouth Disease. *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals*. Available online: http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.01.08_FMD.pdf (accessed on 8 February 2017).
26. Bates, D.; Mächler, M.; Bolker, B.; Walker, S. Fitting linear mixed-effects models using lme4. *J. Stat. Softw.* **2015**, *67*, 1–48. [CrossRef]
27. Grubman, M.J.; Baxt, B. Foot-and-mouth disease. *Clin. Microbiol. Rev.* **2004**, *17*, 465–493. [CrossRef] [PubMed]
28. Genzel, Y. Designing cell lines for viral vaccine production: Where do we stand? *Biotechnol. J.* **2015**, *10*, 728–740. [CrossRef] [PubMed]
29. Jackson, T.; Mould, A.P.; Sheppard, D.; King, A.M. Integrin alphavbeta1 is a receptor for foot-and-mouth disease virus. *J. Virol.* **2002**, *76*, 935–941. [CrossRef] [PubMed]
30. Jackson, T.; Clark, S.; Berryman, S.; Burman, A.; Cambier, S.; Mu, D.; Nishimura, S.; King, A.M. Integrin αvβ8 functions as a receptor for foot-and-mouth disease virus: Role of the β-chain cytodomain in integrin-mediated infection. *J. Virol.* **2004**, *78*, 4533–4540. [CrossRef] [PubMed]
31. Berinstein, A.; Roivainen, M.; Hovi, T.; Mason, P.W.; Baxt, B. Antibodies to the vitronectin receptor (integrin α V β 3) inhibit binding and infection of foot-and-mouth disease virus to cultured cells. *J. Virol.* **1995**, *69*, 2664–2666. [PubMed]
32. Duque, H.; Baxt, B. Foot-and-mouth disease virus receptors: Comparison of bovine α_V integrin utilization by type A and O viruses. *J. Virol.* **2003**, *77*, 2500–2511. [CrossRef] [PubMed]
33. Jackson, T.; Ellard, F.M.; Ghazaleh, R.A.; Brookes, S.M.; Blakemore, W.E.; Corteyn, A.H.; Stuart, D.I.; Newman, J.W.; King, A.M. Efficient infection of cells in culture by type O foot-and-mouth disease virus requires binding to cell surface heparan sulfate. *J. Virol.* **1996**, *70*, 5282–5287. [PubMed]
34. O'Donnell, V.; Larocco, M.; Baxt, B. Heparan sulfate-binding foot-and-mouth disease virus enters cells via caveola-mediated endocytosis. *J. Virol.* **2008**, *82*, 9075–9085. [CrossRef] [PubMed]
35. O'Donnell, V.; LaRocco, M.; Duque, H.; Baxt, B. Analysis of foot-and-mouth disease virus internalization events in cultured cells. *J. Virol.* **2005**, *79*, 8506–8518. [CrossRef] [PubMed]
36. Walther, C.G.; Whitfield, R.; James, D.C. Importance of interaction between integrin and actin cytoskeleton in suspension adaptation of CHO cells. *Appl. Biochem. Biotechnol.* **2016**, *178*, 1286–1302. [CrossRef] [PubMed]
37. Mohapatra, J.K.; Pandey, L.K.; Rai, D.K.; Das, B.; Rodriguez, L.L.; Rout, M.; Subramaniam, S.; Sanyal, A.; Rieder, E.; Pattnaik, B. Cell culture adaptation mutations in foot-and-mouth disease virus serotype A capsid proteins: Implications for receptor interactions. *J.Gen. Virol.* **2015**, *96 Pt 3*, 553–564. [CrossRef] [PubMed]
38. Berryman, S.; Clark, S.; Kakker, N.K.; Silk, R.; Seago, J.; Wadsworth, J.; Chamberlain, K.; Knowles, N.J.; Jackson, T. Positively charged residues at the five-fold symmetry axis of cell culture-adapted foot-and-mouth disease virus permit novel receptor interactions. *J. Virol.* **2013**, *87*, 8735–8744. [CrossRef] [PubMed]
39. Gladue, D.P.; O'Donnell, V.; Baker-Branstetter, R.; Holinka, L.G.; Pacheco, J.M.; Fernandez-Sainz, I.; Lu, Z.; Brocchi, E.; Baxt, B.; Piccone, M.E.; et al. Foot-and-mouth disease virus nonstructural protein 2C interacts with Beclin1, modulating virus replication. *J. Virol.* **2012**, *86*, 12080–12090. [CrossRef] [PubMed]
40. Sweeney, T.R.; Cisnetto, V.; Bose, D.; Bailey, M.; Wilson, J.R.; Zhang, X.; Belsham, G.J.; Curry, S. Foot-and-mouth disease virus 2C is a hexameric AAA+ protein with a coordinated ATP hydrolysis mechanism. *J. Biol. Chem.* **2010**, *285*, 24347–24359. [CrossRef] [PubMed]
41. Wang, J.; Wang, Y.; Liu, J.; Ding, L.; Zhang, Q.; Li, X.; Cao, H.; Tang, J.; Zheng, S.J. A critical role of N-myc and STAT interactor (Nmi) in foot-and-mouth disease virus (FMDV) 2C-induced apoptosis. *Virus Res.* **2012**, *170*, 59–65. [CrossRef] [PubMed]

42. Gladue, D.P.; O'Donnell, V.; Baker-Branstetter, R.; Holinka, L.G.; Pacheco, J.M.; Fernandez-Sainz, I.; Lu, Z.; Ambroggio, X.; Rodriguez, L.; Borca, M.V. Foot-and-mouth disease virus modulates cellular vimentin for virus survival. *J. Virol.* **2013**, *87*, 6794–6803. [CrossRef] [PubMed]
43. Prahlad, V.; Yoon, M.; Moir, R.D.; Vale, R.D.; Goldman, R.D. Rapid movements of vimentin on microtubule tracks: Kinesin-dependent assembly of intermediate filament networks. *J. Cell Biol.* **1998**, *143*, 159–170. [CrossRef] [PubMed]
44. Ben-Ze'ev, A. Cell configuration-related control of vimentin biosynthesis and phosphorylation in cultured mammalian cells. *J. Cell Biol.* **1983**, *97*, 858–865. [CrossRef] [PubMed]
45. O'Donnell, V.; Pacheco, J.M.; Gregg, D.; Baxt, B. Analysis of foot-and-mouth disease virus integrin receptor expression in tissues from naive and infected cattle. *J. Comp. Pathol.* **2009**, *141*, 98–112. [CrossRef] [PubMed]
46. Brown, J.K.; McAleese, S.M.; Thornton, E.M.; Pate, J.A.; Schock, A.; Macrae, A.I.; Scott, P.R.; Miller, H.R.; Collie, D.D. Integrin-$\alpha_v\beta_6$, a putative receptor for foot-and-mouth disease virus, is constitutively expressed in ruminant airways. *J. Histochem. Cytochem.* **2006**, *54*, 807–816. [CrossRef] [PubMed]
47. Neff, S.; Sa-Carvalho, D.; Rieder, E.; Mason, P.W.; Blystone, S.D.; Brown, E.J.; Baxt, B. Foot-and-mouth disease virus virulent for cattle utilizes the integrin $\alpha_v\beta_3$ as its receptor. *J. Virol.* **1998**, *72*, 3587–3594. [PubMed]

Supplementary Materials: Adaption of FMDV Asia-1 to Suspension Culture: Cell Resistance is Overcome by Virus Capsid Alterations

Table S1. Overview about the virus isolates used in this study and their passage history.

Virus Isolate	Passage History*
Asia-1 Shamir/ISR/89 (original)	BHK6 (FLI)
#9 Asia-1	+ production BHK 5, BHK-2P 5 (FLI)
#8 Asia-1	+ BHK21-InVitrus 7, BHK-2P 9 (FLI)
#3 Asia-1	+ BHK21C13 7, BHK-2P 9 (FLI)
Asia TUR 6/2014	BTY 1 (WRL), BHK 25 (FLI)
Asia TUR 6/2014-PT	BTY 1 (WRL), PT 18 (FLI)
Asia HKN 5/2005	BHK 9 (FLI)
Asia PAK 5/2012	BTY 1 (WRL), BHK 8 (FLI)
A_{24} Cruzeiro/BRA/55 (original)	CP 6 BHK 2 (WRL), BHK 4 (FLI)
A_{24}-2P	+ BHK-2P 20 (FLI)
O_1 Manisa/TUR/69	BHK 8 (WRL), BHK 8 (FLI)

* Passage host/passage number (location). Cell lines: BHK: Baby hamster kidney; PT: Ovine cell line; CP: Cattle passage. BHK = adherent BHK21; BHK-2P, production BHK, BHK21-InVitrus, BHK21C13 = suspension cell lines. WRL: FMD World Reference Laboratory, Pirbright, UK; FLI: Friedrich-Loeffler-Institut, Greifswald, Germany.

Table S2. Passaging of FMDV Asia-1 Shamir on different BHK cell lines.

Cells grown in monolayers:

Cell line and medium	Virus passage*	Infection volume	cytopathic effect (CPE)
#1: adherent BHK21C13 in GMEM with 8.5% FBS	1	50 µL	100% after 24h
		NC	no CPE after 24h
	2	30 µL	100% after 24h
		NC	no CPE after 24h
	3	10 µL	100% after 24h
		NC	no CPE after 24h

Cells grown in suspension:

Cell line and medium	Virus passage*	Infection volume	Viability (%)	Total cells/mL ($\times 10^5$)	Viable cells/mL ($\times 10^5$)
#2: BHK-2P in GMEM with 10% FBS	1	5 mL	77	9.9	7.7
		NC	86	12.1	10.4
	2	5 mL	97	15.5	15.1
		NC	100	17.1	17.0
	3	10 mL	93	11.6	10.8
		NC	93	10.9	10.1
	4	10 mL	96	15.6	14.9
		NC	94	17.1	16.1
	5	10 mL	92	12.2	11.2
		NC	97	12.1	11.8
#3: adherent BHK21C13 in Cellvento™ BHK-200	1	5 mL	77	1.8	0.3
		NC	86	1.9	1.8
	2	5 mL	54	4.7	2.5
		NC	100	4.5	4.5
	3	4 mL	18	5.0	0.9
		NC	89	3.4	3.0
#4: BHK-2P in BHK-200 (high passage-p8)	1	5 mL	92	20.0	18.4
		NC	97	17.0	16.5
	2	5 mL	97	29.9	29.0
		NC	96	39.9	38.5
	3	5 mL	96	17.1	16.4
		NC	97	14.2	13.8
	4	5 mL	95	13.7	13.0
		NC	93	16.1	15.0
	5	5 mL	96	15.7	15.1
		NC	99	15.0	14.8
	6	5 mL	73	13.0	9.6
		NC	90	14.7	13.2

Table S2 (continued)

Cell line and medium	Virus passage*	Infection volume	Viability (%)	Total cells/mL (×10⁵)	Viable cells/mL (×10⁵)
#5: BHK-2P short adaptation to BHK-200 (process 4)	1	5 mL	98	23.9	23.5
		NC	97	22.6	21.9
	2	5 mL	97	46.3	45.1
		NC	99	46.2	45.5
	3	5 mL	97	17.1	16.6
		NC	98	23.2	22.7
	4	5 mL	98	23.0	22.6
		NC	94	20.5	19.4
	5	5 mL	99	19.1	18.9
		NC	99	37.2	36.7
#6: BHK21-C in Cellvento™ BHK-200	1	5 mL	97	22.1	21.4
		NC	99	20.4	20.3
	2	5 mL	99	43.2	42.6
		NC	98	52.1	51.2
	3	5 mL	98	20.7	20.7
		NC	98	19.6	19.3
	4	5 mL	99	24.6	24.3
		NC	99	32.6	32.4
	5	5 mL	98	23.7	23.3
		NC	100	25.3	25.2
	6	5 mL	99	51.5	51.0
		NC	98	48.2	47.4
#7: BHK21- Hektor in Cellvento™ BHK-200	1	5 mL	97	17.8	17.3
		NC	99	20.3	20.1
	2	5 mL	97	34.1	32.9
		NC	99	26.8	26.4
	3	5 mL	97	13.7	13.3
		NC	98	16.0	15.6
	4	5 mL	94	19.0	17.9
		NC	97	20.7	20.1
	5	5 mL	96	23.3	22.5
		NC	98	20.4	20.0
	6	5 mL	95	33.5	32.0
		NC	93	24.7	23.0

Table S2 (continued)

Cell line and medium	Virus passage*	Infection volume	Viability (%)	Total cells/mL (×10^5)	Viable cells/mL (×10^5)
#8: BHK21- InVitrus in Cellvento™ BHK-200	1	5 mL	88	17.5	15.3
		NC	98	21.6	21.1
	2	5 mL	84	28.9	24.3
		NC	98	32.7	32.2
	3	5 mL	67	10.4	6.9
		NC	98	15.8	15.6
	4	5 mL	9	11.4	1.0
		NC	96	14.8	14.2
	5	3 mL	5	7.4	0.3
		NC	96	23.9	22.9
#9: production BHK in Cellvento™ BHK-200	1	5 mL	84	27.2	22.9
		NC	96	18.8	18.1
	2	5 mL	56	8.6	4.8
		NC	86	7.3	6.3
	3	5 mL	52	17.3	9.0
		NC	92	13.2	12.2
	4	4 mL	40	49.6	21.1
		NC	97	15.3	14.9

*Cells have different total numbers of passages due to different growth properties.

Table S3. Additional primer mixes used for sequencing.

Primer	Primer Sequence 5'–3'	Locations* 5'–3'	Amplicon size
VP3-2835F	TCG ACG TGT CCC TCG C	2835-2851	366 bp
VP3-3232R	AAGTCTTTGCCGGCG	3217-3232	
VP1-3165F	ATCAGATCACCCACGG	3165-3181	451 bp
VP1-3649R	GTTGCCAGCACACGATG	3632-3649	
3D-7320F	GTTGCAACCCTGATGT	7320-7336	761 bp
3D-8113R	TTCTGCCAATTGCGAC	8097-8113	

* Location in genome of JF739177.

Table S4. Virus passages of Asia-1 Shamir and other serotype Asia-1 isolates on BHK-2P.

First adaption attempt of Asia-1 Shamir

Passage No.	cell number (cells/mL)	infection volume (mL)	cell viability 24 hpi
0	BHK164 (adherent)	50 µL in 10 mL	100% CPE
1	8.3 ×10^5 (undefined)	1.35 mL in 30 mL	99%
2	4.9 ×10^6 (undefined)	1.3	100%
3	4.6 ×10^6 (undefined)	10.0	98%
4	1.0 ×10^6	5.0	97%
5	1.0 ×10^6	5.0	98%
6	1.0 ×10^6	10.0	98%
Infection control	BHK164 (adherent)	1 mL of P6	0% CPE

Second adaption attempt of Asia-1 Shamir

Passage No.	cell number (cells/mL)	infection volume (mL)	cell viability 24 hpi
0	BHK164 (adherent)	50 µL in 10 mL	100% CPE
1	1.0 ×10^6	5.0	98%
2	1.0 ×10^6	15.0	97%
3	1.0 ×10^6	15.0	96%
4	1.0 ×10^6	15.0	96%
5	1.0 ×10^6	15.0	97%
6	1.0 ×10^6	15.0	92%
7	1.0 ×10^6	15.0	99%
8	1.0 ×10^6	15.0	96%
9	1.0 ×10^6	15.0	97%
Infection control	BHK164 (adherent)	1 mL of P9	0% CPE

Third adaption attempt of Asia-1 Shamir

Passage No.	cell number (cells/mL)	infection volume (mL)	cell viability 24 hpi
0	BHK179 (adherent)	50 µL in 10 mL	100% CPE
1	1.0 ×10^6	10.0	99%
2	1.0 ×10^6	15.0	99%
3	1.0 ×10^6	15.0	96%
4	1.0 ×10^6	15.0	98%
5	1.0 ×10^6	15.0	96%
6	1.0 ×10^6	15.0	99%
Infection control	LFBKαvβ6 (adherent)	50 µL of P5	0% CPE

Table S4 (continued)

Adaption attempts of other serotype Asia-1 strains

Asia-1 TUR 6/2014

Passage No.	cell number (cells/mL)	infection volume (mL)	cell viability 24 hpi
0	BHK179 (adherent)	50 µL in 10 mL	100% CPE
1	1.0×10^6	10.0	98%
2	1.0×10^6	15.0	100%
3	1.0×10^6	15.0	99%
4	1.0×10^6	15.0	95%
5	1.0×10^6	15.0	99%
Infection control	LFBKαvβ6 (adherent)	50 µL of P4	0% CPE

Asia-1 TUR 6/2014-PT

Passage No.	cell number (cells/mL)	infection volume (mL)	cell viability 24 hpi
0	BHK179 (adherent)	50 µL in 10 mL	100% CPE
1	1.0×10^6	10.0	99%
2	1.0×10^6	15.0	100%
3	1.0×10^6	15.0	99%
4	1.0×10^6	15.0	100%
5	1.0×10^6	15.0	99%
Infection control	LFBKαvβ6 (adherent)	50 µL of P4	0% CPE

Asia-1 HKN 5/2005

Passage No.	cell number (cells/mL)	infection volume (mL)	cell viability 24 hpi
0	BHK179 (adherent)	50 µL in 10 mL	100% CPE
1	1.0×10^6	10.0	99%
2	1.0×10^6	15.0	99%
3	1.0×10^6	15.0	97%
4	1.0×10^6	15.0	99%
5	1.0×10^6	15.0	95%
6	1.0×10^6	15.0	99%
Infection control	LFBKαvβ6 (adherent)	50 µL of P5	0% CPE

Asia-1 PAK 5/2012

Passage No.	cell number (cells/mL)	infection volume (mL)	cell viability 24 hpi
0	BHK179 (adherent)	50 µL in 10 mL	100% CPE
1	1.0×10^6	10.0	99%
2	1.0×10^6	15.0	99%
3	1.0×10^6	15.0	99%
4	1.0×10^6	15.0	99%
5	1.0×10^6	15.0	96%
6	1.0×10^6	15.0	99%
Infection control	LFBKαvβ6 (adherent)	50 µL of P5	0% CPE

Table S5. Nucleotide and amino acid changes during passaging of Asia-1 in suspension cells.

Genome Region	Virus Isolate			AA Change	Change in Charge/Polarity
	#3 Asia-1	#8 Asia-1	#9 Asia-1		
IRES	T1028Y	-	-		
Leader		C1195T		no	no
VP3	-	-	G2783A	yes, E to K	negative to positive charge
VP1	-	-	A3512G	yes, T to A	polar uncharged to hydrophobic side chain
VP1		A3588G	-	yes, Q to R	polar uncharged side chain to positive charge
VP1	-	-	C3593A	yes, Q to K	polar uncharged side chain to positive charge
VP1		A3594G	-	yes, Q to R	polar uncharged side chain to positive charge
VP1	-	-	G3869A	yes, E to K	negative to positive charge
2C		A4413G		yes, K to R	no
2C		A5261C		yes, K to Q	positive charge to polar uncharged side chain
3A	A5622G	-	-	yes, D to G	negative charge to uncharged
3C	A6449G	-	-	yes, I to V	no
3D	-	-	T7339C	no	no

Mutations that are discussed in detail in the paper are shown in italics. The remaining mutations are included here for the sake of completeness, but are considered irrelevant for the purposes of this study.

III. Influence of cell type and cell culture media on the propagation of foot-and-mouth disease virus with regard to vaccine quality.

Veronika Dill, Bernd Hoffmann, Aline Zimmer, Martin Beer, Michael Eschbaumer

Virology Journal 2018, 15:46

doi: 10.1186/s12985-018-0956-0

Dill *et al. Virology Journal* (2018) 15:46
https://doi.org/10.1186/s12985-018-0956-0

Virology Journal

RESEARCH Open Access

Influence of cell type and cell culture media on the propagation of foot-and-mouth disease virus with regard to vaccine quality

Veronika Dill[1], Bernd Hoffmann[1], Aline Zimmer[2], Martin Beer[1] and Michael Eschbaumer[1*]

Abstract

Background: Suspension culture of BHK cells allows large-scale virus propagation and cost-efficient vaccine production, while the shift to animal-component-free cell culture media without serum is beneficial for the quality and downstream processing of the product. Foot-and-mouth disease virus is still endemic in many parts of the world and high-quality vaccines are essential for the eradication of this highly contagious and economically devastating disease.

Methods: Changes to the viral genome sequence during passaging in an adherent and a suspension cell culture system were compared and the impact of amino acid substitutions on receptor tropism, antigenicity and particle stability was examined. Virus production in suspension cells in animal-component-free media and in serum-containing media as well as in adherent cells in serum-containing media was compared. Infection kinetics were determined and the yield of intact viral particles was estimated in all systems using sucrose density gradient centrifugation.

Results: Capsid protein sequence alterations were serotype-specific, but varied between cell lines. But The A_{24}-2P virus variant had expanded its receptor tropism, but virus neutralization tests found no changes in the antigenic profile in comparison to the original viruses. There were no differences in viral titer between a suspension and an adherent cell culture system, independent of the type of media used. Also, the usage of a serum-free suspension culture system promoted viral growth and allowed an earlier harvest. For serotype O isolates, no differences were seen in the yield of 146S particles. Serotype A preparations revealed a decreased yield of 146S particles in suspension cells independent of the culture media.

Conclusion: The selective pressure of the available surface receptors in different cell culture systems may be responsible for alterations in the capsid coding sequence of culture-grown virus. Important vaccine potency characteristics such as viral titer and the neutralization profile were unaffected, but the 146S particle yield differed for one of the tested serotypes.

Keywords: Animal-component-free media, Foot-and-mouth disease virus, BHK21, Suspension cells, Serum-free media

Background

Foot-and-mouth disease virus (FMDV) is a highly transmissible and extremely contagious RNA virus, infecting domestic as well as wild cloven-hooved animals [1]. Vaccination campaigns are the way of choice to eradicate FMDV in endemic countries and in case of an outbreak in an FMDV-free country, vaccination is a useful strategy to limit spread [2].

Vaccines produced to combat FMDV have a long history, going back to first attempts in the early 1900s and Waldmann's first inactivated vaccine, developed in 1937 [3]. The most common production cell line is the mammalian baby hamster kidney cell (BHK21, clone 13), adapted to grow in suspension by Capstick et al. [4], and processed for large-scale fermenters by Telling and Elsworth [5]. To achieve adaption of the virus to the production cell line, the virus is first passaged in BHK21 adherent cells until a rapid

* Correspondence: michael.eschbaumer@fli.de
[1]Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, Südufer 10, 17493 Greifswald–Insel Riems, Germany
Full list of author information is available at the end of the article

cytopathogenic effect develops, and then further propagated and expanded in stationary or roller systems [6]. In industry practice, viruses are passaged in suspension cell culture to expand the virus to large scale. Preparation of master and working seed stocks for the vaccine production process are the last steps of a successful virus adaption in both cell culture systems [7]. In the vaccine production process, the raw materials, including serum-containing cell culture media, need special attention. Serum as well as other components such as animal tissue hydrolysates are poorly defined, resulting in significant lot-to-lot variation of the product [8, 9]. On top of their substantial costs, animal-derived products can contain viruses, mycoplasmal bacteria or prions, and therefore require special risk assessments by the supplier and the user [9, 10]. Because of this, attempts to find alternatives to serum in vaccine production have been of major importance for many years [11].

Today, cell culture media can be divided in different types based on their content of animal-derived products. Serum-free media (SFM) do not require the addition of serum for optimal cell growth but may contain other additives derived from animals such as lactalbumin, casein, insulin, lipids or sterols [12]. Animal-component-free media (ACFM) are media in which none of the components are animal-derived [11]. Protein-free media (PFM) are free of supplemental polypeptide factors but may contain hydrolyzed peptide fragments from animal or plant sources. Finally, chemically defined media (CDM) comprise well-characterized constituents of low molecular weight and are, in most cases, free of proteins [11, 12]. BHK21 cells have already been adapted to grow in serum-free or animal-component-free cell media for rabies vaccine production [13, 14]. With adaption to serum-free conditions, BHK21 cells switch from anchorage-dependent to suspension growth [13, 14] and fundamental changes in cell structure take place [15, 16]. On the other hand, selective pressures during the adaption of viral strains to BHK21 cells, whether as adherent or as suspension cells, can lead to capsid alterations that influence the antigenicity and stability of the virus particle.

The first part of the study examines the adaption of the virus to an adherent and a suspension cell culture system, the viral sequence changes that take place during subsequent passaging as and their possible impact on receptor tropism, particle stability and antigenicity. The second part of the study compares virus production in an animal-component-free medium and virus production in serum-supplemented growth medium and the possible differences in quality and quantity of the viral harvest.

Methods

Cells

The adherent BHK21C13 cell line (CCLV-RIE 179 in the Collection of Cell Lines in Veterinary Medicine, Friedrich-Loeffler-Institut [FLI], Greifswald, Germany; originally derived from the American Type Culture Collection (ATCC) specimen CCL-10™; short: BHK179) and the adherent BHK21 "clone Tübingen" cell line (CCLV-RIE 164, short: BHK164) were cultured in Minimum Essential Medium Eagle (MEM), supplemented with Hanks' and Earle's salts (Sigma, St. Louis, USA) with 10% fetal bovine serum (FBS) during maintenance and passaging, and with 5% FBS during infection experiments. Cells were incubated in flasks with sealed caps at 37 °C.

The suspension cell line BHK21C13-2P (originally derived from the European Collection of Authenticated Cell Cultures specimen 84,111,301; short: BHK-2P) was either maintained in Glasgow MEM (Thermo Fisher Scientific), supplemented with tryptose phosphate (Sigma-Aldrich) and sodium hydrogen carbonate (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) with 5% FBS or was adapted to grow in the animal-component-free medium Cellvento™ BHK-200 (Merck KGaA, Darmstadt, Germany) in TubeSpin® bioreactors (TPP Techno Plastic Products AG, Trasadingen, Switzerland). The cells were maintained in a shaker incubator with 320 rpm (rpm) at 37 °C, 5% CO_2 and 80% relative humidity.

The Chinese hamster ovary (CHO) cell lines CHO-K1 (ATCC CCL-61, held as CCLV-RIE 134), lacking the known FMDV integrin receptors [17], and the heparan sulfate (HS)-deficient CHO677 [18] (CRL 2244, held as CCLV-RIE 1524) were maintained in Ham's MEM mixed 1:2 with Iscove's Modified Dulbecco's Medium (Thermo Fisher Scientific) and with 10% FBS at 37 °C in sealed flasks.

Viruses and virus titrations

The FMDV isolates A_{24} Cruzeiro and O_1 Manisa were selected from archival stocks at the FLI. Their passage history and origin can be found in Additional file 1: Table S1.

Viral titers were estimated by endpoint titration with the Spearman-Kärber method [19, 20] and expressed as 50% tissue culture infectious dose ($TCID_{50}$) per milliliter. Titrations for virus grown on all cell lines were performed on the adherent BHK164 to avoid biasing the results by titrating BHK179-passaged virus on BHK179 cells.

Virus adaption and passaging

Both virus strains were serially passaged on BHK179 monolayers for 20 passages. In suspension cells, the viruses were passaged until stable adaption to the suspension cell line was achieved. Adaption was defined as a decrease in cell viability to values under 10% within less than 24 h post infection (hpi). Adaption of the virus to growth in BHK-2P as well as passaging the virus on BHK179 was done two times independently. FMDV strain A_{24} Cruzeiro was fully adapted to BHK-2P after 19 passages (16 in the second experiment) and O_1 Manisa after

22 (19) passages. The adapted viruses will be referred to as A_{24}–179, A_{24}-2P, O_1–179 and O_1-2P, respectively.

RNA extraction, RT-PCR and sequencing

FMDV RNA of the original stocks of A_{24} Cruzeiro and O_1 Manisa, of the virus passage 20 in BHK179 and of the final passages in BHK-2P of both adaption experiments was extracted using TRIzol® LS Reagent (Invitrogen, Carlsbad, CA, USA) and the RNeasy® Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturers' instructions. A previously described method was used for RT-PCR and sequencing of the nearly complete open reading frame [21].

The nucleotide sequences were assembled and mapped with Geneious (Biomatters Limited) against the complete published sequence for A_{24} Cruzeiro (GenBank accession no. AY593768) and O_1 Manisa (AY593823) followed by an alignment of original and passaged virus sequences.

To find the passage in which each mutation was fixed in the suspension system, the passages in which a rapid drop in cell viability was observed for the first time were chosen for additional sequencing. For the adherent cell system no such indicator existed and therefore the passages were sequenced in arbitrary intervals.

Structure analysis

Amino acid sequences of the original virus and the final passages in BHK179 and BHK-2P were used to model virus capsid protomers using the Geno3D algorithm [22]. The X-ray crystal structures of A_{24} Cruzeiro [23] (Protein Data Bank accession 1ZBE) and O_1 Manisa [24] (1FOD) served as templates. In total, ten possible structures were generated and the best-fitting model was further analyzed with the UCSF Chimera package [25]. Chimera was developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIGMS P41-GM103311). VIPERdb [26] was used to extract contact information for specific residues.

In-silico analysis

The complete genomes of FMDV strains representing possible vaccine strains [27] as well as representative strains for different topotypes within the seven serotypes were downloaded from GenBank. Multiple sequence alignments for all serotypes were performed using the MUSCLE algorithm as implemented in Geneious and the amino acids at the positions of interest were tabulated.

Acid sensitivity

The protocol of Martín-Acebes et al. [28] was used with modifications. Equal amounts of virus (A_{24} Cruzeiro and O_1 Manisa, original isolates as well as adapted to BHK179 and BHK-2P) were mixed at a final dilution of 1:100 with phosphate-buffered saline (PBS) solutions of different pH within the range of pH values commonly seen in the suspension cell system (7.5, 7.0, 6.8, 6.5). An additional solution with a pH of 5.5 was used as a positive control for FMDV inactivation. The mixtures were incubated for 30 min at room temperature and then neutralized with 1 M Tris-HCl (pH 8.0). The remaining infectivity in each sample was determined by titration on BHK164 cells as described above. Experiments were performed three times independently.

Infectivity testing on CHO cells

A procedure described by Jackson et al. [29] was used to quantify the capacity of the virus strains to infect the FMDV receptor-deficient cell lines CHO-K1 and CHO677. As a modification of the original protocol, the CHO cell preparations were titrated on BHK164. The test was conducted in duplicates and performed three times independently.

Virus neutralization test (VNT)

The VNT was performed on BHK164 cells with A_{24} Cruzeiro, A_{24}–179, A_{24}-2P, O_1 Manisa, O_1–179, and O_1-2P as described by the World Organization for Animal Health (OIE) [30]. A bovine serum, collected 21 days after infection with an earlier passage of the A_{24}Cruzeiro stock virus, was used to neutralize the serotype A virus isolates. Another bovine serum, from an animal infected with an earlier passage of the O_1 Manisa stock virus, also collected at 21 dpi, was used to examine the serotype O virus isolates. The highest dilution in which 50% of the wells did not show any CPE defined the neutralization titer. Titers are expressed as the $\log_{10}$ of the reciprocal of that dilution. To determine the relationship between the original and adapted virus isolates, the r_1 value was calculated by dividing the neutralization titer against the adapted isolate by the neutralization titer against the original virus isolate [30]. All experiments were performed independently in duplicates for a total of three times.

Virus infection kinetics

BHK-2P cells were seeded at a density of 1×10^6 cells/mL and infected with the adapted A_{24}-2P or O_1-2P at an MOI of 0.1. BHK179 cells were cultured in T25 culture flasks until confluency and infected with A_{24}–179 or O_1–179 under the same conditions as the BHK-2P cells. Samples to determine the viral titer were taken after 0 and 4 h and then every 2 h until a total incubation time of 24 h.

Because the determination of cell death and viability is different between adherent and suspension cells, cytopathic effect (CPE, in %) was documented for BHK179 cells, while cell number and cell viability were assessed for BHK-2P

cells. Cell death in suspension cell culture cannot be visually evaluated under a microscope and therefore determination of cell viability is necessary. Additionally, the cell density of an infected culture is compared to an equally seeded negative culture to account for the rapid growth of a healthy suspension culture. Cell numbers and cell viability have been determined by trypan blue staining with an automated cell counter (TC20™, Bio-Rad).

Determination of viral yield

Adherent and suspension cells (cell count 3.7×10^7) were infected at a multiplicity of infection (MOI) of 0.1 and incubated for 20 h. The supernatant was clarified of cell debris by centrifugation for 10 min at 3200×g at 4 °C, followed by purification through a 30% (wt/vol) sucrose cushion in 40 mM sodium phosphate buffer (pH 7.6) with 100 mM NaCl (buffer P as in [31]), centrifuged at 125,755×g in a SW32Ti rotor (Beckman Coulter, Optima LE-70) for 2 h 50 min at 10 °C. Pellets were resuspended in 400 μL buffer P and loaded onto 15% to 45% (wt/vol) sucrose gradients in buffer P. Ultracentrifugation was performed in a SW32Ti rotor at 96,281×g for 3 h at 10 °C. Gradients were fractionated from the bottom of the gradient into one milliliter fractions. All fractions were heated to 70 °C for 30 min before analysis. Absorption at 260 nm was measured twice with a spectrophotometer (NanoDrop™ 2000, Thermo Fisher Scientific). FMDV protein was detected in duplicate by a standard serotype-specific double-antibody sandwich ELISA [30]. The experiment was performed three times.

Statistical analysis and data presentation

Linear mixed-effects models using R (http://www.r-project.org) and lme4 [32] were used to evaluate the differences between treatment groups, with replicates as random effects. The packages car and phia were applied to calculate Wald chi-square tests for fixed effects and their interactions. *P*-values < 0.001 were taken as significant.

Results

Viral sequence changes differ between adaption to adherent or suspension cells for A_{24} Cruzeiro, but not for O_1 Manisa

Viral sequence changes in the capsid coding region were examined during passaging for at least 15 passages in a conventional adherent culture system with serum and in a suspension culture system using animal-component-free media. The O_1 Manisa virus acquired three heterologous mutations within the capsid coding region. All substitutions occurred in VP1 (K41 N, E83K and K210E) and were the same in both culture systems. The first mutation is the substitution of a positively charged lysine at position 41 with a polar uncharged asparagine. This amino acid is flanked by a non-polar phenylalanine at position 39 and a positively charged arginine. It is located at an interface between two VP1 molecules in the five-fold symmetry. Residue E83 of VP1 is exposed on the outer capsid surface in the DE-loop with no interactions at any interfaces. Thirdly, at position 210, the positively charged lysine shifted to a negatively charged glutamic acid. In both culture systems these mutations were already acquired during the first passages (see Additional file 1: Table S1.1 and S1.2). In addition, *in-silico* analyses at the respective positions revealed a degree of conservation for the original amino acids of 97 to 100% (see Additional file 2: Table S2).

A_{24} Cruzeiro also developed amino acid differences in the capsid coding region between low and high passage virus. A_{24}–179 acquired heterologous mutations in VP1 (E194K) and VP3 (C56R). Originally, a negatively charged glutamic acid was situated at position 194; by passage 20, it had been replaced by a positively charged lysine. The *in-silico* analysis revealed a degree of conservation of 93% towards a negatively charged amino acid at this particular position, 7% of examined viruses exhibited a hydrophobic amino acid but none had a positive charge (see Additional file 2: Table S2). On position 56 of VP3, an uncharged cysteine was replaced with a positively charged arginine, another change that contributes to a net increase of positive charges on the surface of the virus capsid. This amino acid change could be found in only 3% of all examined serotype A sequences in the database (see Additional file 2: Table S2). In general, the mutations in A_{24}–179 do not seem to be strictly fixed in the genome and occurred lately in the course of passaging (see Additional file 1: Table S1.3).

In A_{24}-2P, the changes were also located in VP1 and VP3, but at different positions (VP1: E95K, VP3:H85Q). In VP1, a shift from a negatively charged glutamic acid to a positively charged lysine occurred at position 95. This amino acid is in close proximity to the uncharged valine 29 and tyrosine 30 of VP3 and constitutes the interface between two VP1 molecules in the five-fold axis of the molecule. Similar to the mutation in VP1 of A_{24}–179, a negatively charged amino acid at this position is conserved among 73% of serotype A isolates, while 27% display a hydrophobic amino acid. No examined isolate had a positively charged amino acid (see Additional file 2: Table S2). The mutation in VP3 is located in a 3_{10} helix that forms the base of the depression of the HS-binding pocket. Here, a positively charged histidine was replaced by an uncharged glutamine on position 85, which strongly contradicts the conservation of a positively charged amino acid at this position of 100% (see Additional file 2: Table S2). Both mutations were acquired right from the start of the adaption process (see Additional file 1: Table S1.4). The results of this section are summarized in Additional file 3: Table S3. In addition, Fig. 1 illustrates the individual substitutions mapped to a capsid pentamer. For serotype O in particular, the mutations

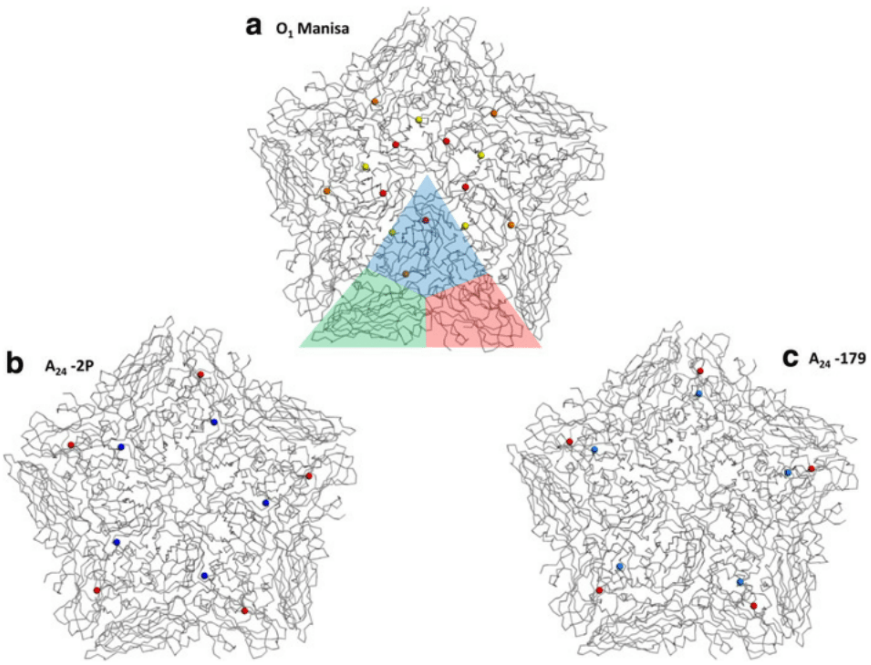

Fig. 1 3D structure with mutations acquired during adaption of FMDV strains A_{24} Cruzeiro and O_1 Manisa. Panels (**a**), (**b**), and (**c**) present detailed structural models of the virus capsid pentamers. The X-ray crystal structure of 1FOD served as template for the 3D models. The structure of a single protomer is highlighted in panel (**a**) (blue: VP1, green: VP2, red: VP3). While O_1 Manisa (panel **a**) acquired the same three mutations in the VP1 region of the capsid (K210E: yellow dots, E83K: orange dots, K41 N: red dots) independent of the culture system, A_{24} Cruzeiro developed different mutations in VP1 (blue dots) and VP3 (red dots) in adherent cell culture (panel **c**) and suspension cell culture (panel **b**). However, these mutations lie in similar locations on the particle

were distributed in a crown-like pattern on the surface in the center of the pentamer (Additional file 4: Figure S1).

Mutations that allow binding to the HS binding pocket lead to increased acid sensitivity

To test if any of the acquired mutations affect the integrity of the viral particle, its stability under acidic conditions was examined. Incubation of the original viruses and their derivatives in buffer solutions of different pH revealed no significant differences in acid stability for the different O_1 Manisa virus populations. Down to a pH of 6.8, no decrease in titer was visible for any of the virus isolates. Variability in virus titer increased at pH 6.5 but the virus populations did not differ significantly from each other. The positive control at pH 5.5 led to a drastic decrease in virus titer. The viral titer for the different A_{24} virus population remained stable down to pH 6.8 as well. However, at pH 6.5, A_{24}–179 underwent a significantly stronger decrease in virus titer than the original virus isolate and A_{24}-2P (Fig. 2).

Capsid mutations extend receptor usage but do not negatively affect neutralization profiles

To examine if the observed sequence changes enable the passaged viruses to use non-integrin cellular receptors, infection experiments with FMDV receptor-deficient cell lines were performed. CHO-K1 cells, which express HS but no relevant integrin receptors, were infected with the original isolates as well as passaged viruses. All six viruses were able to grow on CHO-K1 cells. No significant difference was found between O_1–179 and O_1-2P, but both isolates grew to significantly lower titers in CHO-K1 cells than the original O_1 Manisa ($p < 0.001$). A_{24}-2P infected CHO-K1 cells significantly more effectively than A_{24} Cruzeiro, A_{24}–179 and all O_1 isolates. Furthermore, it was the only virus isolate that was able to infect CHO677 cells, which are devoid of both surface integrins and HS (Table 1).

To determine if any antigenic changes took place during adaption, virus neutralization tests were performed with bovine sera raised against the original A_{24} and O_1 viruses. Titers for A_{24}–179 and A_{24}-2P were similar to the titer obtained with A_{24} Cruzeiro, with r_1-values ≥1.0. The same was true for O_1 Manisa and its derivatives. (Table 1).

The viral yield is similar in adherent and suspension cell culture but the peak is reached earlier in suspension cells.

Virus infection kinetics were performed to compare the pace of viral growth and the overall yield, estimated by

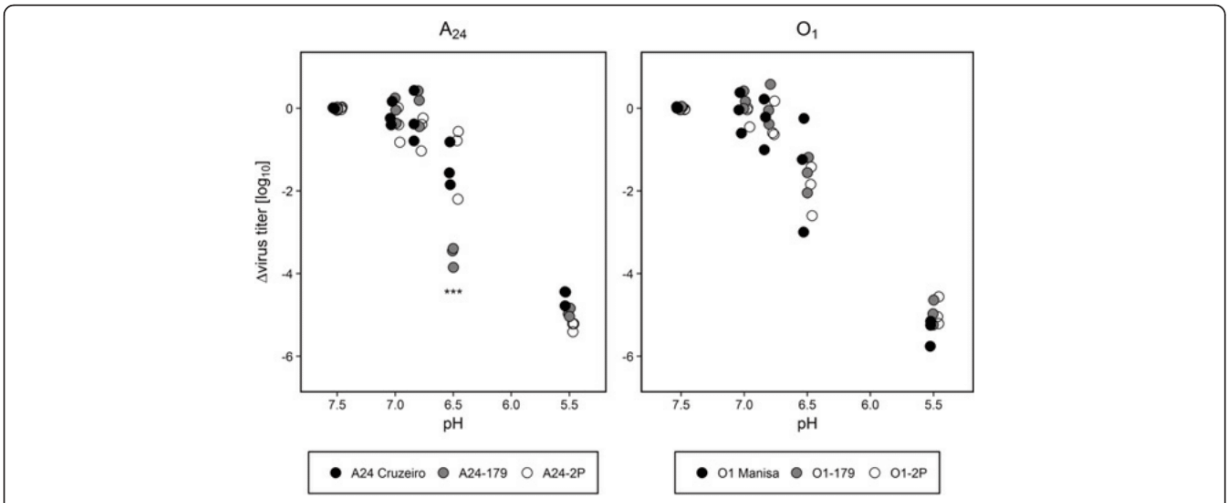

Fig. 2 Sensitivity of O_1 Manisa and A_{24} Cruzeiro and their derivatives to acidic pH Equal amounts of virus of the different passages and culture systems were incubated in buffers of different pH for 30 min, then neutralized and titrated. Titers are expressed relative to the value obtained using PBS at pH 7.5. Experiments were performed three times independently. Significance code: "***" $p \leq 0.001$

endpoint titration, in adherent and suspension BHK cells in serum-containing growth medium and animal-component-free-growth medium. The progression of cell death together with the viral titer was documented to find the best time point for virus harvest in the different cell media systems.

The highest viral titers occur in the moment the cell viability drops drastically in the suspension cell culture system or when the cytopathic effect (CPE) reached 100% in adherent BHK cells. The comparison of both cell culture types shows an earlier cell viability drop and virus release in the suspension cell line for both serotypes compared to the adherent BHK cells. Additionally, the virus infection in the suspension cell system proceeds even faster when using ACFM compared to serum-containing growth medium. After its peak, all tested conditions showed a slight decrease in viral titer over time, independent of cell culture media, cell culture system or serotype used. For serotype A, the maximum titers were reached after 8 h in the suspension cell system with ACFM and after 12 h in the suspension cell system with serum-containing growth medium. The peak of virus titer in adherent BHK cells was seen after 20 h. The progression of serotype O was similar: maximum titers for O_1-2P developed after 12 h in suspension cell culture and after 16 h for O_1–179 in adherent BHK cells. No significant differences in the viral yield were found between the tested conditions (Fig. 3).

The maximum titers that were reached were 8.8 ± 0.4 $\log_{10}$ $TCID_{50}$ per mL for O_1–179 and 8.6 ± 0.1 for A_{24}–179. On BHK-2P, the viral titers were similar between serotypes and independent of the culture medium: 8.3 ± 0.5 $\log_{10}$ $TCID_{50}$/mL for O_1-2P in ACFM, 7.6 ± 0.6 for A_{24}-2P in ACFM and 7.7 ± 0.3 for A_{24}-2P in serum-containing medium.

Table 1 Growth on receptor-deficient cells and neutralization profiles of A_{24} Cruzeiro and O_1 Manisa and their derivatives

Virus isolate	Cell line		VNT	
	CHO-K1	CHO677	Serum titer	r_1-value
A_{24} Cruzeiro	2.8 ± 0.2	negative	3.4 ± 0.2	
A_{24}–179	2.3 ± 0.3	negative	3.6 ± 0.2	1.05
A_{24}-2P	4.0 ± 0.4	2.5 ± 0.6	3.5 ± 0.1	1.02
O_1 Manisa	2.8 ± 0.1	negative	3.2 ± 0.1	
O_1–179	2.0 ± 0.1	negative	3.5 ± 0.2	1.09
O_1-2P	1.9 ± 0.2	negative	3.4 ± 0.1	1.06

Values represent mean virus and neutralization titers and standard deviations, shown as $\log_{10}$ infectious or neutralizing doses per milliliter. The r_1-value was calculated by dividing the neutralization titer against the adapted isolate by the neutralization titer against the original virus isolate

Evaluation of viral particle integrity

Preparations of A_{24}–179 and O_1–179 in a serum-containing adherent cell system, A_{24}-2P in both an ACFM and a serum-containing suspension cell system as well as O_1-2P in an ACFM suspension cell system were analyzed on sucrose density gradients to compare the yield of intact 146S particles, empty 75S capsids and free RNA. The presence of nucleic acid in a fraction was quantified by measuring its absorbance at 260 nm with a spectrophotometer. In the antigen ELISA, the presence of FMDV proteins is indicated by an increased absorbance at 492 nm. Empty particles (75S), which do not contain nucleic acid, do not absorb at 260 nm, but can be detected with the antigen ELISA.

The O_1-2P isolate showed higher amounts of intact 146S virus particles in the ELISA (peak at fraction 9) in

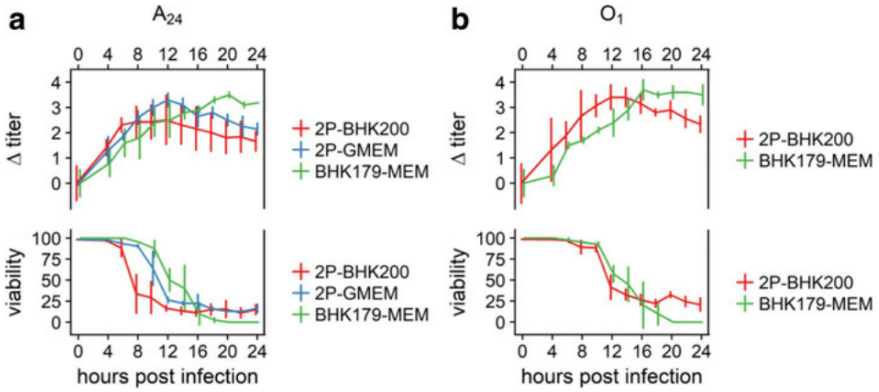

Fig. 3 Virus infection kinetics on monolayer and suspension BHK cells. Virus infection kinetics were performed using the virus variants A_{24}-2P to infect the suspension BHK-2P cell line, maintained in BHK200 (red line) or GMEM + 5% FCS (blue line) and A_{24}–179 to infect the monolayer BHK179 cell line (green line) at an MOI = 0.1 (panel **a**). The virus variant O_1-2P was used to infect the suspension BHK-2P cell line, maintained in BHK200 (red line), and the virus variant O_1–179 was used to infect the monolayer BHK179 cell line (green line) under the same conditions (panel **b**) as described for serotype A. All preparations were sampled for the first time after 4 h and every 2 h thereafter. CPE and cell viability are given in percent (%). Titers are shown in $\log_{10}$ $TCID_{50}$ relative to time 0

comparison to O_1–179, but spectrophotometric measurements revealed a lower 146S peak and high amounts of free RNA for O_1-2P. None of these differences were statistically significant (Fig. 4, panel a, b).

For A_{24} Cruzeiro, the A_{24}–179 isolate had significantly higher amounts of 146S particles (fraction 9) in the spectrophotometric measurements than the A_{24}-2P preparations, independent of the cell culture medium used (Fig. 4, panel d). No significant differences between A_{24}–179 and A_{24}-2P grown in ACFM were found in the antigen ELISA. Although the A_{24}-2P isolate grown in suspension cells with serum-containing media has a

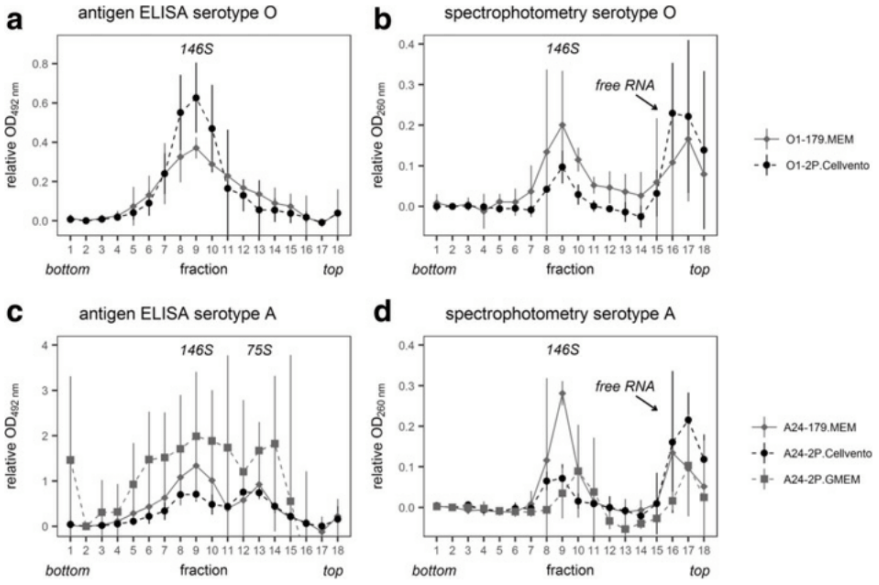

Fig. 4 Sucrose gradient profiles of passaged A_{24} Cruzeiro and O_1 Manisa. Identical numbers of BHK179 and BHK-2P cells, maintained in GMEM + 5% FCS or ACFM were infected with an MOI = 0.1 and virus was harvested after 20 hpi. The harvested virus was concentrated by ultracentrifugation and sedimented through a 15–45% sucrose density gradient. The peaks corresponding to 146S (fraction 9) and empty 75S particles (fraction 13) are indicated. FMDV protein content is shown as absorbance at 492 nm obtained with antibodies specific for O_1 (panel **a**, – 179: solid, grey line, -2P: dotted, black line) and A_{24} (panel **c**, – 179: solid, grey line, -2P: dotted, black line for ACFM; dotted, grey line for serum-containing GMEM) in a standard FMDV antigen ELISA. RNA content corresponds to absorbance at 260 nm as measured with a spectrophotometer (panel **b**: O_1 isolates, panel **d**: A_{24}-isolates, – 179: solid, grey line, -2P: dotted, black line for ACFM; dotted, grey line for serum-containing GMEM). For both assays, absorbances were normalized by subtracting the absorbance reading of fraction 2

higher peak in fraction 9, these data were collected with a different batch of ELISA plates and cannot be directly compared to the earlier preparations.. Formation of empty virus particles (75S, peak at fraction 13) was evident for all serotype A virus preparations (Fig. 4, panel c). For A_{24}–179, the amount of empty particles was reduced compared to the amount of 146S particles, but for both A_{24}-2P preparations the amount of 75S particles was approximately equal to the amount of intact 146S particles.

Discussion

BHK21 cells have been shown to change during passaging and even lose their susceptibility for FMDV [33]. Additionally, the change from adherent to suspension cell culture comes along with profound changes to the cells that the virus needs to adapt to [34]. The first part of this study focused on the sequence changes in the viral genome that take place when adapting to either an adherent or a suspension BHK cell culture system and their influence on virus receptor tropism, antigenicity and particle stability.

The passaging of FMDV O_1 Manisa in either cell culture system resulted in three substitutions in the VP1 capsid protein (K41 N, E83K, K210E). Earlier studies by Gullberg and colleagues found that the switch from K to E at position 210 at the VP1/2A junction in a serotype O FMDV results in the formation of virus particles containing the uncleaved VP1-2A product [31] and is also linked to the E83K substitution within VP1 [35].This substitution is responsible for the inhibition of the cleavage of the VP1/2A junction [35] and provides a selective advantage in the BHK cell culture system, but did not enable the virus to successfully infect CHO cells [36, 37].

The third substitution K41 N is located close to the fivefold symmetry axis of the virus particle at the interface between two VP1 molecules and results in a reduction of positive charge at the interface similar to K210E. Mutations in this particular region have been implicated in the ability to infect cells independently of receptors such as integrin, HS, chondroitin sulfate or sialic acid [38–40]. While it is not clear what selective advantage the inhibition of the VP1/2A junction might have, in sum these mutations seem to allow the use of a receptor on BHK21 cells that is neither integrin nor HS. However, experiments with receptor-deficient CHO cell lines revealed no extended tropism of O_1-2P and O_1–179 in comparison to the original O_1 Manisa isolate.

For A_{24} Cruzeiro, the acquired substitutions E194K in VP1 and C56R in VP3 in A_{24}–179 reflect an adaption for utilization of HS as receptor. According to Fry et al., the HS binding pocket consists of three sites: VP3 residues 55–60 form one of the walls, while residues 84–88 shape the base. The other two walls are composed of residue 133–138 of VP2 and the C-terminus of VP1 (residues 195–197) [23]. The amino acid change from histidine to arginine at position 56 of VP3 has been described as a characteristic feature for HS attachment of serotype O viruses [41]. Together with the second substitution (E194K in VP1), the HS-binding pocket of the capsid acquires a clearly more positive charge. While type A FMDV might show lower affinity to HS than type O [42], the beta-B "knob" regions between residues 55 to 62 of VP3 are structurally very similar between the serotypes [23]. A previous study examining mutations in serotype A capsid proteins after cell culture adaptation also found a switch of C56 to R in a BHK21 culture system, while the E194K mutation was only fixed in strains passaged on IB-RS-2 cells and in a small minority of BHK21 derived isolates [40].

The sequence changes in A_{24}-2P were not as distinct. The switch from a positively charged amino acid to a neutral glutamine (H85Q) at the base of the HS-binding pocket does not support the acquisition of HS as receptor during the course of cell culture adaption. Similar to O_1 Manisa, the A_{24}-2P isolate obtained a positively charged amino acid (E95K) close to the fivefold symmetry axis in VP1. As already discussed for O_1 Manisa, these substitutions suggest that A_{24}-2P uses a yet unknown "third" receptor on BHK-2P suspension cells. This assumption is supported through studies using an A_{24} Cruzeiro mutant (A-SIR #42) that harbors the same E95K change in VP1 [17]. Further studies revealed this amino acid change to be responsible for utilizing Jumonji C-domain containing protein 6 to infect cells in an integrin- and HS-independent way [43]. Indeed, in the present study, A_{24}-2P was the only mutant capable of infecting CHO677 cells, which do neither have surface integrins nor HS.

It has already been shown that adherent BHK cells offer a limited range of surface molecules such as integrins that can be utilized as receptors by FMDV, and BHK cells in suspension culture often have none at all [34]. For this reason, there is a selective pressure in favor of alternative entry mechanisms for the virus. The observed differences between the serotypes may indicate that FMDV type A is more malleable or has a higher mutation rate, resulting in a more variable adaption to different BHK cell lines. Conversely, FMDV serotype O may have a preference for certain mutations that result in a more universal outcome of adaption. This hypothesis is supported by a recently published study by Anil and colleagues, which also showed different mutations occurring in an FMDV serotype A strain depending on passaging in suspension or monolayer BHK cells [44].

Nevertheless, important vaccine quality aspects such as viral antigenicity and particle stability appear to be unaffected by the acquired amino acid substitutions. The r_1-value, which determines the serological relationship

between the original virus and the passaged mutant [30], was higher than 1 for all isolates, indicating that the neutralizing epitopes on the capsid surface are unchanged and immunization with the passaged viruses confers protection like the original isolate does. As for particle stability, one of the main reasons for instability is an increased sensitivity towards low pH. Viral genome release inside the cell is induced through endosomal acidification [28] and there are known sequence mutations that lead to a more labile or a more stable virus capsid. For the O_1 Manisa viruses in the study, no differences in acid sensitivity were observed. In contrast, the virus variant A_{24}–179 showed a significantly stronger decrease in viral titer at pH 6.5 than the original virus or A_{24}-2P. Several studies indicate that virion stability is influenced through amino acid replacements preferentially located at the N terminus of VP1 or the pentameric interface [27, 45, 46]. However, none of the previously described mutations or amino acid substitutions were detected in any of the virus variants generated during passaging in this study.

The switch from media that contain serum and other animal-derived components to a serum-free or even completely animal-component-free system is a major step forward in the production of vaccines. It can bring many advantages such as lower cost, reduced risk of contamination and a cleaner product recovery [9, 12]. The second part of the study examined virus production in an animal-component-free medium compared to virus production in serum-supplemented growth medium and their influence on quality and quantity of the viral harvest.

In the production process of an FMDV vaccine, the virus is harvested as soon as the majority of the cells are dead [8]. Therefore, viral infection kinetics were recorded to find the best time point to harvest the virus. In a monolayer cell culture system, the total cell count is limited due to the available surface area, but growth in a suspension cell system is rapid and unlimited as long as sufficient nutrients and oxygen are available [47]. The maximum viral titers of the kinetic experiments were similar between both cell culture systems. These results are consistent with other studies comparing roller and suspension systems [45]. Furthermore, the maximum viral titers were not influenced by the type of cell culture medium. Kinetic experiments even revealed a quicker virus release when using ACFM. This might be because ACFM contain fewer inhibitory ingredients [46]. On top of reduced biological risks and lower costs through the use of ACFM, the shorter process time also could be an important factors to be taken into account for vaccine producers. The decrease in post-peak titers over time was independent of serum content in the media.

Sucrose density gradient profiles revealed no significant differences in the content of 146S particles between BHK-2P in ACFM and BHK179 in serum-containing media for serotype O preparations. For serotype A preparations, there were no significant differences in contents of 146S particles between the preparation in ACFM and serum-containing growth medium, but the viral yield from the adherent cell culture system exhibited a significantly higher content of 146S particles than the suspension cell preparations. It is also striking that A_{24}-2P yielded 146S and 75S particles in nearly equal amounts, independent of the cell culture media. In addition, all preparations contained high amounts of free RNA. This might lead to the assumption that the packaging of viral RNA into the particle is impaired, which leads to increased free RNA and empty capsid formation. However, not all of the free RNA is of viral origin. The process of purifying the virus from the cell culture supernatant does not completely remove free cellular RNA, which then accumulates in the top fraction of the sucrose gradient.

Conclusion

This study found serotype-specific capsid alterations dependent on the cell line or clone the virus was adapted to. However, cell-specific adaption did not change the neutralization profile of the passaged viruses compared to the original isolates. No differences were found in viral growth and titer between the different cell and media systems. The use of ACFM even appears to support faster virus replication. Differences in the yield of 146S particles, however, were dependent on the cell line, rather than influenced by the culture medium.

Additional files

Additional file 1: Table S1.1. Nucleotide changes in the VP1 coding region of FMDV type O_1 Manisa during serial passaging in adherent BHK21 cells. **Table S1.2.** Nucleotide changes in the VP1 coding region of FMDV type O_1 Manisa during serial passaging in BHK-2P suspension cells. **Table S1.3.** Nucleotide changes in the capsid-coding region of FMDV type A_{24} Cruzeiro during serial passaging in adherent BHK21 cells. **Table S1.4.** Nucleotide changes in the capsid-coding region of FMDV type A_{24} Cruzeiro during serial passaging in BHK-2P suspension cells. (DOCX 17 kb)

Additional file 2: Table S2. *In-silico* analysis covering possible additional vaccine strains (A) and most topotypes within the different serotypes (B) of FMDV. (DOCX 35 kb)

Additional file 3: Table S3. Summary of virus isolates, cell culture systems and mutations acquired during passaging. (DOCX 13 kb)

Additional file 4: Figure S1. Side view of pentamer 3D structure with mutations acquired during adaption of FMDV strains A_{24} Cruzeiro and O_1 Manisa. Panel A shows the crown-like distribution of the acquired mutations in the VP1 region of the capsid of O_1 Manisa (K210E: yellow dots, E83K: orange dots, K41 N: red dots). The substituted amino acids in O_1 Manisa are clustered around the symmetry axis of the pentamer and are more prominent on the capsid surface than the mutations in A_{24}-2P (Panel B) and A_{24}–179 (Panel C) (VP1: blue dots, VP3: red dots). (PPTX 788 kb)

Abbreviations
ACFM: Animal-component-free media; ATCC: American Type Culture Collection; BHK: Baby hamster kidney; CCLV-RIE: Collection of Cell Lines in Veterinary Medicine, Riems; CDM: Chemically defined media; CHO: Chinese hamster ovary; CPE: Cytopathic effect; FBS: Fetal bovine serum; FLI: Friedrich-Loeffler-Institut; FMDV: Foot-and-mouth disease virus; Hpi: Hours post infection; HS: Heparan sulfate; MEM: Minimum Essential Medium Eagle; MOI: Multiplicity of infection; OIE: World Organisation for Animal Health; PBS: Phosphate-buffered saline; PFM: Protein-free media; RNA: Ribonucleic acid; rpm: Revolutions per minute; RT-PCR: Reverse transcription-polymerase chain reaction; SFM: Serum-free media; $TCID_{50}$: 50% tissue culture infectious dose; VNT: Virus neutralization test; wt/vol: Weight/volume

Acknowledgements
Catharina Keßler's help with the sucrose density gradients and Graham Belsham's advice on antigen detection in gradient fractions are gratefully acknowledged.

Funding
The study was funded by Merck Life Science as part of the third-party project "MKS-Growth" (Ri-0367). Merck Life Science had no role in the collection, analysis, or interpretation of data and in the writing of the manuscript.

Availability of data and materials
All data from the current study are available from the corresponding author on request.

Authors' contributions
VD, BH, AZ, MB and ME conceived and designed the experiments. VD performed the experiments and wrote the manuscript. VD and ME analyzed the data. ME edited the manuscript, and all authors read and approved the final manuscript.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
BH, MB and ME declare that they have no competing interests. VD's position was funded by the project. AZ is an employee of Merck Life Science.

Publisher's Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Author details
[1]Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, Südufer 10, 17493 Greifswald–Insel Riems, Germany. [2]Merck KGaA, Merck Life Sciences, Upstream R&D, Frankfurter Straße 250, 64293 Darmstadt, Germany.

Received: 25 September 2017 Accepted: 6 March 2018
Published online: 16 March 2018

References
1. Grubman MJ, Baxt B. Foot-and-mouth disease. Clin Microbiol Rev. 2004;17:465–93.
2. Parida S. Vaccination against foot-and-mouth disease virus: strategies and effectiveness. Expert Rev. Vaccines. 2009;8:347–65.
3. Waldmann O, Köbe K, Pyl G. Die aktive Immunisierung des Rindes gegen Maul-und Klauenseuche mittels Formolimpfstoff. Zent Bakt Parasit Infekt. 1937;138:401–12.
4. Capstick PB, Telling RC, Chapman WG, Stewart DL. Growth of a cloned strain of hamster kidney cells in suspended cultures and their susceptibility to the virus of foot-and-mouth disease. Nature. 1962;195:1163–4.
5. Telling RC, Elsworth R. Submerged culture of hamster kidney cells in a stainless steel vessel. Biotechnol Bioeng. 1965;7:417–34.
6. Hassan AI. Effect of different culture systems on the production of foot and mouth disease trivalent vaccine. Vet World. 2016;9:32–7.
7. Radlett PJ, Pay TW, Garland AJ. The use of BHK suspension cells for the commercial production of foot and mouth disease vaccines over a twenty year period. Dev Biol Stand. 1985;60:163–70.
8. Doel TR. FMD vaccines. Virus Res. 2003;91:81–99.
9. Genzel Y, Fischer M, Reichl U. Serum-free influenza virus production avoiding washing steps and medium exchange in large-scale microcarrier culture. Vaccine. 2006;24:3261–72.
10. European Commission. Commission directive 1999/104/EC of 22 December 1999 amending the annex to council directive 81/852/EEC on the approximation of the laws of the member states relating to analytical, pharmacotoxicoligical and clinical standards and protocols in respect of the testing of veterinary medicinal products. OJEU. 2000;L3:18–20.
11. Merten OW. Development of serum-free media for cell growth and production of viruses/viral vaccines–safety issues of animal products used in serum-free media. Dev Biol (Basel). 2002;111:233–57.
12. Jayme DW, Smith SR. Media formulation options and manufacturing process controls to safeguard against introduction of animal origin contaminants in animal cell culture. Cytotechnology. 2000;33:27–36.
13. Kallel H, Jouini A, Majoul S, Rourou S. Evaluation of various serum and animal protein free media for the production of a veterinary rabies vaccine in BHK-21 cells. J Biotechnol. 2002;95:195–204.
14. Rourou S, Ben Ayed Y, Trabelsi K, Majoul S, Kallel H. An animal component free medium that promotes the growth of various animal cell lines for the production of viral vaccines. Vaccine. 2014;32:2767–9.
15. Amadori M, Berneri C. Genotypic and phenotypic changes of BHK-21 cells grown in suspension cultures. Cytotechnology. 1993;11:S106–8.
16. Amadori M, Volpe G, Defilippi P, Berneri C. Phenotypic features of BHK-21 cells used for production of foot-and-mouth disease vaccine. Biologicals. 1997;25:65–73.
17. Lawrence P, LaRocco M, Baxt B, Rieder E. Examination of soluble integrin resistant mutants of foot-and-mouth disease virus. Virol J. 2013;10:2.
18. Esko JD, Rostand KS, Weinke JL. Tumor formation dependent on proteoglycan biosynthesis. Science. 1988;241:1092–6.
19. Kärber G. Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche. Archiv f experiment Pathol u Pharmakol. 1931;162:480–3.
20. Spearman C. The method of "right and wrong cases" (constant stimuli) without Gauss's formula. Br J Psychol. 1908;2:227–42.
21. Dill V, Beer M, Hoffmann B. Simple, quick and cost-efficient: a universal RT-PCR and sequencing strategy for genomic characterisation of foot-and-mouth disease viruses. J Virol Methods. 2017;246:58–64.
22. Combet C, Jambon M, Deleage G, Geourjon C. Geno3D: automatic comparative molecular modelling of protein. Bioinformatics. 2002;18:213–4.
23. Fry EE, Newman JW, Curry S, Najjam S, Jackson T, Blakemore W, Lea SM, Miller L, Burman A, King AM, Stuart DI. Structure of foot-and-mouth disease virus serotype A10 61 alone and complexed with oligosaccharide receptor: receptor conservation in the face of antigenic variation. J Gen Virol. 2005;86:1909–20.
24. Logan D, Abu-Ghazaleh R, Blakemore W, Curry S, Jackson T, King A, Lea S, Lewis R, Newman J, Parry N, et al. Structure of a major immunogenic site on foot-and-mouth disease virus. Nature. 1993;362:566–8.
25. Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, Ferrin TE. UCSF chimera–a visualization system for exploratory research and analysis. J Comput Chem. 2004;25:1605–12.
26. Carrillo-Tripp M, Shepherd CM, Borelli IA, Venkataraman S, Lander G, Natarajan P, Johnson JE, Brooks CL 3rd, Reddy VS. VIPERdb2: an enhanced and web API enabled relational database for structural virology. Nucleic Acids Res. 2009;37:D436–42.
27. Vaccines : Foot and Mouth Disease [http://www.cfsph.iastate.edu/Vaccines/disease_list.php?disease=foot-and-mouth-disease&lang=en]. Accessed 15 Jan 2018.
28. Martin-Acebes MA, Rincon V, Armas-Portela R, Mateu MG, Sobrino F. A single amino acid substitution in the capsid of foot-and-mouth disease virus can increase acid lability and confer resistance to acid-dependent uncoating inhibition. J Virol. 2010;84:2902–12.
29. Jackson T, Sheppard D, Denyer M, Blakemore W, King AM. The epithelial integrin alphavbeta6 is a receptor for foot-and-mouth disease virus. J Virol. 2000;74:4949–56.
30. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, Chapter 2.1.8: Foot and Mouth Disease [http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.01.08_FMD.pdf].

31. Gullberg M, Polacek C, Botner A, Belsham GJ. Processing of the VP1/2A junction is not necessary for production of foot-and-mouth disease virus empty capsids and infectious viruses: characterization of "self-tagged" particles. J Virol. 2013;87:11591–603.
32. Bates DM, Mächler M, Bolker B, Walker S. Fitting linear mixed-effects models using lme4. J Stat Softw. 2015;67:1–48.
33. Clarke JB, Spier RE. An investigation into causes of resistance of a cloned line of BHK cells to a strain of foot-and-mouth disease virus. Vet Microbiol. 1983;8:259–70.
34. Amadori M, Berneri C, Archetti IL. Immunogenicity of foot-and-mouth disease virus grown in BHK-21 suspension cells. Correlation with cell ploidy alterations and abnormal expression of the alpha 5 beta 1 integrin. Vaccine. 1994;12:159–66.
35. Gullberg M, Polacek C, Belsham GJ. Sequence adaptations affecting cleavage of the VP1/2A junction by the 3C protease in foot-and-mouth disease virus-infected cells. J Gen Virol. 2014;95:2402–10.
36. Zhao Q, Pacheco JM, Mason PW. Evaluation of genetically engineered derivatives of a Chinese strain of foot-and-mouth disease virus reveals a novel cell-binding site which functions in cell culture and in animals. J Virol. 2003;77:3269–80.
37. Maree FF, Blignaut B, de Beer TA, Visser N, Rieder EA. Mapping of amino acid residues responsible for adhesion of cell culture-adapted foot-and-mouth disease SAT type viruses. Virus Res. 2010;153:82–91.
38. Berryman S, Clark S, Kakker NK, Silk R, Seago J, Wadsworth J, Chamberlain K, Knowles NJ, Jackson T. Positively charged residues at the five-fold symmetry axis of cell culture-adapted foot-and-mouth disease virus permit novel receptor interactions. J Virol. 2013;87:8735–44.
39. Baranowski E, Ruiz-Jarabo CM, Sevilla N, Andreu D, Beck E, Domingo E. Cell recognition by foot-and-mouth disease virus that lacks the RGD integrin-binding motif: flexibility in aphthovirus receptor usage. J Virol. 2000;74:1641–7.
40. Mohapatra JK, Pandey LK, Rai DK, Das B, Rodriguez LL, Rout M, Subramaniam S, Sanyal A, Rieder E, Pattnaik B. Cell culture adaptation mutations in foot-and-mouth disease virus serotype a capsid proteins: implications for receptor interactions. J Gen Virol. 2015;96:553–64.
41. Sa-Carvalho D, Rieder E, Baxt B, Rodarte R, Tanuri A, Mason PW. Tissue culture adaptation of foot-and-mouth disease virus selects viruses that bind to heparin and are attenuated in cattle. J Virol. 1997;71:5115–23.
42. Fry EE, Lea SM, Jackson T, Newman JW, Ellard FM, Blakemore WE, Abu-Ghazaleh R, Samuel A, King AM, Stuart DI. The structure and function of a foot-and-mouth disease virus-oligosaccharide receptor complex. EMBO J. 1999;18:543–54.
43. Lawrence P, Rai D, Conderino JS, Uddowla S, Rieder E. Role of Jumonji C-domain containing protein 6 (JMJD6) in infectivity of foot-and-mouth disease virus. Virology. 2016;492:38–52.
44. Anil KU, Sreenivasa BP, Mohapatra JK, Hosamani M, Kumar R, Venkataramanan R. Sequence analysis of capsid coding region of foot-and-mouth disease virus type a vaccine strain during serial passages in BHK-21 adherent and suspension cells. Biologicals. 2012;40:426–30.
45. Spier RE. Reproducibility of yields of foot-and-mouth disease virus from BHK monolayer and suspension cells. Dev Biol Stand. 1976;35:73–8.
46. Frazzati-Gallina NM, Paoli RL, Mourao-Fuches RM, Jorge SA, Pereira CA. Higher production of rabies virus in serum-free medium cell cultures on microcarriers. J Biotechnol. 2001;92:67–72.
47. CELL CULTURE [http://www.scq.ubc.ca/cell-culture/].

Supplementary Materials: Influence of cell type and cell culture media on the propagation of foot-and-mouth disease virus with regard to vaccine quality.

Table S1.1: Nucleotide changes in the VP1 coding region of FMDV type O_1 Manisa during serial passaging in adherent BHK21 cells.

virus isolate		**O_1-179**		
cell system		**BHK179 adherent cell line**		
substitution and position		**A3898G**	**G3517A**	**A3393T**
original		A	G	A
1st adaption	passage 3	G	A	T
	passage 5	G	A	T
2nd adaption	passage 1	G	A	T
	passage 3	G	A	T
	passage 5	G	A	T

Table S1.2: Nucleotide changes in the VP1 coding region of FMDV type O_1 Manisa during serial passaging in BHK-2P suspension cells.

virus isolate		**O_1-2P**		
cell system		**BHK-2P suspension cell line**		
substitution and position		**A3898G**	**G3517A**	**A3393T**
original		A	G	A
1st adaption	passage 5	G	A	T
	passage 6	G	A	T
	passage 7	G	A	T
2nd adaption	passage 1	G	A	T
	passage 2	G	A	T
	passage 3	G	A	T
	passage 4	G	A	T

Table S1.3: Nucleotide changes in the capsid-coding region of FMDV type A_{24} Cruzeiro during serial passaging in adherent BHK21 cells.

virus isolate		**A_{24}-179**	
cell system		**BHK179 adherent cell line**	
substitution and position		**G3803A/ VP1**	**T2726C/ VP3**
original		G	T
1st adaption	passage 3	G	T
	passage 5	R (A/G)	T
	passage 7	A	T
	passage 10	R (A/G)	T
	passage 15	A	Y (T/C)
2nd adaption	passage 1	G	T
	passage 3	G	T
	passage 5	A	T
	passage 7	A	T
	passage 10	R (A/G)	C
	passage 15	A	C

Table S1.4: Nucleotide changes in the capsid-coding region of FMDV type A_{24} Cruzeiro during serial passaging in BHK-2P suspension cells.

virus isolate		A_{24}-2P
cell system		BHK-2P suspension cell line
substitution and position		G3506A/ VP1	T2815A/ VP3
original		G	T
1st adaption	passage 2	A	W (A/T)
	passage 3	A	A
2nd adaption	passage 2	A	A

Table S2: *In-silico* analysis covering possible additional vaccine strains (A) and most topotypes within the different serotypes (B) of FMDV.

S2.A

		O_1-179 / -2P			A_{24}-179		A_{24}-2P		
		K210E	E83K	K41N	E194K / VP1	C56R / VP3	E95K / VP1	H85Q / VP3	
serotype O	AF506822	K	E	K	H	H	E	H	Foot-and-mouth disease virus O strain China/1/99(Tibet), complete genome
	AY593818	K	E	K	H	R	E	H	Foot-and-mouth disease virus O isolate o1campos iso96, complete genome
	AY593819	K	E	K	H	H	E	H	Foot-and-mouth disease virus O isolate o1campos94 iso94, complete genome
	AY593823	K	E	K	H	H	E	H	Foot-and-mouth disease virus O isolate o1manisa iso87, complete genome
	FJ175666	K	E	K	H	H	E	H	Foot-and-mouth disease virus - type O isolate Israel 07-6387, complete genome
	GU566044	K	E	K	H	-	E	H	Foot-and-mouth disease virus - type O isolate SUD/4/99 VP1 (1D) gene, partial cds
	GU566058	K	E	K	H	-	E	H	Foot-and-mouth disease virus - type O isolate SUD/3/2005 VP1 (1D) gene, partial cds
	GU566063	K	E	K	H	-	E	H	Foot-and-mouth disease virus - type O isolate SUD/8/2008 VP1 (1D) gene, partial cds
	HQ009509	K	E	K	H	H	E	H	Foot-and-mouth disease virus - type O strain China/5/99(Fujian), complete genome
	JX040500	K	E	K	H	H	E	H	Foot-and-mouth disease virus - type O isolate TUR/27/2011, complete genome
	KR265075	K	E	K	H	H	E	H	Foot-and-mouth disease virus - type O isolate O/UKG, complete genome
serotype A	KU208000	K	S	K	E	C	E	H	Foot-and-mouth disease virus - type A isolate IND 40/2000 capsid protein gene, partial cds
	KX002202	K	D	K	E	C	V	H	Foot-and-mouth disease virus - type A isolate A/973_SL_Pede polyprotein gene, partial cds
	KX002204	K	D	K	E	C	V	H	Foot-and-mouth disease virus - type A isolate A/AGLopez01 polyprotein gene, partial cds
	KX002205	K	D	K	E	C	V	H	Foot-and-mouth disease virus - type A isolate A/ATLauquen01 polyprotein gene, partial cds
	KY825717	K	D	K	E	C	E	H	Foot-and-mouth disease virus - type A isolate A22/IRQ/24/64 polyprotein gene, partial cds
	KF112902	K	E	K	A	C	E	H	Foot-and-mouth disease virus - type A isolate A/EGY/1/2006 capsid protein gene, partial cds
	FJ755037	K	D	K	E	-	V	H	Foot-and-mouth disease virus - type A isolate A/IRN/27/2005 VP1 (1D) gene, partial cds
	FJ755042	K	D	K	E	-	V	H	Foot-and-mouth disease virus - type A isolate A/IRN/33/2005 VP1 (1D) gene, partial cds
	AY593768	K	E	K	E	C	E	H	Foot-and-mouth disease virus A isolate a24cruzeiro iso71, complete genome
serotype Asia-1	AY304994	K	T	K	D	R	K	H	Foot-and-mouth disease virus Asia 1 IND 63/72, complete genome
	JF739177	K	T	K	D	R	K	H	Foot-and-mouth disease virus - type Asia 1 isolate As1/Shamir/89, complete genome
serotype C	AY593806	K	T	K	Q	T	V	H	Foot-and-mouth disease virus C3 isolate c3ind iso19, complete genome
SAT serotypes	AY593845	K	V	L	-	D	V	N	Foot-and-mouth disease virus SAT 1 isolate sat1bot iso47, complete genome
	KJ999940	-	G	H	-	N	P	H	Foot-and-mouth disease virus - type SAT 2 isolate SAT2/BOT/18/98 P1 polyprotein gene, partial cds
	AY593851	K	A	H	Y	D	H	H	Foot-and-mouth disease virus SAT 3 isolate sat3-3bech iso29, complete genome
	AY593853	K	L	H	Y	D	P	H	Foot-and-mouth disease virus SAT 3 isolate sat3-4bech iso23, complete genome

S2.B		O_1-179 /O_1-2P			A$_{24}$-179		A$_{24}$-2P	
		K210E	E83K	K41N	E194K / VP1	C56R / VP3	E95K / VP1	H85Q / VP3
Serotype A	AY593751	K	D	K	E	C	E	H
	AY593753	K	D	K	E	C	E	H
	AY593757	K	E	K	E	C	E	H
	AY593760	K	D	K	E	C	E	H
	AY593761	K	E	K	E	R	E	H
	AY593771	K	D	K	E	C	E	H
	AY593777	K	D	K	E	C	E	H
	AY593778	R	D	K	E	C	E	H
	AY593780	R	D	K	E	C	E	H
	AY593785	K	D	K	E	C	V	H
	AY593789	K	N	K	E	C	E	H
	AY593793	K	D	K	E	C	E	H
	AY593794	K	D	K	E	C	V	H
	EF117837	K	D	K	E	C	V	H
	HM854025	K	S	K	E	C	E	H
	JF749843	K	E	K	A	C	E	H
	KC440881	K	Q	K	E	C	E	H
	KC440882	K	E	K	E	C	E	H
	KJ754939	K	E	K	E	C	E	H
	KP940474	K	Q	K	E	C	E	H
	KT968663	K	T	Q	E	C	E	H
Serotype O	AF506822	K	E	K	H	H	E	H
	AJ539140	K	E	K	H	H	E	H
	AY593823	K	E	K	H	H	E	H
	AY593826	K	E	K	H	H	E	H
	EF175732	K	E	K	Q	H	E	H
	FJ175666	K	E	K	H	H	E	H
	GU384683	K	E	K	H	H	E	H
	HM229661	K	E	K	H	H	E	H
	JX040500	K	E	K	H	H	E	H
	JX869188	K	E	K	H	H	E	H
	KC440883	K	E	K	H	H	E	H
	KJ825809	K	K	K	H	H	E	H
	KR265075	K	E	K	H	H	E	H
	KU821591	K	E	K	H	H	E	H
	LC036265	K	E	K	H	H	E	H

Table S2.B (continued)

Serotype Asia-1	AY593796	K	T	K	D	R	K	H
	AY593797	K	T	K	D	R	K	H
	AY593800	K	T	K	D	R	K	H
	DQ989309	K	T	K	D	R	K	H
	DQ989321	K	T	K	D	R	K	H
	DQ989323	K	T	K	D	R	K	H
	EF614458	K	T	K	D	R	K	H
	GU125645	K	T	K	D	R	K	H
	GU125646	K	T	K	D	R	K	H
	HQ113233	K	T	K	D	R	K	H
	HQ631363	K	T	K	D	R	K	H
	HQ632774	K	T	K	D	R	K	H
	JN006719	K	T	R	D	R	K	H
	JN006720	K	T	R	D	R	K	H
	KU360085	K	T	K	D	R	K	H
C	AF274010	K	T	E	Q	M	V	H
	KM268897	K	T	K	Q	T	V	H
Serotype SAT 1	AY593839	K	T	L	H	D	E	N
	AY593840	K	T	K	H	D	V	N
	AY593841	K	T	L	H	D	V	N
	AY593842	K	T	L	H	D	V	T
	AY593844	K	T	L	H	D	V	N
	AY593846	K	T	L	H	D	E	N
	HM067706	K	N	L	H	D	L	N
	JF749860	K	T	L	H	D	E	N
	KM268899	K	T	L	H	D	E	N
	KU821590	K	T	L	H	D	V	N
Serotype SAT 2	AF540910	K	E	H	Y	N	P	H
	AY593847	E	E	H	Y	N	P	H
	AY593848	E	T	H	Y	N	P	H
	AY593849	E	E	H	Y	D	P	H
	HM067704	E	N	H	Y	N	P	H
	HM067705	E	E	H	Y	N	P	H
	JF749861	E	A	H	Y	N	P	H
	JF749862	E	K	H	Y	N	P	H
	JF749864	E	E	H	Y	N	P	H
	JX014255	E	D	H	Y	N	P	H
	JX014256	E	D	H	Y	N	P	H
	KC440884	E	D	H	Y	N	P	H
	KM268900	E	D	H	Y	N	P	H
SAT3	AY593850	K	S	H	Y	D	H	C
	AY593851	K	N	H	Y	D	H	S
	AY593852	K	N	H	Y	D	H	S
	AY593853	K	T	H	Y	D	H	S
	KJ820999	K	D	H	Y	D	E	T
	KM268901	K	T	H	Y	D	H	S
	KR108950	K	T	H	Y	D	H	S
	KX375417	K	T	H	Y	D	H	S

Table S2.B (continued)

colour legend:

amino acids with electrically **negative** charged side chains:	**D, E**
amino acids with electrically **positive** charged side chains:	**R, H, K**
amino acids with **polar uncharged** side chains:	**S, T, N, Q**
amino acids with **hydrophobic** side chains:	**A, V, I, L, M, F, Y, W**
special cases:	**C, U, G, P**

Table S3: Summary of virus isolates, cell culture systems and mutations acquired during passaging.

virus	cell culture system	substitution	capsid protein	degree of conservation*	final passage (1st/2nd experiment)
A_{24}-179	adherent	E194K	VP1	93%	20/20
		C56R	VP3	97%	
A_{24}-2P	suspension	E95K	VP1	73%	19/16
		H85Q	VP3	100%	
O_1-179	adherent	K41N	VP1	100%	20/20
		E83K	VP1	96%	
		K210E	VP1	100%	
O_1-2P	suspension	K41N	VP1	100%	22/19
		E83K	VP1	96%	
		K210E	VP1	100%	

*Degree of conservation is based on the results of the *in-silico* analyses at the respective positions for the particular serotypes (Table S2).

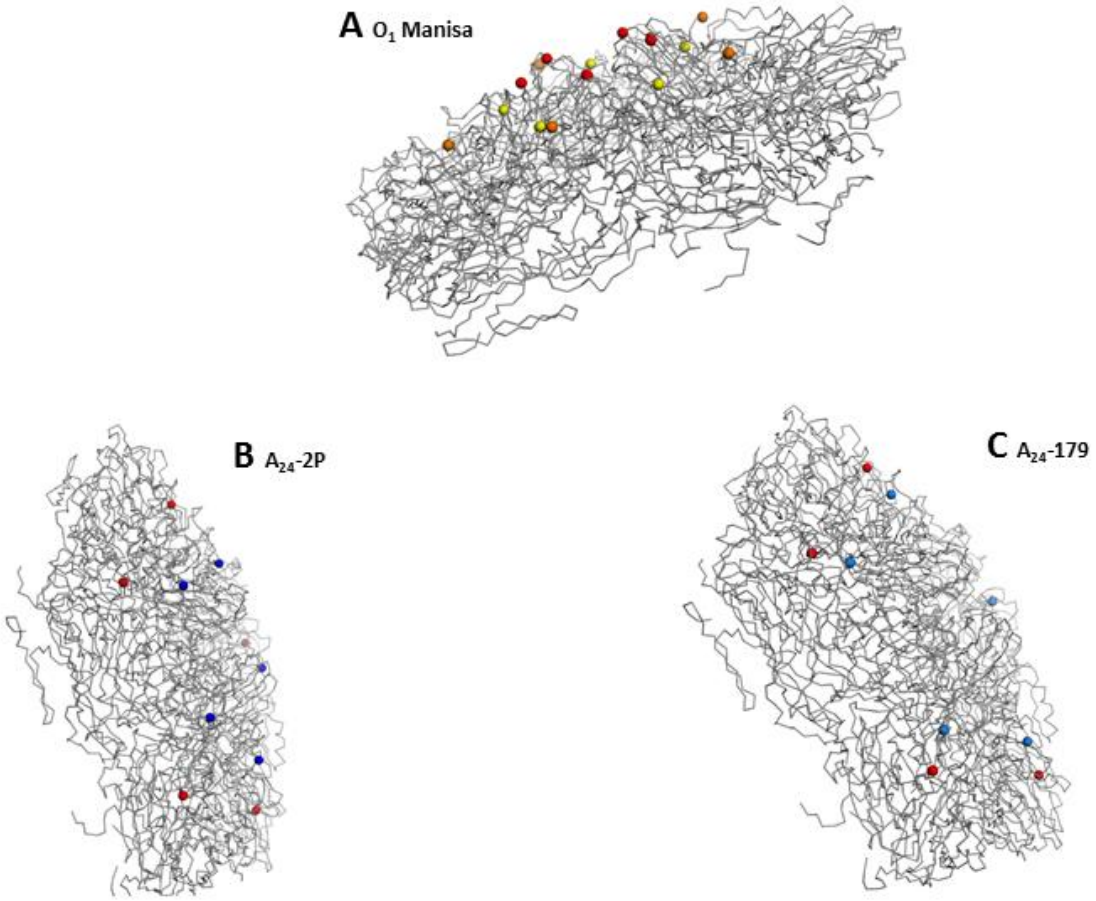

Figure S1: Side view of pentamer 3D structure with mutations acquired during adaption of FMDV strains A_{24} Cruzeiro and O_1 Manisa. Panel A shows the crown-like distribution of the acquired mutations in the VP1 region of the capsid of O1 Manisa (K210E: yellow dots, E83K: orange dots, K41 N: red dots). The substituted amino acids in O1 Manisa are clustered around the symmetry axis of the pentamer and are more prominent on the capsid surface than the mutations in A24-2P (Panel B) and A24–179 (Panel C) (VP1: blue dots, VP3: red dots).

4. Own contributions to publications

I. Veronika Dill, Martin Beer, Bernd Hoffmann. Simple, quick and cost-efficient: A universal RT-PCR and sequencing strategy for genomic characterisation of foot-and-mouth disease viruses. Journal of Virological Methods 246 (2017) 58–64.

Veronika Dill:	Performance of all experiments, analysis and data interpretation, writing of the manuscript
Martin Beer:	Conception of the study, approval of the final manuscript
Bernd Hoffmann:	Conception of the study, primer design, support in writing the manuscript

II. Veronika Dill, Bernd Hoffmann, Aline Zimmer, Martin Beer, Michael Eschbaumer. Adaption of FMDV Asia-1 to Suspension Culture: Cell Resistance Is Overcome by Virus Capsid Alterations. Viruses 2017, 9, 231.

Veronika Dill:	Conception and design of the experiments, performance of all experiments, analysis and data interpretation, writing of the manuscript
Bernd Hoffmann:	Conception and design of the experiments, approval of the final manuscript
Aline Zimmer:	Contribution to reagents and materials, approval of the final manuscript
Martin Beer:	Conception and design of the experiments, approval of the final manuscript
Michael Eschbaumer:	Conception and design of the experiments, support in analyzing the data, editing the manuscript

III. Veronika Dill, Bernd Hoffmann, Aline Zimmer, Martin Beer, Michael Eschbaumer. Influence of cell type and cell culture media on the propagation of foot-and-mouth disease virus with regard to vaccine quality. Virology Journal (2018) 15:46.

Veronika Dill:	Conception and design of the experiments, performance of all experiments, analysis and data interpretation, writing of the manuscript
Bernd Hoffmann:	Conception and design of the experiments, approval of the final manuscript
Aline Zimmer:	Conception and design of the experiments, approval of the final manuscript
Martin Beer:	Conception and design of the experiments, approval of the final manuscript
Michael Eschbaumer:	Conception and design of the experiments, support in analyzing the data, editing the manuscript

In agreement:

Veronika Dill

PD Dr. Rainer G. Ulrich | Prof. Dr. Martin Beer | PD Dr. Sandra Blome

5. Results and Discussion

Due to the rapid mutation rate of FMDV (Domingo et al., 2003) and the associated high genetic diversity, important characteristics of the virus such as particle stability, receptor tropism and immunogenicity can change quickly, both in the natural host and especially during passaging in cell culture (Sa-Carvalho et al., 1997). Thus, these studies describe the development of a new sequencing strategy to easily generate near-complete whole-genome sequences of FMDV for genotyping and molecular epidemiology. Furthermore, reasons for the unsuccessful cultivation of serotype Asia-1 in BHK cell culture were investigated and alternative adaption strategies were demonstrated. Finally, sequence changes during the repeated passaging of FMDV in different BHK cell culture systems were analyzed and their impact on the quality of the virus particles relating to integrity and 146S content was evaluated.

5.1 Molecular tracing and genomic characterisation of FMDV

Numerous approaches to FMDV genome sequencing that use multiple primer panels are described in the literature. However, these studies either include only universal primer sets to generate sequences of the capsid coding region (Xu et al., 2013), are serotype-specific and only cover the VP1 encoding region (Knowles et al., 2016), or are restricted to specific outbreaks and regional virus pools (Abdul-Hamid et al., 2011; Cottam et al., 2006). In this study, a universal primer panel was designed to amplify full-ORF sequences, independent of the serotype and even without any prior knowledge of the FMDV strain involved.

Twelve different FMDV strains, grown in cell culture, have been used to broadly evaluate the final panel of 15 genome-spanning primer combinations (Paper I, Table 1). The assay is based on a double-amplicon strategy to mitigate variations between different virus isolates (Paper I, Figure 1A). To account for the malleability of the virus, two isolates per serotype were chosen: one isolate representing established laboratory strains (often decades old) and one isolate representing strains from more recent outbreaks (Paper I, Table S1). Only one virus sample was used for serotypes C and SAT 3 because of their minor importance in the field. FMDV SAT 3 is mainly restricted to South Africa and serotype C did not cause any major outbreaks during the last 20 years (Saeed et al., 2015). The RNA for each isolate was extracted and a RNA dilution aimed at an approximate Cq value of 20 was selected, detected with two diagnostic real-time RT-PCR assays (Callahan et al., 2002; Oem et al., 2005). The PCR product analysis using gel electrophoresis gave visible bands for all primer pairs and

isolates tested (Paper I, Table 2). The only exception was Mix-5 that did not work with the selected SAT 1 and SAT 3 isolates, probably due to a high percentage of nucleotide mismatches of about 13.5% and 13.1%, respectively, for the forward-primer and 12.7% and 11.4% nucleotide mismatches for the reverse-primer of Mix-5 (Paper I, Table 5). Though, this part of the genome was also covered by Mix-6. For all tested isolates, consensus sequences of high quality, covering the full ORF, could be obtained that were used for genotype prediction (GenBank Accession No KY825717–KY825732). Four samples from animal trials complemented the sample set to test the applicability of the assay for field samples. For this purpose, bovine and ovine saliva, serum of an infected goat and vesicular fluid from an infected pig were used to mirror the most important domestic hosts and diagnostic submissions. The different materials were processed without failure of any of the primer mixes, and high-quality sequences of the viral ORF could be obtained from them as well (Paper I, Table 4 and Figure S2). The analytical sensitivity of the assay was determined with RNA of FMDV O/MOG/7/2010, which had been diluted five times in a 10-fold dilution series. The aforementioned diagnostic real-time RT-PCRs were used to confirm and illustrate the decreasing amount of template. PCR products with varying intensities of the gel bands were produced down to the final dilution of 10^{-5} with all primer mixes (Paper I, Table 3 and Figure S2). Of course, a selection of 12 different FMDV strains does not cover the great variety between different strains of FMDV. To account for the much broader diversity among circulating FMDV strains, an *in-silico* analysis with sequence alignments was conducted, not only for the different serotypes but also for different topotypes and lineages within the serotypes (Paper I, Table S2). In summary, the primers were predicted to show a diversified performance based on the predicted nucleotide mismatches of the particular primer sequences in comparison to the conducted sequence alignments (Paper I, Table 5). More than half of the primers had only one or two nucleotides that did not match the examined sequences (approximately 5% mismatch). The most discrepancies were detected within the SAT serotypes. Here, large sequence variations (between 10 to 30% dissimilarity) were found, reflecting the fact that SAT strains are genetically more diverse than those of other serotypes (Bastos et al., 2003; Samuel and Knowles, 2001).

In an outbreak situation, the identification of the newly emerged virus variant and its serotype affiliation is of great importance to quickly initiate control measures. Later, the understanding of transmission pathways to improve disease control strategies is brought into focus (Abdul-Hamid et al., 2011; Cottam et al., 2008a). On the other hand, developing countries, in which FMDV is still endemic, have only limited assets and restricted access to new technologies for

outbreak investigation. The assay developed here is quick and simple, without the need of complex techniques and expensive equipment. It is also cost efficient compared to other sequencing technologies, although the usage of next-generation sequencing methods is likewise possible with this method. Therefore, it can improve FMDV monitoring and support quicker responses to outbreaks, contributing to the control and eradication of FMDV. The assay was also used for all sequencing in the other studies described below.

5.2 BHK resistance towards FMDV infection is due to insufficient receptor binding capacities

Chemically inactivated virus preparations, formulated into vaccines, are still the instruments of choice to control FMDV in many parts of the world. A crucial point of vaccine development is the adaption of a field virus to cell culture, which is prone to problems (Chamberlain et al., 2015). BHK21 clone 13 cells (BHK21C13) are the cell line of choice for the production of FMD vaccines (Doel, 2003). Unfortunately, BHK cells have varying susceptibility for different FMDV strains (Clarke and Spier, 1980, 1983). This resistance does not affect serotype Asia-1 exclusively, but it seems to be most challenging for this serotype (Clarke and Spier, 1980, 1983). Until now, only a few and also contradictory investigations have been conducted on that topic (Clarke and Spier, 1980; Diderholm and Dinter, 1965).

Because the viral growth cycle can be inhibited at many points, different experiments were performed to elucidate the problem. The experiments were focused on the resistance of the suspension cell line BHK21C13-2P (BHK-2P) to FMDV serotype Asia-1, demonstrated by several unsuccessful infection experiments with different Asia-1 strains (Paper II, Table S4). First of all, the influence of the culture conditions was examined. A virus isolate that replicates well in BHK-2P cells (FMDV serotype A, strain A_{24} Cruzeiro) was compared with the Asia-1 Shamir isolate. Serum is routinely used in cell culture, but the BHK-2P cells are maintained in animal-component-free media (ACFM) (Cellvento BHK200, Merck KGaA) without serum. Therefore, the virus infectivity under these conditions was compared for BHK200 with 5% fetal bovine serum (FBS) and Eagle's Minimum Essential Medium (MEM) with 5% FBS. In all three media, viral titers of both FMDV strains decreased similarly over time (Paper II, Figure 1). Further, virus particle disintegration in dependence of the environmental pH was compared between the strains A_{24} Cruzeiro, O_1 Manisa and Asia-1 Shamir, but again no differences between strains were detectable (Paper II, Figure 2). Lastly, the influence of ammonium chloride was examined, a waste product of cellular metabolism that interferes with endosome acidification and inhibits the release of the viral RNA from the capsid (Carrillo et al., 1984; Martin-Acebes et al., 2010). There were no obvious differences

in sensitivity to ammonium chloride between Asia-1 Shamir and the BHK-2P adapted A_{24} Cruzeiro, although the viral yield of the original A_{24} strain was significantly decreased compared to its adapted variant (Paper II, Figure 3).

The next set of experiments focused on the question whether Asia-1 Shamir is able to replicate inside the BHK-2P cell at all. It was shown that it is indeed possible for Asia-1 Shamir to replicate and produce infectious virus particles, if the viral genome is brought into the cell by chemical transfection (lipofection) (Paper II, Figure 4). This indicates that the cell line is permissive and has the capability to replicate virus, but virus attachment and/or entry is constrained. To compare these critical steps in the viral life cycle between susceptible and resistant BHK cells, a cell line susceptible to Asia-1 Shamir had to be found. The ability of Asia-1 Shamir to infect several different suspension cell lines was tested and three lines (#3, #8, #9) showed good viral growth (Paper II, Table S2). Virus adapted to either one of these susceptible cell lines was then able to grow in and adapt to BHK-2P cells as well (Paper II, Table 1). The finding that cellular resistance can be overcome by the virus through prior adaption to a susceptible cell line, suggested that the problem originates in virus attachment to the cells, related to the presence or quantity of specific cellular receptors. This hypothesis was substantiated by the result of an attachment assay, performed with A_{24} Cruzeiro, the original Asia-1 Shamir and the three adapted virus isolates (#3 Asia-1, #8 Asia-1 and #9 Asia-1) on BHK-2P cells and adherent BHK179 cells. For A24 Cruzeiro, there was no difference in the dose of infectious virus that attached to either BHK179 or BHK-2P cells. The original Asia-1 Shamir, however, showed a significant loss of titer on BHK-2P, while the adapted isolates showed increased viral titers on BHK-2P in comparison to the adherent BHK cells (Paper II, Figure 5). The adherent BHK179 cell line was chosen for these experiments because adherent cells are more likely to display FMDV-compatible integrin receptors on their surface, while suspension cells often show an altered or generally downregulated surface receptor environment (Amadori et al., 1997; Walther et al., 2016). Sequence analysis revealed several mutations in the viral genome of the BHK-2P adapted Asia-1 strains (Paper II, Table S5). Two aa exchanges were shared between all adapted strains: one in the non-structural 2C protein (K285Q) that is important for virus replication (Grubman and Baxt, 2004) and one at amino acid position 110 (Q110K/R) in the VP1 capsid protein (Paper II, Table 2). The aa exchange at position 110 of VP1 was associated with a second exchange in this region (Q108R for #3 and #8 Asia-1, T83A for #9 Asia-1), leading to an increased positive charge on the outside of the virus capsid. Furthermore, the exchanged amino acids are in close spatial proximity around the five-fold symmetry axis of the viral capsid pentamers (Paper II, Figure

6), suggesting new receptor interactions (Berryman et al., 2013; Mohapatra et al., 2015). The #9 Asia-1 isolate contained two more aa exchanges: E59K in VP3 and E202K at the C-terminus of VP1 (Paper II, Table 2), both part of the HS binding site (Fry et al., 2005). The extended receptor tropism of the adapted Asia-1 isolates was confirmed through infection experiments on Chinese hamster ovary K1 cells (CHO-K1), devoid of the primary FMDV receptors, as well as non-integrin, non-HS-displaying CHO cells (CHO677). CHO677 cells are considered a key cell line for finding FMDV variants that might have acquired the capability to use a third alternative receptor (Lawrence and Rieder, 2017). Indeed, all mutants could successfully infect CHO-K1 and CHO677 cells, in contrast to the original Asia-1 Shamir (Paper II, Table 3). However, #9 Asia-1 reached significantly higher titers in CHO677 cells than the other two mutant strains. The results indicate an extended receptor usage for all three adapted isolates and a possible additional role in the usage of a tertiary receptor for the HS-specific aa exchanges in the capsid of #9 Asia-1. The changes on the outer capsid surface, mostly within VP1, the major antigenic protein of the virus capsid (Grubman and Baxt, 2004), also led to an altered neutralization profile. Sera from infected and vaccinated animals still neutralized the adapted #9 isolate (on BHK164 cells) but had no neutralizing activity against isolates #3 and #8 (Paper II, Figure 7). The highly antigenic RGD motif, the crucial receptor binding site for natural infection and a major target for neutralizing antibodies (Nemerow and Stewart, 2001), remained unaffected by the aa exchanges, suggesting that a vaccine formulated from these mutants would lead to a protective immune response against an infection with a field isolate of Asia-1.

The causes of resistance of certain BHK cell lines against FMDV, although not finally clarified, could be narrowed down to an unfavorable receptor repertoire of the resistant cell lines in comparison to susceptible cell lines. Furthermore, a way to overcome the resistance problem by using a FMDV-sensible “wet-nurse” cell line has been found.

5.3 Impact of different culture systems on FMDV harvest, receptor tropism and antigenicity

FMDV serotype O is the most widespread serotype worldwide, while serotype A exhibits the highest antigenic diversity between strains (Parida, 2009; Saeed et al., 2015). Nevertheless, for more than 30 years the FMDV strains O_1 Manisa and A_{24} Cruzeiro have been used for vaccine production (Parida, 2009) and are still considered “high priority strains” that are recommended for inclusion into vaccine antigen banks by the World Reference Laboratory of the Food and Agriculture Organization of the United Nations (WRLFMD) (Saeed et al., 2015; WRLFMD, 2016). For this reason, these strains have been selected to evaluate sequence

changes during virus passaging and to compare their proliferation properties in different cell culture systems.

The FMDV strains O_1 Manisa and A_{24} Cruzeiro have been passaged in parallel 15 to 20 times in adherent BHK179 cells, maintained in MEM with 5% FBS, and in suspension BHK-2P cells, maintained in an ACFM without serum. Sequence analysis of the final virus passages revealed three heterologous aa exchanges in VP1 (K41N, E83K, K210E) for O_1 Manisa, independent of the culture system (Paper III, Figure 1), and no aa exchange in VP3. Earlier studies had showed that cleavage of the VP1/2A junction is inhibited by the aa exchanges E83K and K210E (Gullberg et al., 2013), and that especially E83K creates an advantage for the virus to grow in BHK cells (Maree et al., 2010; Zhao et al., 2003). Between the adherent-cell-passaged A_{24} Cruzeiro (A_{24}-179) and the suspension-cell-passaged A_{24} Cruzeiro (A_{24}-2P), different sequence alterations were evident. While A_{24}-179 acquired exchanges in VP1 at position 194 (E194K) and in VP3 at position 56 (C56R), which are typical alterations for the acquisition of HS as cellular receptor (Fry et al., 2005), the exchanges in A_{24}-2P were located at position 95 of VP1 (E95K) and position 85 of VP3 (H85Q) (Paper III, Figure 1). The E95K exchange within VP1 is close to the fivefold symmetry axis and has been reported in the context of utilizing JMJD6 to infect cells without primary or secondary FMDV receptors (Lawrence et al., 2016). The extended receptor usage of adapted A_{24}-2P was confirmed by infection experiments using the FMDV-receptor-deficient CHO677 cell line (Paper III, Table 1). While all isolates were able to replicate in CHO-K1 cells, A_{24}-2P also grew to significantly higher titers (Paper III, Table 1). Next, virus neutralization tests (VNT) were performed to evaluate the antigenic profile of the adapted virus isolates but no differences to the parental strains were detected (Paper III, Table 1). That the adapted strains maintained the original neutralization profile even after extensive tissue culture adaptation might partially explain why O_1 Manisa and A_{24} Cruzeiro are still used for vaccine production. Because serotype O exhibits comparatively moderate levels of variation, O_1 Manisa vaccines provide coverage in many FMDV O outbreaks, at least in Africa, the Middle East and parts of Asia (Doel, 2003; Parida, 2009). Even for the highly variable FMDV serotype A, A_{24} Cruzeiro vaccines are considered a useful tool in many first-occurrence situations, especially in South America (Parida, 2009; Saeed et al., 2015).

The following set of experiments dealt with virus particle characteristics that are important for vaccine production, compared for the different culture conditions. Proliferation curves revealed the optimal time point for virus harvest. In both culture systems, similar maximum titers of approximately 8.7 $\log_{10}$ $TCID_{50}$ per mL were achieved for both virus strains but the

time of peak cell death varied. In BHK-2P cells, cell viability dropped after 12-16 hours (h), while full cytopathic effect (CPE) in the adherent cell culture system was visible after 20 h of incubation (Paper III, Figure 4). In contrast, viral particle integrity differed between virus grown in suspension cells and virus grown in monolayers (Paper III, Figure 2). The major immunogenic element of an FMD vaccine are the 146S particles (Doel, 2003), which were significantly reduced in preparations of A_{24}-2P in comparison to preparations of A_{24}-179, whereas the amount of free RNA was increased. In addition, serotype A FMDV strains tend to form empty 75S particles in significant amounts (Harmsen et al., 2011; Seki et al., 2009), which was evident for both serotype A preparations. For serotype O, the results were not as distinct. Although a higher content of virus protein was detected by antigen ELISA for isolate O_1-2P, the peak corresponding to the 146S particles was decreased in the spectrophotometric reading, and high amounts of free RNA were evident as well. An assessment of the virus particle stability under acidic conditions did not provide an explanation for the reduced yield of 146S particles and the increased amounts of free RNA in the suspension cell culture system. An increased acid sensitivity was only seen for the A_{24}-179 isolate (Paper III, Figure 3), leading to the assumption that this is linked to the aa exchanges that are necessary for HS binding.

The results presented in this study agree with similar studies of antigen yield and sequence analysis, in which suspension cell culture and monolayer cells for FMDV production were compared (Anil et al., 2012; Spier, 1976). However, this study is the first to compare FMDV antigen production in ACFM and in standard growth media with added serum. The finding that ACFM do not have a detrimental impact on viral yield or antigenicity supports the conversion to alternative vaccine production processes. This is desirable because raw materials of biological origin are poorly defined. Apart from significant variations between different production lots, they can also contain pathogens that compromise product safety and quality (European Commission, 2000; Genzel et al., 2006).

6. Outlook

The relatively small number of isolates used for the validation of the genome sequencing assay should be expanded, with special attention to the SAT serotypes. Comparative analysis of the obtained sequences could uncover regions of the genome that are prone to truncations during sequencing. For these regions, the development of additional backup primers is warranted. International collaborative studies with laboratories in endemic areas could verify the applicability of the assay under real field conditions.

The second study discovered a way to adapt Asia-1 Shamir to a resistant cell line, but also raised new questions. Further work is necessary to get a more detailed view of the differences between resistant and susceptible cell lines regarding e.g. cell metabolism and cell surface proteins. It would be of interest if the “wet nurse”-concept of adapting a certain virus strain to a resistant cell line by first adapting it to a sensible cell line is applicable to other Asia-1 strains and to strains of other serotypes. In addition, studies to prove the antigenic quality and quantity of virus obtained in this way have to be conducted. Animal trials to confirm that a vaccine formulated from these strains elicits a protective immune response could complement the results achieved so far.

The use of ACFM in vaccine production would improve the process due to a minimized risk of contaminants introduced by raw materials. Nevertheless, further experiments should be conducted to clarify the reasons for the difference in 146S content, the most protective immunogenic element. Harvest time points should be optimized to exclude the possibility that viral particles are degraded by host cell enzymes, released into the media after cell death. Further studies should focus on important cell metabolites like ammonium and lactate, since their production is related to the composition of the growth media and might influence the viral yield. The application of laboratory-scale bioreactors, being automatically regulated and containing several liters of cells in suspension and media, would be useful to test the utility of the media in a more realistic production situation.

7. References

Abdul-Hamid, N.F., Firat-Sarac, M., Radford, A.D., Knowles, N.J., King, D.P., 2011. Comparative sequence analysis of representative foot-and-mouth disease virus genomes from Southeast Asia. Virus Genes 43, 41-45.

Acharya, R., Fry, E., Stuart, D., Fox, G., Rowlands, D., Brown, F., 1989. The three-dimensional structure of foot-and-mouth disease virus at 2.9 Å resolution. Nature 337, 709-716.

Alcala, P., Feliu, J.X., Aris, A., Villaverde, A., 2001. Efficient accommodation of recombinant, foot-and-mouth disease virus RGD peptides to cell-surface integrins. Biochem Biophys Res Commun 285, 201-206.

Alexandersen, S., Zhang, Z., Donaldson, A.I., Garland, A.J., 2003. The pathogenesis and diagnosis of foot-and-mouth disease. J Comp Pathol 129, 1-36.

Amadori, M., Berneri, C., Archetti, I.L., 1994. Immunogenicity of foot-and-mouth disease virus grown in BHK-21 suspension cells. Correlation with cell ploidy alterations and abnormal expression of the alpha 5 beta 1 integrin. Vaccine 12, 159-166.

Amadori, M., Volpe, G., Defilippi, P., Berneri, C., 1997. Phenotypic features of BHK-21 cells used for production of foot-and-mouth disease vaccine. Biologicals 25, 65-73.

Anil, K.U., Sreenivasa, B.P., Mohapatra, J.K., Hosamani, M., Kumar, R., Venkataramanan, R., 2012. Sequence analysis of capsid coding region of foot-and-mouth disease virus type A vaccine strain during serial passages in BHK-21 adherent and suspension cells. Biologicals 40, 426-430.

Arzt, J., Baxt, B., Grubman, M.J., Jackson, T., Juleff, N., Rhyan, J., Rieder, E., Waters, R., Rodriguez, L.L., 2011. The pathogenesis of foot-and-mouth disease II: viral pathways in swine, small ruminants, and wildlife; myotropism, chronic syndromes, and molecular virus-host interactions. Transbound Emerg Dis 58, 305-326.

Ayelet, G., Mahapatra, M., Gelaye, E., Egziabher, B.G., Rufeal, T., Sahle, M., Ferris, N.P., Wadsworth, J., Hutchings, G.H., Knowles, N.J., 2009. Genetic characterization of foot-and-mouth disease viruses, Ethiopia, 1981-2007. Emerg Infect Dis 15, 1409-1417.

Bastos, A.D., Haydon, D.T., Sangare, O., Boshoff, C.I., Edrich, J.L., Thomson, G.R., 2003. The implications of virus diversity within the SAT 2 serotype for control of foot-and-mouth disease in sub-Saharan Africa. J Gen Virol 84, 1595-1606.

Belsham, G.J., 2005. Translation and replication of FMDV RNA. Curr Top Microbiol Immunol 288, 43-70.

Berinstein, A., Roivainen, M., Hovi, T., Mason, P.W., Baxt, B., 1995. Antibodies to the vitronectin receptor (integrin alpha V beta 3) inhibit binding and infection of foot-and-mouth disease virus to cultured cells. J Virol 69, 2664-2666.

Bernfield, M., Gotte, M., Park, P.W., Reizes, O., Fitzgerald, M.L., Lincecum, J., Zako, M., 1999. Functions of cell surface heparan sulfate proteoglycans. Annu Rev Biochem 68, 729-777.

Berryman, S., Clark, S., Kakker, N.K., Silk, R., Seago, J., Wadsworth, J., Chamberlain, K., Knowles, N.J., Jackson, T., 2013. Positively charged residues at the five-fold

symmetry axis of cell culture-adapted foot-and-mouth disease virus permit novel receptor interactions. J Virol 87, 8735-8744.

Brooksby, J.B., 1958. The virus of foot-and-mouth disease. Adv Virus Res 5, 1-37.

Callahan, J.D., Brown, F., Osorio, F.A., Sur, J.H., Kramer, E., Long, G.W., Lubroth, J., Ellis, S.J., Shoulars, K.S., Gaffney, K.L., Rock, D.L., Nelson, W.M., 2002. Use of a portable real-time reverse transcriptase-polymerase chain reaction assay for rapid detection of foot-and-mouth disease virus. J Am Vet Med Assoc 220, 1636-1642.

Capstick, P.B., Telling, R.C., Chapman, W.G., Stewart, D.L., 1962. Growth of a cloned strain of hamster kidney cells in suspended cultures and their susceptibility to the virus of foot-and-mouth disease. Nature 195, 1163-1164.

Carrillo, C., Tulman, E.R., Delhon, G., Lu, Z., Carreno, A., Vagnozzi, A., Kutish, G.F., Rock, D.L., 2005. Comparative genomics of foot-and-mouth disease virus. J Virol 79, 6487-6504.

Carrillo, E.C., Giachetti, C., Campos, R.H., 1984. Effect of lysosomotropic agents on the foot-and-mouth disease virus replication. Virology 135, 542-545.

Chamberlain, K., Fowler, V.L., Barnett, P.V., Gold, S., Wadsworth, J., Knowles, N.J., Jackson, T., 2015. Identification of a novel cell culture adaptation site on the capsid of foot-and-mouth disease virus. J Gen Virol 96, 2684-2692.

Clarke, J.B., Spier, R.E., 1980. Variation in the susceptibility of BHK populations and cloned cell lines to three strains of foot-and-mouth disease virus. Arch Virol 63, 1-9.

Clarke, J.B., Spier, R.E., 1983. An investigation into causes of resistance of a cloned line of BHK cells to a strain of foot-and-mouth disease virus. Vet Microbiol 8, 259-270.

Cottam, E.M., Haydon, D.T., Paton, D.J., Gloster, J., Wilesmith, J.W., Ferris, N.P., Hutchings, G.H., King, D.P., 2006. Molecular epidemiology of the foot-and-mouth disease virus outbreak in the United Kingdom in 2001. J Virol 80, 11274-11282.

Cottam, E.M., Thebaud, G., Wadsworth, J., Gloster, J., Mansley, L., Paton, D.J., King, D.P., Haydon, D.T., 2008a. Integrating genetic and epidemiological data to determine transmission pathways of foot-and-mouth disease virus. Proc Biol Sci 275, 887-895.

Cottam, E.M., Wadsworth, J., Shaw, A.E., Rowlands, R.J., Goatley, L., Maan, S., Maan, N.S., Mertens, P.P., Ebert, K., Li, Y., Ryan, E.D., Juleff, N., Ferris, N.P., Wilesmith, J.W., Haydon, D.T., King, D.P., Paton, D.J., Knowles, N.J., 2008b. Transmission pathways of foot-and-mouth disease virus in the United Kingdom in 2007. PLoS Pathog 4, e1000050.

Dhanda, M.R., Gopalakrishnan, V.R., H.S., D., 1957. Note on the occurrence of atypical strains of foot-and-mouth diseases virus in India. . Indian j. vet. sci. 27, 79-84.

Diderholm, H., Dinter, Z., 1965. A line of polyoma-transformed BHK21 cells insusceptible to foot-and-mouth disease virus. Virology 26, 369-372.

Doel, T.R., Chong, W.K., 1982. Comparative immunogenicity of 146S, 75S and 12S particles of foot-and-mouth disease virus. Arch Virol 73, 185-191.

Doel, T.R., 2003. FMD vaccines. Virus Res 91, 81-99.

Domingo, E., Baranowski, E., Escarmis, C., Sobrino, F., 2002. Foot-and-mouth disease virus. Comp Immunol Microbiol Infect Dis 25, 297-308.

Domingo, E., Escarmis, C., Baranowski, E., Ruiz-Jarabo, C.M., Carrillo, E., Nunez, J.I., Sobrino, F., 2003. Evolution of foot-and-mouth disease virus. Virus Res 91, 47-63.

European Commission, 2000. Commission Directive 1999/104/EC of 22 December 1999 amending the Annex to Council Directive 81/852/EEC on the approximation of the laws of the Member States relating to analytical, pharmacotoxicoligical and clinical standards and protocols in respect of the testing of veterinary medicinal products. OJEU L3, 18-20.

Fry, E.E., Newman, J.W., Curry, S., Najjam, S., Jackson, T., Blakemore, W., Lea, S.M., Miller, L., Burman, A., King, A.M., Stuart, D.I., 2005. Structure of Foot-and-mouth disease virus serotype A10 61 alone and complexed with oligosaccharide receptor: receptor conservation in the face of antigenic variation. J Gen Virol 86, 1909-1920.

Genzel, Y., Fischer, M., Reichl, U., 2006. Serum-free influenza virus production avoiding washing steps and medium exchange in large-scale microcarrier culture. Vaccine 24, 3261-3272.

Gloster, J., Sellers, R.F., Donaldson, A.I., 1982. Long distance transport of foot-and-mouth disease virus over the sea. Vet Rec 110, 47-52.

Grubman, M.J., Baxt, B., 2004. Foot-and-mouth disease. Clin Microbiol Rev 17, 465-493.

Gullberg, M., Polacek, C., Botner, A., Belsham, G.J., 2013. Processing of the VP1/2A junction is not necessary for production of foot-and-mouth disease virus empty capsids and infectious viruses: characterization of "self-tagged" particles. J Virol 87, 11591-11603.

Han, S.C., Guo, H.C., Sun, S.Q., 2015. Three-dimensional structure of foot-and-mouth disease virus and its biological functions. Arch Virol 160, 1-16.

Harmsen, M.M., Fijten, H.P., Westra, D.F., Coco-Martin, J.M., 2011. Effect of thiomersal on dissociation of intact (146S) foot-and-mouth disease virions into 12S particles as assessed by novel ELISAs specific for either 146S or 12S particles. Vaccine 29, 2682-2690.

Jackson, T., Ellard, F.M., Ghazaleh, R.A., Brookes, S.M., Blakemore, W.E., Corteyn, A.H., Stuart, D.I., Newman, J.W., King, A.M., 1996. Efficient infection of cells in culture by type O foot-and-mouth disease virus requires binding to cell surface heparan sulfate. J Virol 70, 5282-5287.

Jackson, T., Sheppard, D., Denyer, M., Blakemore, W., King, A.M., 2000. The epithelial integrin alphavbeta6 is a receptor for foot-and-mouth disease virus. J Virol 74, 4949-4956.

Jackson, T., Mould, A.P., Sheppard, D., King, A.M., 2002. Integrin alphavbeta1 is a receptor for foot-and-mouth disease virus. J Virol 76, 935-941.

Jackson, T., Clark, S., Berryman, S., Burman, A., Cambier, S., Mu, D., Nishimura, S., King, A.M., 2004. Integrin alphavbeta8 functions as a receptor for foot-and-mouth disease virus: role of the beta-chain cytodomain in integrin-mediated infection. J Virol 78, 4533-4540.

Jamal, S.M., Belsham, G.J., 2013. Foot-and-mouth disease: past, present and future. Veterinary Research 44, 1-14.

James, A.D., Rushton, J., 2002. The economics of foot and mouth disease. Rev Sci Tech 21, 637-644.

Juleff, N., Windsor, M., Reid, E., Seago, J., Zhang, Z., Monaghan, P., Morrison, I.W., Charleston, B., 2008. Foot-and-mouth disease virus persists in the light zone of germinal centres. PLoS One 3, e3434.

King, D.P., Burman, A., Gold, S., Shaw, A.E., Jackson, T., Ferris, N.P., 2011. Integrin subunit expression in cell cultures used for the diagnosis of foot-and-mouth disease. Vet Immunol Immunopathol 140, 259-265.

Knight-Jones, T.J., Rushton, J., 2013. The economic impacts of foot and mouth disease - what are they, how big are they and where do they occur? Prev Vet Med 112, 161-173.

Knowles, N.J., Samuel, A.R., Davies, P.R., Kitching, R.P., Donaldson, A.I., 2001. Outbreak of foot-and-mouth disease virus serotype O in the UK caused by a pandemic strain. Vet Rec 148, 258-259.

Knowles, N.J., Samuel, A.R., 2003. Molecular epidemiology of foot-and-mouth disease virus. Virus Res 91, 65-80.

Knowles, N.J., Wadsworth, J., Bachanek-Bankowska, K., King, D.P., 2016. VP1 sequencing protocol for foot and mouth disease virus molecular epidemiology. Rev Sci Tech 35, 741-755.

Lawrence, P., LaRocco, M., Baxt, B., Rieder, E., 2013. Examination of soluble integrin resistant mutants of foot-and-mouth disease virus. Virol J 10, 2.

Lawrence, P., Rai, D., Conderino, J.S., Uddowla, S., Rieder, E., 2016. Role of Jumonji C-domain containing protein 6 (JMJD6) in infectivity of foot-and-mouth disease virus. Virology 492, 38-52.

Lawrence, P., Rieder, E., 2017. Insights into Jumonji C-domain containing protein 6 (JMJD6): a multifactorial role in foot-and-mouth disease virus replication in cells. Virus Genes 53, 340-351.

Logan, D., Abu-Ghazaleh, R., Blakemore, W., Curry, S., Jackson, T., King, A., Lea, S., Lewis, R., Newman, J., Parry, N., et al., 1993. Structure of a major immunogenic site on foot-and-mouth disease virus. Nature 362, 566-568.

Maree, F.F., Blignaut, B., de Beer, T.A., Visser, N., Rieder, E.A., 2010. Mapping of amino acid residues responsible for adhesion of cell culture-adapted foot-and-mouth disease SAT type viruses. Virus Res 153, 82-91.

Martin-Acebes, M.A., Rincon, V., Armas-Portela, R., Mateu, M.G., Sobrino, F., 2010. A single amino acid substitution in the capsid of foot-and-mouth disease virus can increase acid lability and confer resistance to acid-dependent uncoating inhibition. J Virol 84, 2902-2912.

Mateu, M.G., 1995. Antibody recognition of picornaviruses and escape from neutralization: a structural view. Virus Res 38, 1-24.

Mohapatra, J.K., Pandey, L.K., Rai, D.K., Das, B., Rodriguez, L.L., Rout, M., Subramaniam, S., Sanyal, A., Rieder, E., Pattnaik, B., 2015. Cell culture adaptation mutations in foot-and-mouth disease virus serotype A capsid proteins: implications for receptor interactions. J Gen Virol 96, 553-564.

Neff, S., Sa-Carvalho, D., Rieder, E., Mason, P.W., Blystone, S.D., Brown, E.J., Baxt, B., 1998. Foot-and-mouth disease virus virulent for cattle utilizes the integrin alpha(v)beta3 as its receptor. J Virol 72, 3587-3594.

Nemerow, G.R., Stewart, P.L., 2001. Antibody neutralization epitopes and integrin binding sites on nonenveloped viruses. Virology 288, 189-191.

O'Donnell, V., Pacheco, J.M., Gregg, D., Baxt, B., 2009. Analysis of foot-and-mouth disease virus integrin receptor expression in tissues from naive and infected cattle. J Comp Pathol 141, 98-112.

Oem, J.K., Kye, S.J., Lee, K.N., Kim, Y.J., Park, J.Y., Park, J.H., Joo, Y.S., Song, H.J., 2005. Development of a Lightcycler-based reverse transcription polymerase chain reaction for the detection of foot-and-mouth disease virus. J Vet Sci 6, 207-212.

OIE 2017a. Chapter 2.1.8. Foot and Mouth Disease (Infection with Foot and Mouth Disease Virus). In Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (http://www.oie.int/international-standard-setting/terrestrial-manual/access-online/).

OIE 2017b. Chapter 1.3. Diseases, Infections and Infestations Listed by the OIE. In Terrestrial Animal Health Code (http://www.oie.int/index.php?id=169&L=0&htmfile=chapitre_diagnostic_tests.htm).

Parida, S., 2009. Vaccination against foot-and-mouth disease virus: strategies and effectiveness. Expert Rev Vaccines 8, 347-365.

Pettersen, E.F., Goddard, T.D., Huang, C.C., Couch, G.S., Greenblatt, D.M., Meng, E.C., Ferrin, T.E., 2004. UCSF Chimera--a visualization system for exploratory research and analysis. J Comput Chem 25, 1605-1612.

Rodriguez, L.L., Gay, C.G., 2011. Development of vaccines toward the global control and eradication of foot-and-mouth disease. Expert Rev Vaccines 10, 377-387.

Ruiz-Saenz, J., Goez, Y., Tabares, W., Lopez-Herrera, A., 2009. Cellular receptors for foot and mouth disease virus. Intervirology 52, 201-212.

Sa-Carvalho, D., Rieder, E., Baxt, B., Rodarte, R., Tanuri, A., Mason, P.W., 1997. Tissue culture adaptation of foot-and-mouth disease virus selects viruses that bind to heparin and are attenuated in cattle. J Virol 71, 5115-5123.

Saeed, A., Kanwal, S., Arshad, M., Ali, M., Shaikh, R.S., Abubakar, M., 2015. Foot-and-mouth disease: overview of motives of disease spread and efficacy of available vaccines. J Anim Sci Technol 57, 10.

Samuel, A.R., Knowles, N.J., 2001. Foot-and-mouth disease virus: cause of the recent crisis for the UK livestock industry. Trends Genet 17, 421-424.

Sangar, D.V., Rowlands, D.J., Cavanagh, D., Brown, F., 1976. Characterization of the minor polypeptides in the foot-and-mouth disease particle. J Gen Virol 31, 35-46.

Sanner, M.F., Olson, A.J., Spehner, J.C., 1996. Reduced surface: an efficient way to compute molecular surfaces. Biopolymers 38, 305-320.

Schaftenaar, W., 2002. Use of vaccination against foot and mouth disease in zoo animals, endangered species and exceptionally valuable animals. Rev Sci Tech 21, 613-623.

Seki, C., Robiolo, B., Periolo, O., Iglesias, M., D'Antuono, A., Maradei, E., Barros, V., La Torre, J., Mattion, N., 2009. Rapid methodology for antigenic profiling of FMDV field strains and for the control of identity, purity and viral integrity in commercial virus vaccines using monoclonal antibodies. Vet Microbiol 133, 239-251.

Sellers, R.F., Parker, J., 1969. Airborne excretion of foot-and-mouth disease virus. J Hyg (Lond) 67, 671-677.

Spier, R.E., 1976. Reproducibility of yields of foot-and-mouth disease virus from BHK monolayer and suspension cells. Dev Biol Stand 35, 73-78.

Stenfeldt, C., Belsham, G.J., 2012. Detection of foot-and-mouth disease virus RNA in pharyngeal epithelium biopsy samples obtained from infected cattle: investigation of possible sites of virus replication and persistence. Vet Microbiol 154, 230-239.

Stenfeldt, C., Diaz-San Segundo, F., de Los Santos, T., Rodriguez, L.L., Arzt, J., 2016. The Pathogenesis of Foot-and-Mouth Disease in Pigs. Front Vet Sci 3, 41.

Stewart, P.L., Nemerow, G.R., 2007. Cell integrins: commonly used receptors for diverse viral pathogens. Trends Microbiol 15, 500-507.

Thompson, D., Muriel, P., Russell, D., Osborne, P., Bromley, A., Rowland, M., Creigh-Tyte, S., Brown, C., 2002. Economic costs of the foot and mouth disease outbreak in the United Kingdom in 2001. Rev Sci Tech 21, 675-687.

Vallée, H., Carré, H., 1922. Sur la pluralité du virus aphteux. Comptes rendus hebdomadaires des séances de l'Académie des sciences. 174, 1498–1500.

Waldmann, O., Trautwein, K., 1926. Experimentelle Untersuchungen über die Pluralitat des Maul- und Klauenseuche Virus. Berliner tierärztliche Wochenschrift 42, 569–571.

Walther, C.G., Whitfield, R., James, D.C., 2016. Importance of Interaction between Integrin and Actin Cytoskeleton in Suspension Adaptation of CHO cells. Appl Biochem Biotechnol 178, 1286-1302.

Wang, G., Wang, Y., Shang, Y., Zhang, Z., Liu, X., 2015. How foot-and-mouth disease virus receptor mediates foot-and-mouth disease virus infection. Virol J 12, 9.

WRLFMD 2016. OIE/FAO Foot-and-Mouth Disease Reference Laboratory Network Annual Report 2016 (http://www.wrlfmd.org/ref_labs/ref_lab_reports/OIE-FAO%20FMD%20Ref%20Lab%20Network%20Report%202016.pdf).

Xu, L., Hurtle, W., Rowland, J.M., Casteran, K.A., Bucko, S.M., Grau, F.R., Valdazo-Gonzalez, B., Knowles, N.J., King, D.P., Beckham, T.R., McIntosh, M.T., 2013. Development of a universal RT-PCR for amplifying and sequencing the leader and capsid-coding region of foot-and-mouth disease virus. J Virol Methods 189, 70-76.

Zell, R., Delwart, E., Gorbalenya, A.E., Hovi, T., King, A.M.Q., Knowles, N.J., Lindberg, A.M., Pallansch, M.A., Palmenberg, A.C., Reuter, G., Simmonds, P., Skern, T., Stanway, G., Yamashita, T., Consortium, I.R., 2017. ICTV Virus Taxonomy Profile: *Picornaviridae*. Journal of General Virology.

Zhao, Q., Pacheco, J.M., Mason, P.W., 2003. Evaluation of genetically engineered derivatives of a Chinese strain of foot-and-mouth disease virus reveals a novel cell-binding site which functions in cell culture and in animals. J Virol 77, 3269-3280.

8. List of publications

1. Scientific articles

1.1 Scientific articles, included into this thesis

Dill V, Beer M, Hoffmann B (2017). Simple, quick and cost-efficient: A universal RT-PCR and sequencing strategy for genomic characterisation of foot-and-mouth disease viruses. Journal of Virological Methods 246: 58–64.

Dill V, Hoffmann B, Zimmer A, Beer M, Eschbaumer M (2017). Adaption of FMDV Asia-1 to Suspension Culture: Cell Resistance Is Overcome by Virus Capsid Alterations. Viruses 9: 231.

Dill V., Hoffmann B, Zimmer A, Beer M, Eschbaumer M (2018). Influence of cell type and cell culture media on the propagation of foot-and-mouth disease virus with regard to vaccine quality. Virology Journal 15:46.

1.2 Miscellaneous scientific articles

Dill V, Eschbaumer M, Beer M, Hoffmann B (2017). Inter-laboratory validation of foot-and-mouth disease diagnostic capability in Germany. Veterinary Microbiology 203: 62–67.

Goller KV, Dill V, Madi M, Martin P, Van der Stede Y, Vandenberge V, HaasB, Van Borm S, Koenen F, Kasanga CJ, Ndusilo N, Beer M, Liu L, Mioulet V, Armson B, King DP, Fowler VL (2017). Rapid and simple detection of foot-and-mouth-disease virus: Evaluation of a cartridge-based molecular detection system for use in basic laboratories. Transboundary and Emerging Diseases 1-7.

2. Oral presentations

* = presenting author

Goller K, Dill V*, Martin P, Blome S, Beer M, Haas B. "Cartridge-based real-time molecular diagnostic assays for the rapid and simple detection of African swine fever and foot-and-mouth disease virus". Xth International Congress for Veterinary Virology and 9th Annual Meeting of EPIZONE; 31th August to 3rd September 2015, Montpellier, France

Dill V*, Hoffmann B, Zimmer A, Beer M, Eschbaumer M (2017). "Adaption of FMDV Asia-1 to Suspension Culture: Cell Resistance Is Overcome by Virus Capsid Alterations." New Developments in Inflammation, Infection and Immunology (NID$_3$) Conference 2017, 10th November 2017, Borstel, Germany

3. Poster

Dill V*, Haas B, von Hagen J. "Analysis of Foot-and-Mouth Disease Virus regarding the Influence of Chemical Defined Media on the Virus Particle". Bioprocess International Conference, Merck (company internal conference); 27th April 2015, Darmstadt, Germany

Dill V*, Beer M, Hoffmann B. "Simple, quick and cost-efficient: A universal RT-PCR for amplifying and sequencing the genome of foot-and-mouth disease virus".1st Summer School: "Infection Biology"; 28th to 30th September 2016, Greifswald, Germany

Zoli M*, Höper D, Dill V, Beer M. "Identification of gene signatures associated with foot-and-mouth disease virus infection by transcriptome analysis". 1st Summer School: "Infection Biology"; 28th to 30th September 2016, Greifswald, Germany

Dill V, Ehret J*, Eschbaumer M, Zimmer A. "Cell culture media for veterinary vaccine production of FMDV in BHK cells". R&D Summit, Merck (company internal conference), 31st October to 5th November 2016, St. Louis, USA

Dill V*, Beer M, Hoffmann B. "Simple, quick and cost-efficient: A universal RT-PCR and sequencing strategy for genomic characterisation of foot-and-mouth disease viruses". 11th Annual Meeting of EPIZONE; 19th to 21st September 2017, Paris, France

9. Eigenständigkeitserklärung

Hiermit erkläre ich, dass diese Arbeit bisher von mir weder an der Mathematisch-Naturwissenschaftlichen Fakultät der Ernst-Moritz-Arndt-Universität Greifswald noch einer anderen wissenschaftlichen Einrichtung zum Zwecke der Promotion eingereicht wurde.

Ferner erkläre ich, dass ich diese Arbeit selbstständig verfasst und keine anderen als die darin angegebenen Hilfsmittel und Hilfen benutzt und keine Textabschnitte eines Dritten ohne Kennzeichnung übernommen habe.

Greifswald, den 30.11.2017

Veronika Dill