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Foot-and-Mouth Disease (FMD) Carrier State in Livestock Population and its Diagnosis

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ABSTRACT

Approximately, 50% of Foot and Mouth Disease (FMD) affected bovines persistently harbours the virus in the oro-pharyngeal epithelium for a prolonged period of time after recovery. The phenomenon is known as FMDV persistence and the animals as FMDV carriers. The virus can be isolated from oropharyngeal fluid (OPF) during a period more than 28 days after infection. Although direct transmission of virus from carrier to healthy animals has not been established, but these animals as a risk, for emergence of new outbreak cannot be overlooked. Therefore, trade policies consider the carriers as contagious and import of livestock from enzootic zones is restricted. FMDV persistence also complicates the retrieval of FMD-free status as the country or zone must evidence complete clearance of virus source. Thus, the detection of carrier animals after an outbreak or during export and import is essentially needed. This can be achieved by detecting the viral antigen and genome, or viral antibodies. Various methods such as virus isolation, RT-PCR, mucosal antibody detection ELISA etc. have been developed for this purpose. However, each test has some advantages and limitations. This article discusses about the persistence of FMDV in carrier animals and various methods for its detection.

HIGHLIGHTS

- The article describes the FMDV persistence in carrier animals and various methods for its detection.
- About half of the FMD affected bovines persistently harbours the virus in the oro-pharyngeal epithelium for a prolonged period after recovery.
- The diagnosis of carrier animals after an outbreak can be achieved by detecting the viral antigen genome.

Keywords: Foot-and-Mouth Disease, Carrier, Oropharyngeal fluid, ELISA, RT-PCR

Foot-and-Mouth Disease Virus (FMDV) is the causative organism of Aphthus fever which is a prototype of the genus *Aphthovirus* in the family *Picornaviridae*. It is a single stranded RNA (ssRNA) virus and exists in 7 serotypes {O, A, C, Asia-1 and Southern African Territories (SAT-1, SAT-2 and SAT-3), which are distinct immunologically (Knowels and Samuel, 2003). These serotypes of FMDV were divided into different genotypes, lineages and sub-lineages. The geographically confined

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genotypes are known as topotypes. Serotype O, A and C constitute 11, 3 and 3 topotypes, respectively. A single topotype was assigned to Asia 1 serotype viruses, whereas SAT-1, -2 and-3 viruses constitutes 9, 14 and 5 topotypes, respectively (Biswal *et al*., 2012). The genome of FMDV is surrounded by a protein shell known as capsid which comprises 60 copies of capsomere, and each capsomere contains 4 structural proteins (SP) viz. VP1, VP2, VP3 and VP4. The ssRNA genome consists of three parts i.e. the 5′ untranslated region (5UTR), a long open reading frame (ORF) and the 3′ untranslated region (3′ UTR) (Lin and Flint, 2000). The 5′UTR consists of an 'S' fragment at its 5′ end, a poly C tract, a series of RNA pseudoknot structures, a *cis*-acting replication element (*cre*) and the internal ribosome entry site (IRES). The 3′UTR constitutes 2 stem loop structures (SLI & SLII) and a Poly 'A' tract (Fig. 1). The long ORF consists of 4 regions i.e., L, P1, P2 and P3, encoding a large polyprotein. This protein is excised by the viral proteases to generate different structural and non-structural proteins. The L region encodes for 2 overlapping L-proteins, Lab & Lb. Four SPs are encoded by the P1 region and these proteins forms the capsomere of viral capsid and P2 and P3 region encodes for different Non-structural proteins (NSPs) which are concerned with the various functions associated with virus life cycle .

FMDV is commonly transmitted by direct exposure to the affected animals either by mechanical transfer of virus through damaged skin or intact mucous membrane or by aerosol transmission (droplet infection) (Biswal *et al*., 2012). It can also be transmitted indirectly through contaminated fomites. Ruminants are mostly acquiring the infection in the form of aerosolized virus through respiratory route while pigs are mostly infected by consumption of food contaminated with virus (Arzt *et al*., 2011b). After aerosol inoculation in cattle., early viral replication initiates at the nasopharyngeal epithelium overlying mucosal associated lymphoid tissue (MALT) particularly at the dorsal surface of soft palate and roof of pharynx, which are considered as the primary site of FMDV replication (Stenfeldt *et al*., 2015; Stenfeldt *et al*., 2018) but the tonsil plays a greater role in primary virus multiplication in sheep. However, in pigs, subsequent to oral exposure, primary infection occurs within the epithelial crypts of oro-pharyngeal and laryngo-pharyngeal tonsils. After entry into the animal body, the virus adheres to the host epithelial cells of nasopharynx (Schneider-Schaulies, 2000) by the cell membrane surface receptors (integrins) after which the virus is processed into the endosomal-lysosomal pathway, ultimately leading to mobilization of viral RNA into host cell cytoplasm. The entire replication cycle occurs in the cytoplasm and new virions are generated. After replication at the primary site, virus enters into the bloodstream (viremia) resulting in widespread distribution of FMDV in different organs and tissues, particularly the cornified stratified squamous epithelium of oral cavity and skin including the feet, teats and snout, which are the secondary sites of FMDV replication (Alexandersen *et al*., 2003). At these sites, the vesicular lesions appear due to massive amplification of the virus within the keratinocytes (Arzt *et al*., 2009). The predominant clinical signs of FMD include acute febrile reaction, sudden drop in milk yield, formation of vesicles at the secondary sites, ropy salivation, lameness etc. (Alexandersen *et al*., 2003).

FMDV infection elicits a rapid and strong humoral immune response where FMDV-specific neutralizing antibody appears in the serum within 4-7 days of exposure to virus (Eschbaumer *et al*., 2016; Stenfeldt *et al*., 2011) and the viremia is cleared with resolution of clinical symptoms within 2 weeks of infection. However, the virus persists for a prolonged period and multiply locally at the epithelial region of upper respiratory tract particularly at the pharyngeal region in approximately 50% of affected cattle, a condition known as 'FMDV persistent infection' or 'the carrier state'.

The carrier state

Approximately, half of the affected cattle, whether immunized or non-immunized, exhibit an asymptomatic persistence of FMDV in the oropharyngeal and nasopharyngeal epithelium after clinical recovery. This phenomenon is known as FMDV Persistence and the animals are known as FMDV carriers that are described as the animals from which FMDV can be isolated from oropharyngeal fluid (OPF) after 28 days of infection. In contrast to ruminants, pigs eliminate the infection within 21-28 days and don't develop carrier state (Stenfeldt *et al.*, 2016b; Parida *et al.*, 2007) except some studies where viral genome was identified in the blood from recovered swine and viral genome as well as viral proteins were identified in lymphoid tissues for 30-60 days post-

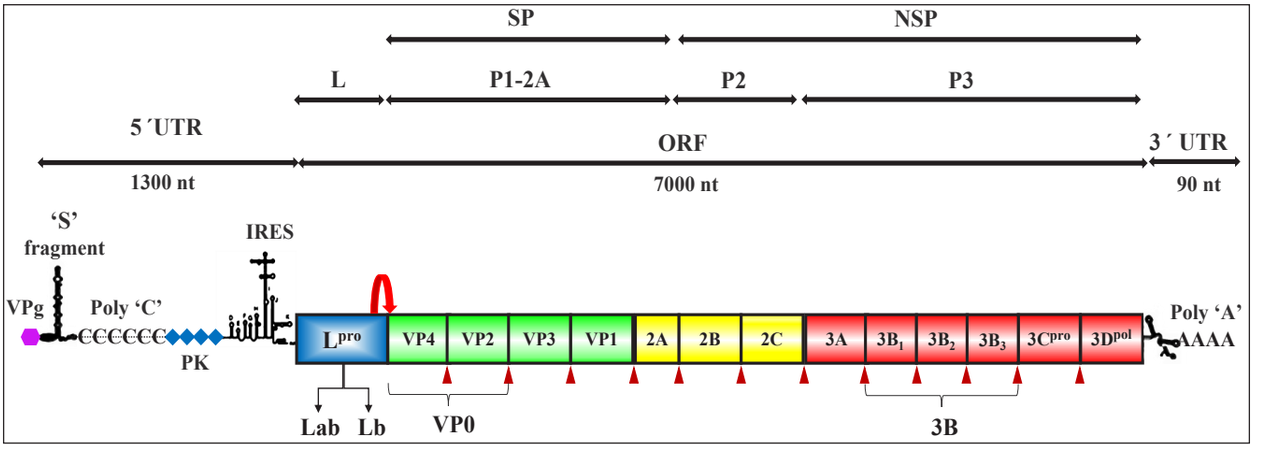

Fig. 1: Genome organization of FMD virus. The FMDV genome comprises 5´UTR, one large ORF and 3´UTR. The FMDV RNA is covalently linked to a small peptide called VPg (violet), that is made in 3 various forms (encoded by the $3B_{1-3}$) and each act as the primer for RNA synthesis. So, RNA genome is linked covalently to aVPg peptide after its synthesis. The 5´UTR consist of various structural elements viz. a small 'S' fragment, a poly C tract, pseudoknots (PK) and the internal ribosome entry site (IRES). The Open Reading Frame (ORF) encodes for the polypeptides P1-2A, P2 and P3, which are again cleaved by $3C^{pro}$ to yield 4 structural and 8 non-structural proteins. The cleavage sites of $3C^{pro}$ are indicated by red arrowheads. The 3´UTR includes 2 stem loop structures followed by a poly A tract. UTR: Untranslated region, SP: Structural protein, NSP: Non-structural protein

infection (Stenfeldt *et al.*, 2016b). Van Bekkum *et al.,* (1959) first documented the carrier state by demonstrating the evidence of virus in contagious form in the "saliva" or OPF by using probang cup from convalescent cattle, up to several months after infection. Later, many experiments have confirmed the persistence of virus in non-vaccinated as well as in vaccinated animals, irrespective of previously occurring clinical FMD (Pacheco *et al.*, 2015; Parthiban *et al.*, 2015a). FMDV persistency has been demonstrated to occur in cattle, sheep, goats, Asian buffalo and various wildlife species, most commonly in African buffalo (Weaver *et al.*, 2013). Very few studies have been carried out to determine persistence of FMDV in small ruminants. However, prevalence of FMDV persistence was shown to be higher in sheep as compared to goat (Arzt *et al.*, 2011a). Llamas (*Llama glama*) does not show FMDV persistence. Among wildlife species, African buffalo was found to be the most common species to become FMDV carrier in which the virus may persist for up to 5 years post infection (Alexandersen *et al.*, 2002) and has a major role in the virus circulation in ecosystems within buffalo population (Robinson *et al.*, 2016). Other wild ruminants such as deer and impala also get acute infection by the virus but they are less likely to develop carrier state (Bastos *et al.*, 2000). The period of virus persistence is influenced by host factors, strains and serotypes of infected FMDV and it varies from months to years (Biswal *et al.*, 2019; Tenzin *et al.*, 2008; Hayer *et al.*, 2018). The maximum duration of the carrier state in different species was reported as follows: cattle- 3.5 years, sheep- 9 months, goat- 4 months, African buffalo- 5 years (Bertram *et al*., 2018b). Occurrence of virus persistence in small ruminants is less frequent and for a shorter period lasting for 1 to 5 months only (Sutmoller and Olascoaga, 2002).

Anatomic localization of FMDV in carrier animals

Continued recovery of FMDV from OPF previously suggested the oro-pharynx as the site of FMDV persistence. However, many investigations on anatomic localization of FMDV persistence in cattle reported that the virus detection is confined to the nasopharyngeal tissue (Alexandersen *et al.*, 2002), retropharyngeal and submandibular lymph nodes (Juleff *et al.*, 2008; Zhang and Alexandersen, 2004). Presence of viral genome in the basal layer of dorsal soft palate epithelium was also confirmed by *in situ* hybridization (Zhang and Kitching, 2001). Identification of structural (VP1) and non-structural (3D) antigen in the epithelium of same site by immuno-microscopy in carrier cattle also evidenced the localization

of FMDV (Stenfeldt *et al.*, 2016a). From these evidences, it was concluded that the pharynx, especially, the dorsal soft palate and the epithelium adjoining the nasopharynx was the viral replication site in carrier cattle. In carrier sheep, virus was mostly recovered from tonsils than mucosal epithelium specifically in the crypts of epithelium within the oropharyngeal and laryngopharyngeal tonsils (Stenfeldt *et al.*, 2019). In African buffalo, pharyngeal tonsil, palatine tonsil and nasopharyngeal mucosa were the persistence site for FMDV (Maree *et al.*, 2016).

Mechanism of FMDV persistence

Various studies have been carried out to explain the mechanism of FMD virus persistence including host response to FMDV (Zhang *et al.*, 2004), viral antigenic variation due to gene mutation (Horsington and Zhang, 2007a) or differences in the innate immune response (Stenfeldt *et al.*, 2012). However, the interaction between innumerable virus and host factors in the persistent phase complicates the study on the mechanism of virus persistence (O'Donnell *et al.*, 2014). Immune system plays an important role in persistent infection in ruminants (Arzt *et al.*, 2011b). This fact was supported by the findings that cattle which receive a lesser vaccine dose are more often evidenced virus persistence and increasing the dosage of vaccine reduced the frequency of carriers in vaccinated cattle (Parida *et al.*, 2006; Parthiban *et al.*, 2015a). In contrast, many studies have also reported no variations in the frequency of virus persistence between vaccinated and naïve animals and immunization does not protect the animals from becoming carriers (Eschbaumer *et al.*, 2016; Bertram *et al.*, 2018a). The virus escapes the immune system mainly by interfering with the innate immune mechanism (Golde *et al.*, 2011) and alter the cytokine signalling pathways and/or employ a tissue specific reduced cell death mechanism and produce a different non-cytolytic infection (Zhu *et al.*, 2013; Pacheco *et al.*, 2015). This inhibitory effect on cytokine pathway results in malfunctioning of antigen presenting cells (Grubman *et al.*, 2008). Similar to other viral disease, acute phase of FMDV infection provoke proinflammatory as well as innate immune responses (Stenfeldt *et al.*, 2012; Arzt *et al.*, 2014; Perez-Martin *et al.*, 2012; Zhu *et al.*, 2013;) where as in the persistent phase, FMDV suppress or modulate the expression of many cytokine genes like IFN-α/β, IL-12 and RANTES (Pacheco *et al.*, 2015). While comparing the RNA levels of cytokines and toll-like receptor in lymphoid tissues of dorsal soft palate, Zhang *et al.* (2006) found significantly higher TNF-α RNA in carrier animals whereas all other genes of cytokines and Toll-like receptors had similar expressions in both carrier and non-carrier cattle. Another study reported a substantial reduction in detection of viral antigen and genome in FMDV persistently infected cells treated with IFN-γ, which suggested that this cytokine may have some function in virus clearance in vivo (Zhang *et al.*, 2002; Oh *et al.*, 2012). A proposed mechanism for strong predilection of FMDV in dorsal soft palate and dorsal nasopharyngeal region was that these sites have no layer of dead cells on their epithelium i.e., they are non-cornified and if they possess the suitable receptors may enhance the entry of virus which would be very effective in virus uptake and also may have significant importance in virus persistence (Alexandersen *et al.*, 2002). It was also proposed that during persistent infection, the virus may able to suppress the activation of CD8+ T cells which when activated recognises the infected cells (Alexandersen *et al.,* 2002) or may prevent the detection of virus affected cells by downregulation of expression of MHC class I which prevents the normal cytolytic action and creates an intracellular environment encouraging for prolonged virus persistence. However, no other report was found in the support of this mechanism. Antigenic variation due to viral gene mutation may be a factor for the development of persistent phase. This antigenic variation along with FMDV capsid protein mutation may be a reason for inhibition of virus clearance by IgA in OPF of carrier animals (Arzt *et al.*, 2011b). VP2 region of FMDV is highly immunological and mutation within these sites affect the antigenicity of the viral protein and may be a factor for FMDV persistence. This was supported by Horsington and Zhang (2007b), who confirmed that substitution in the B-C loop of VP2 may be involved with virus persistence. A lot of evidences suggested that the factors associated with the animal's immune response are the chief determinants of FMDV persistence. However, the mechanisms associated with the establishment, maintenance and resolution of the virus persistence remain undermined and requires more detailed study.

Host Response to FMDV infection

FMDV infection provokes a rapid humoral as well as cell

mediated immune response which is serotype and strain specific and induce immunity to counter there-infection with viruses which are identical in their antigenicity with the previously infected viruses. Early FMDV infection is associated with a rise in B-lymphocyte derived IgM as the first neutralizing antibody within 3-4 days of infection and this antibody response peaks at 10-14 days post-infection (Windsor *et al.*, 2011; Juleff *et al.*, 2009; Golde *et al.*, 2008) and then declines. This transient IgM response is followed by a sustained IgG antibody response, detected first at 4-7 dpi and turn out to be the predominant neutralizing antibody by 2 weeks post infection (Eschbaumer *et al.*, 2016). IgG1 antibody response is higher than IgG2 in both vaccinated as well as naturally infected animals. These immune cells bind to the virus surface and enhances the opsonization and engulfment by the phagocytic cells present in the secondary lymphoid organs and quickly eliminate viremia. The CD4+ helper T-cells recognises the epitopes in structural and non-structural antigens and induce B-cell activation and antibody production (Blanco *et al.*, 2001). It also contributes to maintain a suitable microenvironment essential for a collaborative immune response (Sobrino *et al.*, 2001).

Stenfeldt *et al.* (2017) found a greater quantity of CD3+ and CD8+ lymphocytes in the nasopharyngeal mucosa in the cattle that had recently eliminated the infection in comparison to persistently infected cattle. There are also evidences of transient increase in interferon activity (IFN-I/III) in concurrence with viremia in acute FMDV infection (Eschbaumer *et al.*, 2016; Stenfeldt *et al.*, 2011; Windsor *et al.*, 2011; Arzt *et al.*, 2014). Additionally, there is also an initial rise in the concentration of acute phase proteins (haptoglobin and serum amyloid A) in serum, which return towards normal during the carrier state (Stenfeldt *et al.*, 2011). However, the antibody mediated response along with phagocytosis clears the viremia whereas direct cell-mediated response effectively clears the intracellular virus from the epithelial tissues (Stenfeldt and Arzt, 2020).

Genetic and antigenic variations in FMD Carriers

Antigenic variation act as a major complication in control and eradication of FMD as it is responsible for the failure of cross-protection among the serotypes and sometimes among the strains within the serotypes (Brito *et al.*, 2013; Ramirez-Carvajal *et al.*, 2018). There are many evidences of genetic and antigenic variations in the FMDV isolated from carrier animals (Farooq *et al.*, 2018; Pauszek *et al.*, 2017; Arzt *et al.*, 2019). However, many studies have reported no definite mutations in the viral RNA were constantly linked with FMDV persistence (Parthiban *et al.*, 2015a; Arzt *et al.*, 2019; Ramirez-Carvajal *et al.*, 2018; Parthiban *et al.*, 2015b). Alterations in FMDV genome affecting its antigenicity occurs continuously during persistent infection (Bertram *et al.*, 2018b; Biswal *et al.*, 2019) but such changes leading to evolution of novel viral lineages are unknown (Cortey *et al.*, 2019). The factors responsible for significant genomic variations are lesser reliability of the RNA replication apparatus (Alexandersen *et al.*, 2002), absence of constraints on certain segments as well as potential selection mechanism on other segments (Knowles *et al.*, 2001). Recombination among several FMDV RNA replicating within a particular cell is also a contributing factor for genetic variation but its role in generating in vivo variants has very limited evidence. One example of this type of recombinant is O1 Burgwedel strain in 1987 which was reported due to recombination between O1 Kaufbeuren (vaccine strain) and a C1 type. Two investigations have recognized 2 distinct amino acid substitution viz. VP1 Q172R and VP2 Y80H constantly in the viruses isolated from carrier animals (Horsington and Zhang, 2007a; Pauszek *et al.*, 2016). Selection pressure is a major factor in viral evolution is which may vary from species to species or may vary in different stages of infection viz. acute clinical infection and persistent infection, within the same host (Brito *et al.*, 2017). Many studies described FMDV as a quasispecies, described as a group of viruses driven by various selection pressures (Sierra *et al.*, 2000). One study reported the presence and absence of virus populations combined with substitution at the major antigenic site indicating the occurrence of antigenic selection process in carrier animals (Arzt *et al.*, 2019). Biswal *et al*. (2019) isolated genome sequences of FMDV from carrier cattle and buffalo and performed the phylogenetic and parsimony analysis. He demonstrated separate clustering of cattle derived isolates from buffalo derived isolates suggesting the species-specific selection pressure on evolution of FMDV. Another result from this study was, buffalo derived isolates descended in a single lineage from the outbreak virus whereas cattle derived isolates descended in five separate lineages from the outbreak virus which suggested differential selection pressures between the host species (Biswal *et al.*, 2019).

Although many studies have investigated about the variation in genome and antigenicity of FMDV during the carrier state but the contribution of viral genomic alterations to the establishments and continuance of persistent infection are still unclear. However, the developments in next generation sequencing as well as advanced bioinformatics tool may clarify the phenomena in future.

Transmission of FMDV from carrier to healthy animals

Some historical field outbreaks studies were thought to have been originated due to spreading of viruses from a persistently infected animal to a susceptible animal (Horsington and Zhang, 2007a; Pauszek *et al.*, 2016; Brito *et al.*, 2017). Arzt *et al.* (2018) in his experiment reported the development of clinical FMD in naïve cattle in which unprocessed OPF from persistently infected cattle was inoculated. However, many experimental studies could not establish the disease transmission from carrier animals to susceptible sentinels (Bertram *et al.*, 2018a; Tenzin *et al.*, 2008; Parthiban *et al.*, 2015a). The suspicion for transmission was aroused due to an outbreak in FMD free areas after introduction of healthy convalescent cattle from region with previous history of FMD outbreak and also from some evidences of subclinical infection of neonatal calves born to carrier cattle. In UK, FMD epizootic had occurred in 1922 to 1924 due to migration of a clinically recovered bull and a heifer from a farm where FMD outbreak had occurred 8 months before, into a district where no disease was observed (Sutmoller and Olascoaga, 2002). Involvement of carrier cattle in FMD outbreak in Zimbabwe caused by SAT-2 serotype was also evidenced. However, it may be unique to SAT serotype viruses and was not reported for other serotypes (Sutmoller and Olascoaga, 2002). Recently, Ranjan *et al.* (2018) detected serotype 'O' FMDV RNA by RT-mPCR in the OPF from six out of twelve healthy calves of 6 months age which were born from carrier cattle in two dairy farms in India.This silent infection in calves might have occurred because of vertical transmission of virus during pregnancy (Ranjan *et al.*, 2016b) or due to horizontal infection from carrier dams. Several OPF sampling of vaccinated Asian buffalo (*Bubalus bubalis*) in 30 livestock farms during a period of 12 months in Pakistan have reported multiple introductions of different serotypes and lineages in all the farms with no noticeable clinical signs, which indicated the subclinical virus circulations together with new strain introduction without exhibition of clinical FMD (Farooq *et al.*, 2018). This result was well correlated with the findings of other studies which had reported a greater prevalence of FMDV in OPF of cattle and Asian buffalo in the absence of clinical FMD (Klein *et al.*, 2008; Jamal *et al.*, 2012). Bertram *et al.* (2018a) during his study in 2018 also found similar kind of observation where carrier cattle were sub-clinically infected with new strains of FMDV in Vietnam and this superimposed subclinical infection had a significant role in prolongation of carrier state. A general observation from different studies is that transmission of FMDV from carrier African buffalo to susceptible species is more frequent than transmission from carrier cattle. Some studies have reported the transmission of disease from persistently infected African buffalo to cattle in Zimbabwe, both naturally, in outbreaks of 1989 and 1991 with SAT-1 serotypes as well as experimentally (Stenfeldt and Arzt, 2020). Carrier African buffalo bulls transmitting the disease sexually to domestic cows was reported, where SAT serotype viruses were also isolated from the semen as well as sheath washes of an infected African buffalo and the virus in the sheath wash isolated from the prepucial epithelium.

Many researchers had performed controlled experiments to demonstrate the initiation of clinical disease by carrier but the disease transmission could not be established by close contact exposure of susceptible animals with carrier cattle (Sutmoller and Olascoaga, 2002). However, one recent study reported that harvesting of oropharyngeal fluid from carrier animals and deposition of these fluid into the nasopharyngeal region of naïve cattle caused fulminating FMD (Arzt *et al.*, 2018).

The apparent lack of contagiousness of virus in carrier cattle may be due to neutralization effect of surface immunoglobulins on virus released in OPF. This hypothesis was strengthenedby demonstration of increased infectivity of virus in tissue culture of OPF samples treated with fluorocarbon compound (Trichlorotrifluoroethane, TTE) that separateantibodies that attached to virus (Stenfeldt *et al.*, 2016a). Although, carrier cattle shed very low amount of virus in OPF, evidences on the contagiousness of the virus associated with carrier animals have justified the significance of carrier state at the individual animal level (Stenfeldt and Arzt, 2020).

Economic importance of FMDV Carrier state

The transmission of FMD from carrier animal to susceptible livestock remained controversial. The countries must demonstrate a complete clearance of FMDV source to gain FMD-free status as per the international regulations on FMD laid by OIE (Afonso *et al.*, 2012). Thus, globally FMDV carrier state is considered as a danger to the livestock sector because the persistence of FMDV complicates the retrieval of FMD-free status. This is the reason for which FMD-free countries usually impose trade restrictions on livestock and livestock products from countries or zones where circulation of FMDV is still evidenced (Pacheco *et al.*, 2015). However, the global trade restriction imposed on FMDV persistence is mainly due to two considerations. First, the risk of transmission from asymptomatic carriers and trade policies considers the carriers as contagious, a reason for which FMD-free countries restricts import of livestock from enzootic zones. Second, the FMD-free countries slaughter the exposed animals when come across with invasion of FMDV, as vaccination does not prevent the carrier state. This trade embargo prohibits the developing and under developed countries from participating in global trade for livestock and livestock products in international markets (Knight-Jones and Rushton, 2013). With regard to infectiousness, the within host evolution of FMDV strains during persistent infection is also a possible hazard which may generate novel virus variants that may undermine the host immunity to initiate a new outbreak (Biswal *et al.*, 2019). This is one reason for depopulating the affected as well as the vaccinated cattle by the FMD-free countries for control and eradication of FMD. Depopulation, quarantine and global trade restrictions have a strong financial effect on endemic countries with no assurance of prevention of disease transmission to FMD-free countries (Mansley *et al.*, 2011; Bertram *et al.*, 2018a).

DETECTION OF FMD CARRIER ANIMALS

In order to make a country or a zone FMD free, the extinction of carrier state is essential, as the country must evidence a complete clearance of virus source. For this, identification of FMD carrier animals subsequent to an outbreak or during livestock import and export, is a preliminary step which can be done either by detection of viral agents (antigen/genome) or antibodies specific to FMDV.

Viral agent detection

The World Organisation for Animal Health recommended various methods for the detection of FMD viral agents (antigen/genome) in FMD affected animals. However, in carrier animals, virus isolation (VI) method is most commonly used for viral antigen detection and nucleic acid recognition methods (RT-PCR, Real time RT-PCR etc.) for viral genome detection.

Virus Isolation (VI)

Presently, the live FMDV in clinical sample is only detected in cell culture through virus isolation (VI) (Ranjan *et al.*, 2016a). Live virus in OPF samples from carrier animals can also be isolated using the same technique. Various cell culture systems of bovine, ovine and porcine origin may be used to grow FMDV (Biswal *et al.*, 2012). Primary bovine thyroid (BTY) cells are highly sensitive to different serotypes but its preparation is difficult and expensive, and also it loses FMDV susceptibility after multiple passages. BTY cells are more sensitivity to the FMDV than primary lamb kidney (LK) cells but the LK cells are susceptible to infection even after cryopreservation (Biswal *et al.*, 2012). Immortalized cell lines such as baby hamster kidney (BHK-21) cells and IB-RS-2 cells, can also use but they have a low sensitivity than primary cells (Ferris *et al.*, 2006). However, development of LFBK-αvβ6 stable line was an outstanding cell line for isolation of FMDV (LaRocco *et al.*, 2013) which can be used for diagnosis of FMD carrier animals by isolating the virus from oro-pharyngeal epithelium. The cytopathic effect (CPE) develops and detected within 48 hours in cell culture medium. If no CPE is demonstrated within 48 hours, then fresh cell culture medium should be taken and inoculated with cell lysate after freezing and thawing, and again the CPE is detected after another 48 hours. In OPF samples, pre-treatment with an equal volume of fluorocarbons such as trichloro trifluoroethane (TTE) mediates the release of antibody complexed virus and improves the infectivity of OPF in cell culture, thus increasing the rate of virus detection. However, this is a time-consuming method and requires about 2-4 days to isolate the virus (Sharma *et al.*, 2015). Additionally, the difficulties accompanying with maintaining a constant supply of cell lines and cell culture contamination are major drawbacks of this method of viral antigen detection (Jamal and Belsham, 2013).

Nucleic Acid Recognition (NAR) methods

Reverse transcription-polymerase chain reaction (RT-PCR) can be used to amplify the FMDV genome in clinical samples like OPF (Ranjan *et al.*, 2014) because of its quick, highly sensitive and reliability in diagnosis of FMD carrier animals. Primers specific for different serotypes which target different regions of viral genome including the 5′ UTR, ORF and the 3′ UTRwere designed for the detection of all seven serotypes of FMDV by RT-PCR. However, none of these primers can detect the disease and capable of virus typing when evaluated on a number of samples which represents all the seven serotypes (Jamal and Belsham, 2013).

To enhance the diagnostic sensitivity, multiplex RT-PCR (RT-mPCR) was developed by incorporating multiple sets of forward primers specific to different serotypes which target the 1D region and a common reverse primer (NK61) that targets the 2B region (Bao *et al.*, 2008; Mohapatra *et al.*, 2011b). FMDV persistence and multiplication in oro-pharyngeal region can also be evidenced by detecting its genome in OPF using RT-mPCR technique. However, the major drawback associated with this technique is the generation of false positive results because of carry-over of PCR amplicons (Hoffmann *et al.*, 2009). To avoid cross contamination and for more feasibility of the test, a thermo-stable RT-PCR mixture was developed (Sharma *et al.*, 2014a) where the constituents of the reaction mixture were mixed in a vial and lyophilized. Nuclease free water is added to these vials before use. Recently, another type called direct boil RT-mPCR was developed where the clinical sample was mixed with DEPC treated ultra-purified water and boiled at 95°C in order to release the viral RNA (Biswal *et al.*, 2017). This method of RNA extraction was found to be an alternative to commercial RNA extraction kit and reduces the cost of diagnosis.

A one step, reverse transcription loop-mediated isothermal amplification assay (RT-LAMP) targeting the 3D region of FMDV genome was developed that enabled the FMDV to be detected within an hour without thermal cycling (Ranjan *et al.*, 2014; Madhanmohan *et al.*, 2013). Recently, one study modified the previously described RT-LAMP assay where an internal positive control (IPC) was developed and incorporated to alleviate interference of non extracted field samples and reduces false negatives.

Real-time PCR is now considered as the gold standard in nucleic acid detection due to its enhanced rapidity, higher analytical sensitivity, higher quantitative performance, absence of post-PCR processing i.e., electrophoresis etc. DNA-binding fluorophores, the 5' endonuclease, adjacent linear and hairpin oligoprobes, and the self-fluorescing amplicons are 5 important components of real-time PCR which enable to amplify and quantify a specific nucleic acid sequence simultaneously (Mackay *et al.*, 2002). The primers and probes which are unique to a highly conserved region in the P3 gene were used in the first universal rRT-PCR which has reached an analytical sensitivity at 1×10^2 TCID50 for all the serotypes. Several types of real-time RT-PCR (Moonen*et al.*, 2003; Saduakassova *et al.*, 2018) and portable real-time RT-PCR (Donaldson *et al.*, 2001; Hearps *et al.*, 2002) were also developed to detect the viral genome. Although OIE Terrestrial Manual, 2018 presently suggests the utilization of 'universal' primers and probes that target the conserved segments within 5′UTR (Reid *et al.*, 2001) or 3D regions (Callahan *et al.*, 2002), the assays specific to different serotypes were also created (Reid *et al.*, 2014; Bachanek-Bankowska *et al.*, 2016). Reid *et al.* (2014) carried out 3D and 5′UTR rRT-PCRs using various samples with FMDV O, A, and Asia-1 serotypes and FMD negative samples and demonstrated no-false positive results and a detection rate of 91% and 96% in these tests, respectively. The diagnostic performance of 5′UTR and 3D rRT-PCR was also examined in many experiments. The real-time quantitative PCR (RT-qPCR) assay has been used as the primary diagnostic test for the detection of FMD carrier animals and has a greater role in the disease control programme (Zhang and Alexandersen, 2003).

Antibody detection

Other than the detection of viral antigen or genome, FMDV persistence can also be well diagnosed by the detection of antibody response by serological test, particularly during post-outbreak period (Rémond *et al.*, 2002). These tests for FMD are carried out for certification of animals before import or export, confirmation of suspected cases, demonstration of vaccine efficacy and to evidence non-existence of infection (OIE, 2018). The serological tests are performed to detect antibodies against FMDV structural proteins (SP) and non-structural proteins (NSP).

Detection of antibody against Structural Protein of FMDV

The test to identify antibodies against SP are specific

to different serotypes and identifies antibodies induced by both natural infection as well as vaccination. Virus neutralization test (VNT) is the most common method recommended for carrier animal detection which detects antibodies against FMDV structural protein.

Virus Neutralization Test (VNT)

VNT recognizes the antibodies against structural protein of FMDV and is specific to different serotypes. This test is recommended for screening of FMD carrier status while certifying the animals or animal products prior to export/import (OIE, 2018). A variety of cell lines can be used in VNT, but the BHK or IBRS-2 cells give superior results than PK-2 cells (Moonen *et al.*, 2004). VNT is slow, labour intensive and subject to contamination. A high-level bio=containment facility is also required for the test to be carried out, which is often an expensive obstacle to eliminate (Poonsuk *et al.*, 2018). The ELISA is advantageous over VNT because ELISA is rapid, inactivated antigens can be used, and can also be carried out using small volumes of post-vaccination sera (Paton *et al.*, 2005).

Detection of antibody against Non-Structural Protein of FMDV

Identification of FMDV infected animals is critically essential in control and eradication of the disease particularly for those countries which are free from FMD or with sporadic outbreak. It is important to differentiate between recently vaccinated animals from those animals which were previously infected with FMDV for trade purpose, as approximately 50% of the infected animal exhibit virus persistence and become carrier animals. These animals are not reliable as they can be act as a nidus of a new outbreak, although transmission from carriers to naïve animals has not been demonstrated experimentally. Multiplication of virus during the infection induces the generation of antibodies in response to structural as well as non-structural proteins because similar to SPs, some NSPs (mainly proteases and RNA polymerases) are also immunogenic (Rémond *et al.*, 2002). Although, detection of FMDV specific antibody against the structural proteins can be suitable for diagnosis, but there should be no history of vaccination as purified vaccine induces antibody production in response to the structural proteins only. Thus, detection of NSP specific antibody is preferred to differentiate infected from vaccinated animals (DIVA). Earlier, the assays used for the recognition of NSP specific antibodies depend on radioimmuno-precipitation or enzyme linked immune-electrotransfer blot assays. However, many ELISA have been developed and preferred over these assays as they are not suitable for quick screening of samples in large numbers. An immune-diffusion test using the viral polymerase also called as virus infection associated (VIA) was the first test to be developed for DIVA. However, after repeated vaccination with conventional vaccine, animals may develop antibody to VIA antigen. Therefore, a number of antigenic NSPs like 3ABC, 3AB, 3A, 3B, 3C, 2A, 2B, 2C were identified and used as potential antigen in various types of ELISAs (Poonsuk *et al.*, 2018). The leader protease (L protein) is not preferred for diagnosis purpose as it is very less immunogenic where as other NSPs are used. Among all the NSPs, 3ABC gene seems to be the most trustworthy indicator of FMDV replication (Grubman, 2005; Henderson, 2005) and detection of antibodies against this NSP was found very sensitive as well as specific tool for DIVA assessment while compared with the immuno-blotting test (EITB) against 5 bioengineered NSPs viz. 3A, 3B, 2C, 3D and 3ABC (Bergmann *et al.*, 2000). However, this OIE recommended assay for identification of antibodies against NSP is available only from PANAFTOSA, Brazil to the laboratories South American. A competitive ELISA to detect NSP specific Abs was designed using a recombinant 3ABC protein (antigen) and the 3B-specific mAb (Yang *et al.*, 2015). Chitray *et al.* (2018) developed a SAT-serotype specific 3ABC-NSP ELISA for DIVA assessment. In addition to 3ABC ELISAs, many NSP assayswere developed which identifies the antibodies against recombinant 2B (Biswal *et al.*, 2014), 3AB (Mohapatra *et al.*, 2011a), 3B (Chung *et al.*, 2018), and 3D (Mahajan *et al.*, 2014). Some in-house DIVA assays including 1 competitive and 4 indirect ELISAs using 3AB3, 3ABC, and truncated 2C (2Ct) were designed in India (Mahajan *et al.*, 2013; Mohapatra *et al.*, 2011a; Sharma *et al.*, 2012) and their performance was compared with the two commercially available ELISA kits (PrioCheck® FMDV-NS and Svanovir FMDV 3ABC-Ab ELISA kit). These in-house DIVA assays were equally effective in differentiating infected individuals from the vaccinated population (Sharma *et al.*, 2014b). In one study, all three copies of 3B NSP were expressed in a prokaryotic system and an indirect ELISA was developed

(r3B I-ELISA) which has a diagnostic sensitivity and specificity of about 92.1% and 98.1%, respectively (Mohapatra *et al.*, 2014). In another experiment, an indirect ELISA was designed to identify the antibodies against 3B NSP in infected cattle with 97% sensitivity but a lower specificity (80%) than in-house r3AB3 I-ELISA (Mahajan *et al.*, 2015).

Mucosal antibody detection

IgA is the secretory and excretory antibody (Ranjan *et al.*, 2016a) and is the main antibody isotype in saliva and probang sample. Previously, it has also been recognized that the mucosal antibody i.e., IgA was secreted predominantly from the pharyngeal mucosa in animals exposed to live FMDV. FMDV infected cattle regularly mount an IgA antibody response in saliva or oro-pharyngeal fluid, in contrast, this antibody is not produced after vaccination (Parida *et al.*, 2006). Similar findings were also reported in pigs where parenterally immunized pigs do not produce mucosal antibodies, in contrast, they elicit a greater level of antibody response if get infected subsequent to vaccination (Pacheco *et al.*, 2010). However, one experimental study demonstrated a strong IgA response (local and systemic) after immunizing pigs with a dendrimeric peptide (B_4T) that contains a single copy of FMDV T-cell epitope and branched out into 4 copies of a B-cell epitope (Cubillos *et al.*, 2008). Jain *et al.* (2019) developed an IgA ELISA using field samples and found that more carrier animals were detected by this assay in comparison to qRT-PCR. Recently, Biswal *et al.* (2021) developed and validated a mucosal IgA ELISA for detection of carrier animals with enhanced level of sensitivity and specificity with detection level of 96.9%. Thus, an assay detecting IgA are suitable to conduct sero-surveillance to know about the virus circulation as well as for detecting FMDV persistently infected animals (Biswal *et al.*, 2008; Jain *et al.*, 2019).

Detection of FMDV in the OPF collected through the probang cup

Previously it had been well established that FMDV multiply locally in the nasopharyngeal and oropharyngeal region. Van Bekkum *et al.* (1959) first documented the carrier state by demonstrating the existence of infectious virion in the "saliva" or oesophageal-pharyngeal fluid by using probang cup from convalescent cattle, up to several months after infection. Later, this probang cup was modified, standardized and used in an optimized approach to detect FMDV in oropharyngeal fluid from carrier cattle. Since then, this approach was followed for the detection of carrier status in animals by testing this OPF for live virus, by VI or Viral genome detection by PCR.

Physiological consideration of OPF collection

When the animal respires, the soft palate is placed below the anterior end of epiglottis (Fig. 2A). This placing of the palate creates a hollow passage in the respiratory tract and the air from the nasal cavities is pass via nasopharyngeal regions and enters into the trachea and lungs. However, in swallowing animals, there is realignment of structures due to the swallowing reflex where the soft palate is deviated dorsally and the epiglottis blocks the tracheal entrance (Fig. 2B). This realignment of structures leads to creation of a passage for feed from the mouth cavity into the oesophageal opening (Stenfeldt and Arzt, 2020).

The probang cup is inserted into the oral cavity for collection of OPF, which stimulates a swallowing reflex and allowing the entry of the metallic probang cup to the dorsal nasopharynx and caudo-dorsal soft palate (Fig. 2C). The mucosal epithelium of the dorsal part of nasopharynx and the posterior end of dorsal soft palate is scrapped by repeated insertion and retraction of the probang cup. The scrapping of this region can be evidenced by visualizing the bruise at the posterior aspect of soft palate after post-mortem examination of the animal. Sampling using a probang cup is a semi-invasive process, because it is associated with the scrapping of epithelial mucosa with the edges of the metal cup.

Limitation of carrier animal detection from OPF collected through probang cup

Although this approach of OPF collection followed by virus isolation and viral genome detection is widely used for detection of FMDV carrier animals, this approach encounters with various major constraints viz. discontinuous release of virus as well as the low virus titres, release of virus-antibody complexes in OPF which limits virus isolation, chances of getting false negative results due to virus inactivation while sampling and shipment to the laboratory, and the carrier state might be simulated, in that FMDV RNA can be encapsidated into coat proteins of

bovine enteroviruses. Furthermore, the collection of OPF using probang cup is a cumbersome process and also it is not practicable to test all the OPF samples while screening a large animal population by virus isolation and PCR (Biswal *et al.*, 2022).

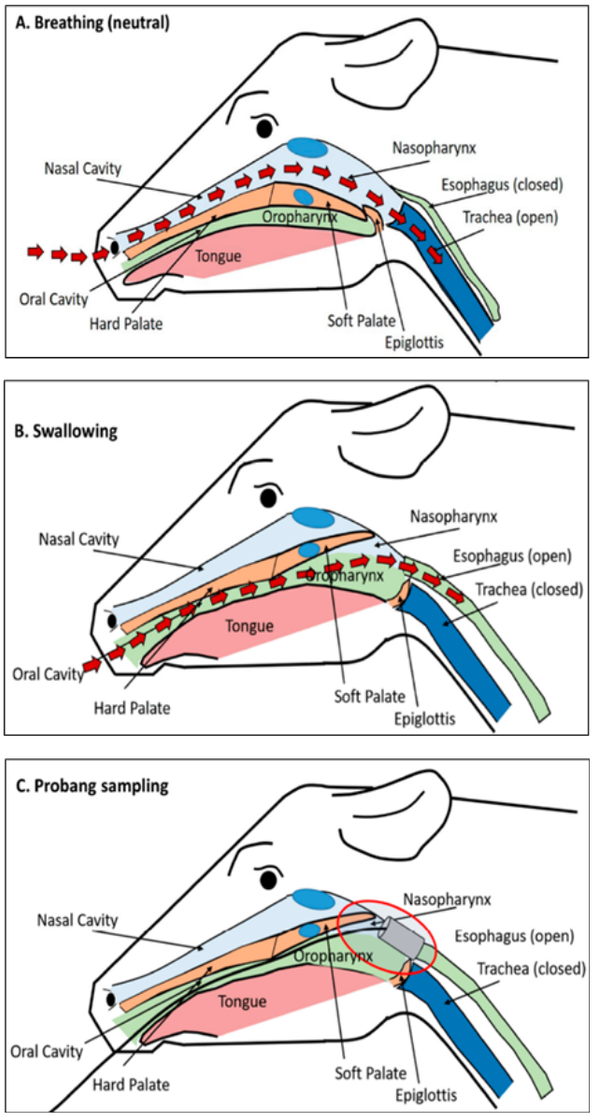

Fig. 2: Anatomic and physiologic considerations of detection of FMDV in probang samples adopted from Stenfeldt and Arzt, 2020. **(A)** During breathing, the posterior part of the soft palate is placed below the tip of the epiglottis creating an open path for passage of air from the nasal cavity into lungs (path of red arrows). **(B)** During swallowing, the soft palate is elevated towards the dorsal nasopharynx, the epiglottis closes so that feed can pass from the oral cavity into the opening of oesophagus. **(C)** Insertion of probang cup into the oral cavity stimulates a swallowing reflex which allows access of the metal cup to the dorsal nasopharynx as well as the caudo-dorsal soft palate

FUTURE PROSPECTIVE

FMD carrier animals have been remained as a constraint in the international market during export of livestock and livestock products. It jeopardizes the declaration of FMD free status as it evidenced virus circulation and remained as a source of infection. Therefore, detection and removal of the FMD carrier animals in a geographical area is necessary. A more sensitive and specific diagnostic assay should be developed which can overcome the limitations of conventional methods used nowadays. More research can be done to develop serological assays which can identify the antibodies of acute infection against the non-structural protein of FMDV. A comprehensive eradication policy should be suggested to the government authority to remove the carrier animals from the herds, so that a virus free geographical area can be maintained.

CONCLUSION

Approximately, half of the FMD infected animals remain as carriers in which the virus persists and multiply in the oro-pharyngeal epithelium. Although transmission of the virus has not been demonstrated under experimental condition, substantial field evidences suggested the virus in carrier animals as infective and few field outbreak has been proposed to be originated from FMD carrier animals. Therefore, FMDV persistency is considered as a threat to livestock economy and the FMD free countries put trade barriers on the enzootic countries. This article reviews some anatomic, physiologic, and economic impact of FMDV persistency and focuses more on its diagnosis. Thus, FMD carrier animals must be considered as threat in FMD endemic countries, and should be detected and removed to maintain a virus free country.

REFERENCES

Afonso, C.L., Miller, P.J., Grund, C., Koch, G., Peeters, B.P., Selleck, P.W. and Srinivas, G.B. 2012. Manual of diagnostic tests and vaccines for terrestrial animals. *World Organization for Animal Health, Paris, France*.

Alexandersen, S., Zhang, Z. and Donaldson, A.I. 2002. Aspects of the persistence of foot-and-mouth disease virus in animals—the carrier problem. *Microbes Infect*., **4**(10): 1099–1110.

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

Arzt, J., Baxt, B., Grubman, M.J., Jackson, T., Juleff, N., Rhyan, J., Rieder, E., Waters, R. and Rodriguez, L.L. 2011a. 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**(4): 305–326.

Arzt, J., Belsham, G.J., Lohse, L., Bøtner, A. and Stenfeldt, C. 2018. Transmission of foot-and-mouth disease from persistently infected carrier cattle to naive cattle via transfer of oropharyngeal fluid. *Msphere*, **3**(5).

Arzt, J., Fish, I., Pauszek, S.J., Johnson, S.L., Chain, P.S., Rai, D.K., Rieder, E., Goldberg, T.L., Rodriguez, L.L. and Stenfeldt, C. 2019. The evolution of a super-swarm of foot-and-mouth disease virus in cattle. *PloS one*, **14**(4): e0210847.

Arzt, J., Gregg, D.A., Clavijo, A. and Rodriguez, L.L. 2009. Optimization of immunohistochemical and fluorescent antibody techniques for localization of Foot-and-mouth disease virus in animal tissues. *J. Vet. Diagn. Investig.*, **21**(6): 779–792.

Arzt, J., Juleff, N., Zhang, Z. and Rodriguez, L.L. 2011b. The pathogenesis of foot-and-mouth disease I: viral pathways in cattle. *Transbound. Emerg. Dis.*, **58**(4): 291–304.

Arzt, J., Pacheco, J.M., Smoliga, G.R., Tucker, M.T., Bishop, E., Pauszek, S.J., Hartwig, E.J., De Los Santos, T. and Rodriguez, L.L. 2014. Foot-and-mouth disease virus virulence in cattle is co-determined by viral replication dynamics and route of infection. *Virology*, **452**: 12–22.

Bachanek-Bankowska, K., Mero, H.R., Wadsworth, J., Mioulet, V., Sallu, R., Belsham, G.J., Kasanga, C.J., Knowles, N.J. and King, D.P. 2016. Development and evaluation of tailored specific real-time RT-PCR assays for detection of foot-and-mouth disease virus serotypes circulating in East Africa. *J. Virol. Methods*, **237**: 114–120.

Bao, H.-F., Li, D., Guo, J.-H., Lu, Z.-J., Chen, Y.-L., Liu, Z.-X., Liu, X.-T. and Xie, Q.-G. 2008. A highly sensitive and specific multiplex RT-PCR to detect foot-and-mouth disease virus in tissue and food samples. *Arch. Virol.*, **153**(1): 205–209.

Bastos, A.D.S., Boshoff, C.I., Keet, D.F., Bengis, R.G. and Thomson, G.R. 2000. Natural transmission of foot-and-mouth disease virus between African buffalo (*Syncerus caffer*) and impala (*Aepyceros melampus*) in the Kruger National Park, South Africa. *Epidemiol. Infect.*, **124**(3): 591–598.

Bergmann, I.E., Malirat, V., Neitzert, E., Beck, E., Panizzutti, N., Sánchez, C. and Falczuk, A. 2000. Improvement of a serodiagnostic strategy for foot-and-mouth disease virus surveillance in cattle under systematic vaccination: a combined system of an indirect ELISA-3ABC with an enzyme-linked immunoelectrotransfer blot assay. *Arch. Virol.*, **145**(3): 473–489.

Bertram, M.R., Delgado, A., Pauszek, S.J., Smoliga, G.R., Brito, B., Stenfeldt, C., Hartwig, E.J., Jumbo, S.D., Abdoulmoumini, M. and Marie, A.A.O. 2018a. Effect of vaccination on cattle subclinically infected with foot-and-mouth disease virus in Cameroon. *Prev. Vet. Med*, **155**: 1–10.

Bertram, M.R., Vu, L.T., Pauszek, S.J., Brito, B.P., Hartwig, E.J., Smoliga, G.R., Hoang, B.H., Phuong, N.T., Stenfeldt, C. and Fish, I.H. 2018b. Lack of transmission of foot-and-mouth disease virus from persistently infected cattle to naïve cattle under field conditions in Vietnam. *Front. Vet. Sci.*, **5**: 174.

Biswal, J.K., Jena, B.R., Ali, S.Z., Ranjan, R., Mohapatra, J.K. and Singh, R.P. 2022. One-step SYBR green-based real-time RT-PCR assay for detection of foot-and-mouth disease virus circulating in India. *Virus Genes*, **58**(2): 113-121.

Biswal, J.K., Jena, S., Mohapatra, J.K., Bisht, P. and Pattnaik, B. 2014. Detection of antibodies specific for foot-and-mouth disease virus infection using indirect ELISA based on recombinant nonstructural protein 2B. *Arch. Virol.*, **159**(7): 1641–1650.

Biswal, J.K., Nardo, A.D., Taylor, G., Paton, D.J. and Parida, S. 2021. Development and Validation of a Mucosal Antibody (IgA) Test to Identify Persistent Infection with Foot-and-Mouth Disease Virus. *Viruses*, **13**(5): 814.

Biswal, J.K., Paton, D.J., Taylor, G. and Parida, S. 2008. Detection of persistently FMD infected cattle by Salivary IgA test. *EU FMD Reports: The Global control of FMD-Tools, ideas and ideals–Erice, Italy*: 14–17.

Biswal, J.K., Ranjan, R., Das, B., Subramaniam, S. and Pattnaik, B. 2017. The direct boil RT-mPCR: A simple and rapid method for detection of foot-and-mouth disease virus genome in clinical samples without nucleic acid extraction. *Indian J. Vet. Pathol.*, **41**(1): 12.

Biswal, J.K., Ranjan, R., Subramaniam, S., Mohapatra, J.K., Patidar, S., Sharma, M.K., Bertram, M.R., Brito, B., Rodriguez, L.L. and Pattnaik, B. 2019. Genetic and antigenic variation of foot-and-mouth disease virus during persistent infection in naturally infected cattle and Asian buffalo in India. *PloS one*, **14**(6): e0214832.

Biswal, J.K., Sanyal, A., Rodriguez, L.L., Subramaniam, S., Arzt, J., Sharma, G.K., Hammond, J.M., Parida, S., Mohapatra, J.K. and Mathapati, B.S. 2012. Foot-and-mouth disease: Global status and Indian perspective. *Indian J. Anim. Res.*, **82**(2): 109–131.

Blanco, E., Garcia-Briones, M., Sanz-Parra, A., Gomes, P., De Oliveira, E., Valero, M.L., Andreu, D., Ley, V. and Sobrino, F. 2001. Identification of T-cell epitopes in nonstructural proteins of foot-and-mouth disease virus. *J. Virol.*, **75**(7): 3164–3174.

Brito, B., Pauszek, S.J., Eschbaumer, M., Stenfeldt, C., de Carvalho Ferreira, H.C., Vu, L.T., Phuong, N.T., Hoang, B.H., Tho, N.D. and Dong, P.V. 2017. Phylodynamics of foot-and-mouth disease virus O/PanAsia in Vietnam 2010–2014. *Vet. Res.*, **48**(1): 1–12.

Brito, B.P., Perez, A.M., Jamal, S.M., Belsham, G.J., Pauszek, S.J., Ahmed, Z. and Rodriguez, L.L. 2013. Foot-and-Mouth Disease Virus Serotype O Phylodynamics: Genetic Variability Associated with Epidemiological Factors in Pakistan. *Transbound. Emerg. Dis.*, **60**(6): 516–524.

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. and 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**(11): 1636–1642.

Chitray, M., Grazioli, S., Willems, T., Tshabalala, T., De Vleeschauwer, A., Esterhuysen, J.J., Brocchi, E., De Clercq, K. and Maree, F.F. 2018. Development and validation of a foot-and-mouth disease virus SAT serotype-specific 3ABC assay to differentiate infected from vaccinated animals. *J. Virol. Methods*, **255**: 44–51.

Chung, C.J., Clavijo, A., Bounpheng, M.A., Uddowla, S., Sayed, A., Dancho, B., Olesen, I.C., Pacheco, J., Kamicker, B.J., Brake, D.A., Bandaranayaka-Mudiyanselage, C.L., Lee, S.S., Rai, D.K. and Rieder, E. 2018. An improved, rapid competitive ELISA using a novel conserved 3B epitope for the detection of serum antibodies to foot-and-mouth disease virus. *J. Vet. Diagn. Investig.: Official Publication of the American Association of Veterinary Laboratory Diagnosticians, Inc*, **30**(5): 699–707.

Cortey, M., Ferretti, L., Pérez-Martín, E., Zhang, F., de Klerk-Lorist, L.-M., Scott, K., Freimanis, G., Seago, J., Ribeca, P. and van Schalkwyk, L. 2019. Persistent infection of African buffalo (Synceruscaffer) with Foot-and-Mouth Disease Virus: limited viral evolution and no evidence of antibody neutralization escape. *J. Virol.* ,**93**(15): e00563–19.

Cubillos, C., de la Torre, B.G., Jakab, A., Clementi, G., Borrás, E., Bárcena, J., Andreu, D., Sobrino, F. and Blanco, E. 2008. Enhanced mucosal immunoglobulin A response and solid protection against foot-and-mouth disease virus challenge induced by a novel dendrimeric peptide. *J. Virol.*, **82**(14): 7223–7230.

Donaldson, A.I., Hearps, A. and Alexandersen, S. 2001. Evaluation of a portable, "real-time" PCR machine for FMD diagnosis. *The Vet. Rec.*, **149**(14): 430.

Eschbaumer, M., Stenfeldt, C., Rekant, S.I., Pacheco, J.M., Hartwig, E.J., Smoliga, G.R., Kenney, M.A., Golde, W.T., Rodriguez, L.L. and Arzt, J. 2016. Systemic immune response and virus persistence after foot-and-mouth disease virus infection of naive cattle and cattle vaccinated with a homologous adenovirus-vectored vaccine. *BMC Vet. Res.*, **12**(1): 1–18.

Farooq, U., Ahmed, Z., Naeem, K., Bertram, M., Brito, B., Stenfeldt, C., Pauszek, S.J., LaRocco, M., Rodriguez, L. and Arzt, J. 2018. Characterization of naturally occurring, new and persistent subclinical foot-and-mouth disease virus infection in vaccinated Asian buffalo in Islamabad Capital Territory, Pakistan. *Transbound. Emerg. Dis.*, **65**(6): 1836–1850.

Ferris, N.P., King, D.P., Reid, S.M., Hutchings, G.H., Shaw, A.E., Paton, D.J., Goris, N., Haas, B., Hoffmann, B. and Brocchi, E. 2006. Foot-and-mouth disease virus: a first inter-laboratory comparison trial to evaluate virus isolation and RT-PCR detection methods. *Vet. Microbiol.*, **117**(2–4): 130–140.

Golde, W.T., de Los Santos, T., Robinson, L., Grubman, M.J., Sevilla, N., Summerfield, A. and Charleston, B. 2011. Evidence of activation and suppression during the early immune response to foot-and-mouth disease virus. *Transbound. Emerg. Dis.*, **58**(4): 283–290.

Golde, W.T., Nfon, C.K. and Toka, F.N. 2008. Immune evasion during foot-and-mouth disease virus infection of swine. *Immunol. Rev.*, **225**(1): 85–95.

Grubman, M.J. 2005. Development of novel strategies to control foot-and-mouth disease: marker vaccines and antivirals. *Biologicals: J. Int. Assoc. Biol. Stand.*, **33**(4): 227–234.

Grubman, M.J., Moraes, M.P., Diaz-San Segundo, F., Pena, L. and De Los Santos, T. 2008. Evading the host immune response: how foot-and-mouth disease virus has become an effective pathogen. *FEMS Microbiol. Immunol.*, **53**(1): 8–17.

Hayer, S.S., Ranjan, R., Biswal, J.K., Subramaniam, S., Mohapatra, J.K., Sharma, G.K., Rout, M., Dash, B.B., Das, B. and Prusty, B.R. 2018. Quantitative characteristics of the foot-and-mouth disease carrier state under natural conditions in India. *Transbound. Emerg. Dis.*, **65**(1): 253–260.

Hearps, A., Zhang, Z. and Alexandersen, S. 2002. Evaluation of the portable Cepheid Smart Cycler real-time PCR machine for the rapid diagnosis of foot-and-mouth disease. *The Vet. Rec.* ,**150**(20): 625–628.

Henderson, L.M. 2005. Overview of marker vaccine and differential diagnostic test technology. *Biologicals: J. Int. Assoc. Biol. Stand.*, **33**(4): 203–209.

Hoffmann, B., Beer, M., Reid, S.M., Mertens, P., Oura, C.A.L., van Rijn, P.A., Slomka, M.J., Banks, J., Brown, I.H., Alexander, D.J. and King, D.P. 2009. A review of RT-PCR

technologies used in veterinary virology and disease control: sensitive and specific diagnosis of five livestock diseases notifiable to the World Organisation for Animal Health. *Vet. Microbiol.*, **139**(1–2): 1–23.

Horsington, J. and Zhang, Z. 2007a. Consistent change in the B–C loop of VP2 observed in foot-and-mouth disease virus from persistently infected cattle: Implications for association with persistence. *Virus Res.*, **125**(1): 114–118.

Horsington, J. and Zhang, Z. 2007b. Analysis of foot-and-mouth disease virus replication using strand-specific quantitative RT-PCR. *J. Virol. Methods*, **144**(1–2): 149–155.

Jain, B., Tewari, A., Batra, K. and Kadian, S.K. 2019. New approaches for post-vaccination surveillance of foot-and-mouth disease. *Acta Virol.*, **63**(1): 45–52.

Jamal, S.M. and Belsham, G.J. 2013. Foot-and-mouth disease: past, present and future. *Vet. Res.*, **44**(1): 116.

Jamal, S.M., Ferrari, G., Hussain, M., Nawroz, A.H., Aslami, A.A., Khan, E., Murvatulloev, S., Ahmed, S. and Belsham, G.J. 2012. Detection and genetic characterization of foot-and-mouth disease viruses in samples from clinically healthy animals in endemic settings. *Transbound. Emerg. Dis.*, **59**(5): 429–440.

Juleff, N., Windsor, M., Lefevre, E.A., Gubbins, S., Hamblin, P., Reid, E., McLaughlin, K., Beverley, P.C., Morrison, I.W. and Charleston, B. 2009. Foot-and-mouth disease virus can induce a specific and rapid CD4+ T-cell-independent neutralizing and isotype class-switched antibody response in naive cattle. *J. Virol.* ,**83**(8): 3626–3636.

Juleff, N., Windsor, M., Reid, E., Seago, J., Zhang, Z., Monaghan, P., Morrison, I.W. and Charleston, B. 2008. Foot-and-mouth disease virus persists in the light zone of germinal centres. *PloS one*, **3**(10): e3434.

Klein, J., Hussain, M., Ahmad, M., Afzal, M. and Alexandersen, S. 2008. Epidemiology of foot-and-mouth disease in Landhi Dairy Colony, Pakistan, the world largest Buffalo colony. *Virol. J.*, **5**(1): 53.

Knight-Jones, T.J.D. and 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**(3–4): 161–173.

Knowles, N.J. and Samuel, A.R. 2003. Molecular epidemiology of foot-and-mouth disease virus. *Virus Res.*, **91**(1): 65–80.

Knowles, N.J., Davies, P.R., Henry, T., O'Donnell, V., Pacheco, J.M. and 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**(3): 1551–1556.

LaRocco, M., Krug, P.W., Kramer, E., Ahmed, Z., Pacheco, J.M., Duque, H., Baxt, B. and Rodriguez, L.L. 2013. A continuous bovine kidney cell line constitutively expressing bovine αvβ6 integrin has increased susceptibility to foot-and-mouth disease virus. *J. Clin. Microbiol.*, **51**(6): 1714–1720.

Lin, H.-J.L. and Flint, S.J. 2000. Identification of a cellular repressor of transcription of the adenoviral late IVa2 gene that is unaltered in activity in infected cells. *Virology*, **277**(2): 397–410.

Mackay, I.M., Arden, K.E. and Nitsche, A. 2002. Real-time PCR in virology. *Nucleic Acids Res.*, **30**(6): 1292–1305.

Madhanmohan, M., Nagendrakumar, S.B., Manikumar, K., Yuvaraj, S., Parida, S. and Srinivasan, V.A. 2013. Development and evaluation of a real-time reverse transcription-loop-mediated isothermal amplification assay for rapid serotyping of foot-and-mouth disease virus. *J. Virol. Methods*, **187**(1): 195–202.

Mahajan, S., Jk, M., Lk, P., Gk, S. and B, P. 2014. Indirect ELISA using recombinant nonstructural protein 3D to detect foot and mouth disease virus infection associated antibodies. *Biologicals: J. Int. Assoc. Biol. Stand.*, **43**(1): 47–54.

Mahajan, S., Mohapatra, J.K., Pandey, L.K., Sharma, G.K. and Pattnaik, B. 2013. Truncated recombinant non-structural protein 2C-based indirect ELISA for FMD sero-surveillance. *J. Virol. Methods*, **193**(2): 405–414.

Mahajan, S., Mohapatra, J.K., Pandey, L.K., Sharma, G.K. and Pattnaik, B. 2015. Indirect ELISA using recombinant nonstructural protein 3D to detect foot and mouth disease virus infection associated antibodies. *Biologicals: Biologicals: J. Int. Assoc. Biol. Stand.*, **43**(1): 47–54.

Mansley, L.M., Donaldson, A.I., Thrusfield, M.V. and Honhold, N. 2011. Destructive tension: mathematics versus experience—the progress and control of the 2001 foot and mouth disease epidemic in Great Britain. *Revue Scientifique et Technique-OIE*, **30**(2): 483.

Maree, F., de Klerk-Lorist, L.-M., Gubbins, S., Zhang, F., Seago, J., Pérez-Martín, E., Reid, L., Scott, K., van Schalkwyk, L. and Bengis, R. 2016. Differential persistence of foot-and-mouth disease virus in African buffalo is related to virus virulence. *J. Virol.*, **90**(10): 5132–5140.

Mohapatra, A.K., Mohapatra, J.K., Pandey, L.K., Sanyal, A. and Pattnaik, B. 2014. Diagnostic potential of recombinant nonstructural protein 3B to detect antibodies induced by foot-and-mouth disease virus infection in bovines. *Arch. Virol.*, **159**(9): 2359–2369.

Mohapatra, J.K., Pandey, L.K., Sanyal, A. and Pattnaik, B. 2011a. Recombinant non-structural polyprotein 3AB-based serodiagnostic strategy for FMD surveillance in bovines irrespective of vaccination. *J. Virol. Methods*, **177**(2): 184–192.

Mohapatra, J.K., Pandey, L.K., Sharma, G.K., Barik, S.K., Pawar, S.S., Palsamy, R. and Pattnaik, B. 2011b. Multiplex

PCR for rapid detection of serotype A foot-and-mouth disease virus variants with amino acid deletion at position 59 of the capsid protein VP3. *J. Virol. Methods*, **171**(1): 287–291.

Moonen, P., Boonstra, J., van der Honing, R.H., Leendertse, C.B., Jacobs, L. and Dekker, A. 2003. Validation of a Light Cycler-based reverse transcription polymerase chain reaction for the detection of foot-and-mouth disease virus. *J. Virol. Methods*, **113**(1): 35–41.

Moonen, P., Jacobs, L., Crienen, A. and Dekker, A. 2004. Detection of carriers of foot-and-mouth disease virus among vaccinated cattle. *Vet. Microbiol.*, **103**(3–4): 151–160.

O'Donnell, V., Pacheco, J.M., Larocco, M., Gladue, D.P., Pauszek, S.J., Smoliga, G., Krug, P.W., Baxt, B., Borca, M.V. and Rodriguez, L. 2014. Virus–host interactions in persistently FMDV-infected cells derived from bovine pharynx. *Virology*, **468**: 185–196.

Oh, Y., Fleming, L., Statham, B., Hamblin, P., Barnett, P., Paton, D.J., Park, J.-H., Joo, Y.S. and Parida, S. 2012. Interferon-γ induced by *in vitro* re-stimulation of CD4+ T-cells correlates with *in vivo* FMD vaccine induced protection of cattle against disease and persistent infection. *PloS one*, **7**(9): e44365.

OIE. 2018. Foot and mouth disease (infection with foot and mouth disease virus), OIE Terrestrial Manual, Chapter 3.1.8.

Pacheco, J.M., Butler, J.E., Jew, J., Ferman, G.S., Zhu, J. and Golde, W.T. 2010. IgA antibody response of swine to foot-and-mouth disease virus infection and vaccination. *Clin. Vaccine Immunol.: CVI*, **17**(4): 550–558.

Pacheco, J.M., Smoliga, G.R., O'Donnell, V., Brito, B.P., Stenfeldt, C., Rodriguez, L.L. and Arzt, J. 2015. Persistent foot-and-mouth disease virus infection in the nasopharynx of cattle; tissue-specific distribution and local cytokine expression. *PloS one*, **10**(5): e0125698.

Parida, S., Anderson, J., Cox, S.J., Barnett, P.V. and Paton, D.J. 2006. Secretory IgA as an indicator of oro-pharyngeal foot-and-mouth disease virus replication and as a tool for post vaccination surveillance. *Vaccine*, **24**(8): 1107–1116.

Parida, S., Fleming, L., Oh, Y., Mahapatra, M., Hamblin, P., Gloster, J., Doel, C., Gubbins, S. and Paton, D.J. 2007. Reduction of foot-and-mouth disease (FMD) virus load in nasal excretions, saliva and exhaled air of vaccinated pigs following direct contact challenge. *Vaccine*, **25**(45): 7806–7817.

Parthiban, A.R., Mahapatra, M. and Parida, S. 2015b. Complete genome sequences of serotype O foot-and-mouth disease viruses recovered from experimental persistently infected cattle. *Genome Announc.*, **3**(4).

Parthiban, A.R., Mahapatra, M., Gubbins, S. and Parida, S. 2015a. Virus excretion from foot-and-mouth disease virus carrier cattle and their potential role in causing new outbreaks. *PloS one*, **10**(6): e0128815.

Paton, D.J., Valarcher, J.F., Bergmann, I., Matlho, O.G., Zakharov, V.M., Palma, E.L. and Thomson, G.R. 2005. Selection of foot and mouth disease vaccine strains--a review. *Revue Scientifique Et Technique (International Office of Epizootics)*, **24**(3): 981–993.

Pauszek, S.J., Bertram, M.R., Vu, L.T., Hartwig, E.J., Smoliga, G.R., Brito, B., Stenfeldt, C., VanderWaal, K., Fish, I.H. and Hung, V.V. 2017. Genome sequences of seven foot-and-mouth disease virus isolates collected from serial samples from one persistently infected carrier cow in Vietnam. *Genome Announc.*, **5**(34).

Pauszek, S.J., Eschbaumer, M., Brito, B., de Carvalho Ferreira, H.C., Vu, L.T., Phuong, N.T., Hoang, B.H., Tho, N.D., Dong, P.V. and Minh, P.Q. 2016. Site-specific substitution (Q172R) in the VP1 protein of FMDV isolates collected from asymptomatic carrier ruminants in Vietnam. *Vir. Rep.*, **6**: 90–96.

Perez-Martin, E., Weiss, M., Diaz-San Segundo, F., Pacheco, J.M., Arzt, J., Grubman, M.J. and de los Santos, T. 2012. Bovine type III interferon significantly delays and reduces the severity of foot-and-mouth disease in cattle. *J. Virol.*, **86**(8): 4477–4487

Poonsuk, K., Giménez-Lirola, L. and Zimmerman, J.J. 2018. A review of foot-and-mouth disease virus (FMDV) testing in livestock with an emphasis on the use of alternative diagnostic specimens. *Anim. Health Res. Rev.*, **19**(2): 100–112.

Ramirez-Carvajal, L., Pauszek, S.J., Ahmed, Z., Farooq, U., Naeem, K., Shabman, R.S., Stockwell, T.B. and Rodriguez, L.L. 2018. Genetic stability of foot-and-mouth disease virus during long-term infections in natural hosts. *PLoS One*, **13**(2): e0190977.

Ranjan, R., Biswal, J.K., Sharma, G.K. and Pattnaik, B. 2016a. A Review on Foot-and-mouth disease: pathology, diagnosis and its management. *Indian J. Vet. Pathol.*, **40**(2): 105.

Ranjan, R., Biswal, J.K., Subramaniam, S., Dash, B.B., Singh, K.P., Arzt, J., Rodriguez, L.L. and Pattnaik, B. 2018. Evidence of subclinical foot-and-mouth disease virus infection in young calves born from clinically recovered cow under natural condition. *Trop. Anim. Health Prod.*, **50**(5): 1167–1170.

Ranjan, R., Biswal, J.K., Subramaniam, S., Singh, K.P., Stenfeldt, C., Rodriguez, L.L., Pattnaik, B. and Arzt, J. 2016b. Foot-and-mouth disease virus-associated abortion and vertical transmit ssion following acute infection in cattle under natural conditions. *PLoS One*, **11**(12): e0167163.

Ranjan, R., Kangayan, M., Subramaniam, S., Mohapatra, J.K., Biswal, J.K., Sharma, G.K., Sanyal, A. and Pattnaik, B. 2014. Development and evaluation of a one step reverse transcription-loop mediated isothermal amplification assay

(RT-LAMP) for rapid detection of foot and mouth disease virus in India. *Virus dis.*, **25**(3): 358–364.

Reid, S.M., Ferris, N.P., Hutchings, G.H., Zhang, Z., Belsham, G.J. and Alexandersen, S. 2001. Diagnosis of foot-and-mouth disease by real-time fluorogenic PCR assay. *The Vet. Rec.*, **149**(20): 621–623.

Reid, S.M., Mioulet, V., Knowles, N.J., Shirazi, N., Belsham, G.J. and King, D.P. 2014. Development of tailored real-time RT-PCR assays for the detection and differentiation of serotype O, A and Asia-1 foot-and-mouth disease virus lineages circulating in the Middle East. *J. Virol. Methods*, **207**: 146–153.

Rémond, M., Kaiser, C. and Lebreton, F. 2002. Diagnosis and screening of foot-and-mouth disease. *Comp. Immunol. Microbiol. Infect. Dis.*, **25**(5–6): 309–320.

Robinson, L., Knight-Jones, T.J.D., Charleston, B., Rodriguez, L.L., Gay, C.G., Sumption, K.J. and Vosloo, W. 2016. Global Foot-and-Mouth Disease Research Update and Gap Analysis: 7–Pathogenesis and Molecular Biology. *Transbound. Emerg. Dis.*, **63**: 63–71.

Saduakassova, M.A., Sultanov, A.A., Kutumbetov, L.B., Wadsworth, J., A Wood, B., Knowles, N.J., King, D.P. and Bachanek-Bankowska, K. 2018. Development and evaluation of a novel real-time RT-PCR to detect foot-and-mouth disease viruses from the emerging A/ASIA/G-VII lineage. *J. Virol. Methods*, **252**: 37–41.

Schneider-Schaulies, J. 2000. Cellular receptors for viruses: links to tropism and pathogenesis. *J. Gen. Virol.*, **81**(6): 1413–1429.

Sharma, G.K., Mahajan, S., Das, B., Ranjan, R., Kanani, A., Sanyal, A. and Pattnaik, B. 2014a. Comparison of stabilisers for development of a lyophilised multiplex reverse-transcription PCR mixture for rapid detection of foot and mouth disease virus serotypes. *Revue Scientifique Et Technique (International Office of Epizootics)*, **33**(3): 859–867.

Sharma, G.K., Mahajan, S., Matura, R., Subramaniam, S., Ranjan, R., Biswal, J., Rout, M., Mohapatra, J.K., Dash, B.B. and Sanyal, A. 2015. Diagnostic assays developed for the control of foot-and-mouth disease in India. *World J. Virol.*, **4**(3): 295

Sharma, G.K., Mohapatra, J.K., Mahajan, S., Matura, R., Subramaniam, S. and Pattnaik, B. 2014b. Comparative evaluation of non-structural protein-antibody detecting ELISAs for foot-and-mouth disease sero-surveillance under intensive vaccination. *J. Virol. Methods*, **207**: 22–28.

Sharma, G.K., Mohapatra, J.K., Pandey, L.K., Mahajan, S., Mathapati, B.S., Sanyal, A. and Pattnaik, B. 2012. Immunodiagnosis of foot-and-mouth disease using mutated recombinant 3ABC polyprotein in a competitive ELISA. *J. Virol. Methods*, **185**(1): 52–60.

Sierra, S., Dávila, M., Lowenstein, P.R. and Domingo, E. 2000. Response of foot-and-mouth disease virus to increased mutagenesis: influence of viral load and fitness in loss of infectivity. *J. Virol.*, **74**(18): 8316–8323.

Sobrino, F., Sáiz, M., Jiménez-Clavero, M.A., Núñez, J.I., Rosas, M.F., Baranowski, E. and Ley, V. 2001. Foot-and-mouth disease virus: a long known virus, but a current threat. *Vet. Res.*, **32**(1): 1–30.

Stenfeldt, C. and Arzt, J. 2020. The Carrier conundrum; a review of recent advances and persistent gaps regarding the carrier state of foot-and-mouth disease virus. *Pathogens*, **9**(3): 167.

Stenfeldt, C., Eschbaumer, M., Pacheco, J.M., Rekant, S.I., Rodriguez, L.L. and Arzt, J. 2015. Pathogenesis of primary foot-and-mouth disease virus infection in the nasopharynx of vaccinated and non-vaccinated cattle. *PLoS One*, **10**(11): e0143666.

Stenfeldt, C., Eschbaumer, M., Rekant, S.I., Pacheco, J.M., Smoliga, G.R., Hartwig, E.J., Rodriguez, L.L. and Arzt, J. 2016a. The foot-and-mouth disease carrier state divergence in cattle. *J. Virol.*, **90**(14): 6344–6364.

Stenfeldt, C., Eschbaumer, M., Smoliga, G.R., Rodriguez, L.L., Zhu, J. and Arzt, J. 2017. Clearance of a persistent picornavirus infection is associated with enhanced pro-apoptotic and cellular immune responses. *Sci. Rep.*, **7**(1): 1–15.

Stenfeldt, C., Hartwig, E.J., Smoliga, G.R., Palinski, R., Silva, E.B., Bertram, M.R., Fish, I.H., Pauszek, S.J. and Arzt, J. 2018. Contact challenge of cattle with foot-and-mouth disease virus validates the role of the nasopharyngeal epithelium as the site of primary and persistent infection. *Msphere*, **3**(6).

Stenfeldt, C., Heegaard, P.M., Stockmarr, A. and Belsham, G.J. 2012. Modulation of cytokine mRNA expression in pharyngeal epithelial samples obtained from cattle infected with foot-and-mouth disease virus. *J. Comp. Pathol.*, **146**(2–3): 243–252.

Stenfeldt, C., Heegaard, P.M., Stockmarr, A., Tjørnehøj, K. and Belsham, G.J. 2011. Analysis of the acute phase responses of Serum Amyloid A, Haptoglobin and Type 1 Interferon in cattle experimentally infected with foot-and-mouth disease virus serotype O. *Vet. Res.*, **42**(1): 66.

Stenfeldt, C., Pacheco, J.M., Singanallur, N.B., Vosloo, W., Rodriguez, L.L. and Arzt, J. 2019. Virulence beneath the fleece; a tale of foot-and-mouth disease virus pathogenesis in sheep. *Plos one*, **14**(12): e0227061.

Stenfeldt, C., Pacheco, J.M., Smoliga, G.R., Bishop, E., Pauszek, S.J., Hartwig, E.J., Rodriguez, L.L. and Arzt, J. 2016b. Detection of foot-and-mouth disease virus RNA and

capsid protein in lymphoid tissues of convalescent pigs does not indicate existence of a carrier state. *Transbound. Emerg. Dis.*, **63**(2): 152–164.

Sutmoller, P. and Olascoaga, R.C. 2002. Unapparent foot and mouth disease infection (sub-clinical infections and carriers): implications for control. *Revue scientifique et technique-Office international des épizooties*, **21**(3): 519–524.

Tenzin, Dekker, A., Vernooij, H., Bouma, A. and Stegeman, A. 2008. Rate of foot-and-mouth disease virus transmission by carriers quantified from experimental data. *Risk Analysis: An International Journal*, **28**(2): 303–309.

Van Bekkum, J.G., Frenkel, H.S., Frederiks, H.H.J. and Frenkel, S. 1959. Observations on the carrier state of cattle exposed to foot-and-mouth disease virus. *Tijdschr. Diergeneeskd*, **84**: 1159–1164.

Weaver, G.V., Domenech, J., Thiermann, A.R. and Karesh, W.B. 2013. Foot and mouth disease: a look from the wild side. *J. Wildl. Dis.*, **49**(4): 759–785.

Windsor, M.A., Carr, B.V., Bankowski, B., Gibson, D., Reid, E., Hamblin, P., Gubbins, S., Juleff, N. and Charleston, B. 2011. Cattle remain immunocompetent during the acute phase of foot-and-mouth disease virus infection. *Vet. Res.*, **42**(1): 108.

Yang, M., Parida, S., Salo, T., Hole, K., Velazquez-Salinas, L. and Clavijo, A. 2015. Development of a competitive enzyme-linked immunosorbent assay for detection of antibodies against the 3B protein of foot-and-mouth disease virus. *Clin. Vaccine Immunol.*, **22**(4): 389–397.

Zhang, Z. and Alexandersen, S. 2003. Detection of carrier cattle and sheep persistently infected with foot-and-mouth disease virus by a rapid real-time RT-PCR assay. *J. Virol. Methods*, **111**(2): 95–100

Zhang, Z. and Alexandersen, S. 2004. Quantitative analysis of foot-and-mouth disease virus RNA loads in bovine tissues: implications for the site of viral persistence. *J. Gen. Virol.*, **85**(9): 2567–2575.

Zhang, Z., Bashiruddin, J.B., Doel, C., Horsington, J., Durand, S. and Alexandersen, S. 2006. Cytokine and Toll-like receptor mRNAs in the nasal-associated lymphoid tissues of cattle during foot-and-mouth disease virus infection. *J. Comp. Pathol.*, **134**(1): 56–62.

Zhang, Z., Murphy, C., Quan, M., Knight, J. and Alexandersen, S. 2004. Extent of reduction of foot-and-mouth disease virus RNA load in oesophageal–pharyngeal fluid after peak levels may be a critical determinant of virus persistence in infected cattle. *J. Gen. Virol.*, **85**(2): 415–421.

Zhang, Z.D. and Kitching, R.P. 2001. The localization of persistent foot and mouth disease virus in the epithelial cells of the soft palate and pharynx. *J. Comp. Pathol.*, **124**(2–3): 89–94.

Zhang, Z.D., Hutching, G., Kitching, P. and Alexandersen, S. 2002. The effects of gamma interferon on replication of foot-and-mouth disease virus in persistently infected bovine cells. *Arch. Virol.*, **147**(11): 2157–2167.

Zhu, J.J., Arzt, J., Puckette, M.C., Smoliga, G.R., Pacheco, J.M. and Rodriguez, L.L. 2013. Mechanisms of foot-and-mouth disease virus tropism inferred from differential tissue gene expression. *PloS one*, **8**(5): e64119.