A REVIEW OF FOOT-AND-MOUTH DISEASE VIRUS (FMDV) TESTING IN LIVESTOCK WITH AN EMPHASIS ON THE USE OF ALTERNATIVE DIAGNOSTIC SPECIMENS

Running title: FMDV testing and alternative specimens

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Abstract

Foot-and-mouth disease virus (FMDV) remains an important pathogen of livestock more than 120 years after it was identified, with annual costs from production losses and vaccination estimated at €5.3 to €17 billion EURO ($6.5 to $21 billion USD) in FMDV-endemic areas. Control and eradication are difficult because FMDV is highly contagious, genetically and antigenically diverse, infectious for a wide variety of species, able to establish subclinical carriers in ruminants, and widely geographically distributed. For early detection, sustained control, or eradication, sensitive and specific FMDV surveillance procedures compatible with high through-put testing platforms are required. At present, surveillance relies on the detection of FMDV-specific antibody or virus, most commonly in individual animal serum, vesicular fluid or epithelial specimens. However, FMDV and/or antibody are also detectable in other body secretions and/or specimens, e.g., buccal and nasal secretions, respiratory exhalations (aerosols), mammary secretions, urine, feces, and environmental samples. These alternative specimens offer non-invasive diagnostic alternatives to individual animal sampling and the potential for more efficient, responsive, and cost-effective surveillance. Herein we review FMDV testing methods for contemporary and alternative diagnostic specimens and their application to FMDV surveillance in livestock (cattle, swine, sheep, and goats).

Keywords: foot-and-mouth disease virus, FMDV, surveillance, review, diagnosis, specimen

1.0 Introduction

Foot-and-mouth disease virus (FMDV) is a member of family *Picornaviridae*, genus *Aphthovirus* (Bachrach, 1977; Rodrigo and Dopazo, 1995; Rueckert, 1996). FMDV was the first virus of vertebrates to be identified, i.e., Loeffler and Frosch (1897) collected vesicular fluid, passed it through ceramic filters impermeable to bacteria, and reproduced clinical signs in cattle exposed to the filtrate. FMDV consists of a single-stranded, positive-sense RNA genome of approximately 8,500 bases organized in three major regions (5′ non-coding regulatory region, polyprotein coding region, and 3′ non-coding regulatory region), with a polyadenylated 3′-end and a small, covalently linked protein (VPg) at the 5′-end. Polyproteins are post-translationally cleaved by viral protease into four structural proteins (VP1, VP2, VP3, and VP4) and 8 nonstructural proteins (L, 2A, 2B, 2C, 3A, 3B, 3C, and 3D) (Ryan et al., 1989). Structural proteins VP1, VP2, and VP3 assemble to form an icosahedral structure that is internally bound by VP4. Nonstructural proteins function in virus replication and interactions with host cell factors and for processing of the structural proteins (Domingo et al., 2002; Grubman and Baxt, 2004).

The classic clinical signs of FMDV infection (vesicles on the mouth and feet), were first described by Hieronymous Fracastorius (1546) after observing an outbreak in cattle near Verona, Italy (Mahy, 2005). FMDV is infectious for most animals in the order *Artiodactyla* (even-toed ungulates), but especially cattle, buffalo, swine, sheep, and goats (Alexandersen and Mowat, 2005; Bastos et al., 2000; Burrows, 1968; Gibbs et al., 1975a,b; Kitching et al., 2002a,b). In addition, more than 70 wildlife species are known to be susceptible to FMDV, including white-tailed deer (*Odocoileus virginianus*) (Fenner et al., 1993; Moniwa et al., 2012; Snowdon, 1968).

FMDV in wildlife species is a serious concern because of the problems entailed in eradicating the virus from such populations. In the United States, 20,000 mule deer (*Odocoileus hermionus*) were killed in Stanislav National Forest to control the 1924-1926 FMDV outbreak in California.

The virus is highly contagious and, depending on the route of exposure, ≤ 10 tissue culture infectious doses are sufficient to infect and produce clinical disease in susceptible ruminants (Alexandersen et al., 2003b; Sellers, 1971). Although incubation time can be considerably longer depending on dose and route of infection, viremia typically appears 24 to 48 hours post exposure and vesicles in the mouth and on the feet, thereafter (Baxt and Mason, 1995; Yilma, 1980). In an FMDV outbreak, transmission within and between populations can be rapid due to the short *in vivo* replication cycle (4 to 6 hours) and acute onset of shedding (1 to 3 days) (Donaldson et al., 1987; Grau et al., 2015; Grubman and Baxt, 2004). The most common route of FMDV transmission is direct contact, however, transmission can occur over significant distances due to aerosol and mechanical dissemination of virus through water, feed, and fomites (Brooksby, 1982; Thomson et al., 2003). Clinically healthy FMDV carriers (reported up to 3.5 years in cattle, 9 months in sheep, and 4 months in goats) occur in both naïve and vaccinated ruminants, complicating control and eradication efforts (Alexandersen et al., 2002a; Alexandersen et al., 2003b; Kitching, 1998; Pereira, 1981).

Infection elicits a rapid immune response, but as a result of extensive antigenic variation, immunity against one FMDV isolate does not necessarily protect against others (Bedson et al., 1927, Galloway et al., 1948; Gebauer et al., 1988; Salt, 1993; Sutmoller, 2003; van Bekkum et al., 1959). Variation in VP1, VP2, and VP3 proteins made it possible for early investigators to

use cross-neutralization tests to classify serotypes. In 1922, Vallée and Carré reported the presence of what is known today as serotype O in France and serotype A in Germany. Shortly thereafter, Waldmann and Trautwein, (1926) reported what is now identified as serotype C in Germany (Brown, 2003). Three more serotypes (South African Territories; SAT 1, SAT 2, and SAT 3) were discovered in South Africa by Brooksby et al. (1958) and Asia 1 was identified in Pakistan in 1957 (Brooksby and Roger, 1957). Antigenic variation is a challenge to FMDV control because it has the potential to complicate vaccinology and diagnostics.

Depending on the geographic region, serotype-specific, inactivated FMDV vaccines are used to control clinical disease in endemic areas, but have also been used in FMDV eradication campaigns, e.g. Uruguay, Argentina, and Paraguay (Sumption et al., 2008). Outbreaks have occurred in every livestock-containing region of the world with the exception of New Zealand. According to the World Animal Health Organization (OIE, 2017), 66 countries are free of FMDV without vaccination, 9 countries are free of FMDV with vaccination and the remainder are endemically infected or lack reliable data upon which to base their true status.

Originally, FMDV used in vaccine production was derived from fluid collected from vesicular lesions on virus-inoculated cattle, just as was done previously for the production of smallpox vaccine virus (vaccinia virus) (Fenner et al.., 1990; Sutmoller et al., 2003). Thus, Vallée et al. (1926) attempted to produce a FMDV vaccine using formaldehyde-inactivated fluid and loose epithelial tissues from vesicles on calves. Thereafter, Frenkel (1947) used macroscopic slices of tongue epithelium to propagate virus and prepare formaldehyde-inactivated vaccine. This approach was used by Rosenbusch et al. (1948) to produce enough FMDV vaccine to vaccinate

more than 2 million cattle in Argentina (Brown, 2003). Over time, various cell lines e.g. pig kidney (IBRS-2, MVPK-1), porcine kidney (LFBK) or baby hamster kidney fibroblast (BHK-21), were used in diagnostics or for FMDV propagation (Capstick et al., 1962; Mohapatra et al., 2015; Snowdon, 1966; Swaney, 1976). Among these cell lines, BHK-21 has been used for large-scale production of FMDV vaccine (Doel, 2003). In addition, a variety of contemporary vaccine technologies have been evaluated under experimental conditions, e.g. subunit, vector expression of subunit components, and DNA vaccines.

Protective immunity is directed toward structural proteins (Longjam et al., 2011). Therefore, elimination of non-structural proteins (NSPs) (L, 2A, 2B, 2C, 3A, 3B, 3C, and 3D) during vaccine production results in vaccinates without antibodies against these proteins, i.e., DIVA (differentiating infected from vaccinated animals) vaccines. That is, DIVA-vaccinated animals produce antibodies against FMDV structural proteins, but not against NSPs, whereas FMDV-infected animals produce antibodies against both structural and NSPs. Implementation of a DIVA strategy based on the detection of antibodies against NSPs in infected animals is used to monitor the on-going success of FMDV eradication and to maintain "FMD-free with vaccination" status (Bergman et al., 2004). However, it has been observed that inadequately purified FMDV vaccines can contain enough residual NSP to induce anti-NSP antibody and produce false positive ELISA results (Uttenthal et al., 2010).

Whether the goal is early detection, sustained control, or eradication, diagnostically and analytically sensitive and specific (but affordable) FMDV surveillance tools are mandatory.

Herein we review FMDV testing methods, contemporary and alternative diagnostic specimens, and their application in FMDV surveillance in livestock (cattle, swine, sheep, and goats).

2.0 Tests and testing

Prior to the development of the complement fixation test (1929), FMDV infection was diagnosed primarily by clinical signs, i.e., the presence of vesicles on epithelial surfaces of the feet, mouth, nasal regions, and mammary glands (Bachrach, 1968). However, diagnosis based on clinical signs is complicated by the fact that other viral infections, e.g., swine vesicular disease virus (SVDV), vesicular stomatitis virus (VSV), and vesicular exanthema of swine virus (VESV), may produce lesions which are indistinguishable from FMDV. Today, the detection of FMDV infections relies on the detection of FMDV-specific antibody (virus neutralization, antibody ELISA) or on the detection of the virus and/or viral components (virus isolation, antigen-capture ELISA, or reverse transcription-polymerase chain reaction (RT-PCR)). These techniques are reviewed below.

2.1 Virus detection

2.1.1 Direct complement fixation test

Prior to the development of techniques for virus isolation, Ciuca (1929) showed that the direct complement fixation test could be used to detect FMDV and serotype isolates. The method was based on the fact that guinea pig-derived complement is bound by virus-antibody complexes. If virus-antibody binding does not occur, the free complement will lyse sheep red blood cells (RBC) in the presence of anti-sheep RBC antibody. It was possible to identify FMDV serotypes using the direct complement fixation test because FMDV antibodies are serotype specific. Later,

Traub and Mohlmann (1943) used the direct complement fixation test to serotype FMDV in cattle. The direct complement fixation test is best used early in infection because it requires a high concentration of virus in the test specimen; thus, it is not useful when vesicles begin to resolve (Rice and Brooksby, 1953). Further, serum with pro- or anti-complementary activity will affect the test results (Ferris and Dawson, 1988).

2.1.2 Virus isolation

FMDV isolation was first described by Frenkel (1947) using primary bovine tongue epithelial cells, but Sellers (1955) and Bachrach et al. (1955) adapted primary bovine and swine kidney cells to FMDV diagnostics. Historically, bovine thyroid cells were considered the best primary cells for FMDV isolation, but more recently, continuous cell lines, e.g., IBRS-2, MVPK-1 clone 7, LFBK, BHK21, and BHK21-CT, have been widely used (Dinka et al., 1977; Ferris et al., 2006a,b; House et al., 1989; Nair, 1987). Among several stable cell lines, bovine kidney cells expressing β6 and αV and integrin subunits (LFBK-αVβ6) were highly susceptible to all FMDV serotypes (LaRocco et al., 2013). The availability of cell culture techniques and the realization that FMDV could be grown *in vitro* made typing of FMDV isolates more practical (Rweyemamu, 1982).

Virus isolation is the only way to confirm the presence of live FMDV, despite well-recognized challenges: (1) working with infectious FMDV presents a significant biosafety risk; (2) cell cultures lose susceptibility to the virus over time; (3) cell lines lose permissiveness to the virus over passages; (4) antibodies present in samples from infected animals may completely or partially neutralize FMDV; (5) virus isolation is much less analytically sensitive than RT-PCR

(Alexandersen et al., 2003a); (6) cytopathic effect can be caused by a variety of factors, not just FMDV, thus positive results must be confirmed using other methods.

Propagating virus on cell culture requires technical skill, adequate laboratory facilities, and more time than molecular assays. The diagnostic sensitivity of FMDV isolation varies among laboratories, virus serotype, and the cells used in the procedure (Alexandersen et al., 2003a). Ferris et al. (2006a) evaluated five European FMDV reference laboratories using a set of vesicular samples from FMDV-infected cattle (serotypes O, A, Asia 1, and SAT 2), SVDV-infected pigs, and negative control samples from cattle and pigs. Among primary cells, bovine thyroid cells provided the highest rate of FMDV isolation (94%) compared to primary lamb kidney cells (69%). The rate of isolation also varied among continuous cell lines: 69% for IBRS-2, 56% for BHK21, and 25% for BHK21-CT. In addition, primary bovine thyroid cells and IBRS-2 cells were susceptible to all FMDV serotypes, whereas primary lamb kidney cells, BHK21, and BHK21-CT cells were not susceptible to FMDV serotype SAT2. Data from more recent studies suggested that newer cell lines are highly susceptible to FMDV, but only partial comparisons among cell lines have been done. Brehm et al. (2009) compared primary bovine thyroid cells, IBRS-2, BHK21, and ZZ-R 127 (fetal goat) cell lines using FMDV isolates representing all 7 serotypes. Although less sensitive than primary bovine thyroid cells, cell line ZZ-R 127 was more sensitive than the other cell lines included in the comparison. Similarly, LaRocco et al. (2013) found the LFBK-αVβ6 continuous cell line to more susceptible to FMDV than primary lamb kidney, IBRS-2, and BHK21 cells.

2.1.3 Antigen-capture ELISA

The OIE (2012) recommends the use of FMDV antigen-capture ELISA for the detection of viral antigen and identification of viral serotype in clinical specimens and culture isolates (Ferris and Donaldson, 1992; Roeder and Le Blanc Smith, 1987). Crowther and Elzein (1979a,b; 1980) initially reported the use of antigen-capture ELISA to detect FMDV in cell culture and later applied the test to the detection of FMDV in cattle epithelial tissues. Currently, antigen-capture ELISAs based on polyclonal antibodies or various monoclonal antibodies targeting structural or non-structural proteins are available (Ferris and Dawson, 1988; Hamblin et al., 1984; Roeder and Le Blanc Smith, 1987). Antigen-capture ELISA is capable of rapidly testing large numbers of samples, i.e., results can be obtained in 3 to 4 hours (Alexandersen et al., 2003a; Grubman and Baxt, 2004). However, the antigenic variability within and between serotypes further compromises the limited analytical sensitivity of the antigen-capture ELISA format. Studies showed that 70% to 80% of cell culture-positive samples and 63% to 71% of RT-PCR positive oral/nasal swabs were detected by Ag-capture ELISA (Alexandersen et al., 2003a; Morioka et al., 2014).

2.1.4 Antigen-capture lateral-flow assay

FMDV antigen-capture lateral flow assays or rapid chromatographic strip tests allow rapid on-site diagnosis in areas where the disease is endemic and in reference laboratories when a rapid result is needed. These assays detect FMDV antigens in vesicular fluids or epithelial suspension from infected animals using monoclonal or polyclonal antibodies (Ferris et al., 2009; 2010; Jiang et al., 2011; Oem et al., 2009; Reid et al., 2001). Oem et al. (2009) reported that a monoclonal antibody-based lateral-flow assay showed 87% diagnostic sensitivity and 99% diagnostic

specificity for the detection of FMDV serotypes O, A, Asia1, and C when testing epithelial suspension specimens.

2.1.5 Reverse transcription-polymerase chain reaction (RT-PCR)

Relative to other virus detection methods, RT-PCR is considered to offer shorter turn-around time plus higher diagnostic and analytical sensitivity and specificity (Alexandersen et al., 2003a; Callens et al., 1998; King et al., 2006; Moss and Haas, 1999; Reid et al., 1998; Reid et al., 1999; Reid et al., 2000; Shaw et al., 2004). Although FMDV is highly resistant to degradation in the environment, RT-PCR can detect nucleic acid from both infectious or inactivated virus, thereby reducing the impact of sample handling deficiencies on virus detection (Cottral, 1969; Longjam et al., 2011). The FMDV genome is heterogeneous. To avoid false negative results, RT-PCR primers and probes must target nucleic acid sequences that are broadly conserved across all serotypes. For surveillance, RT-PCR can be used in parallel with virus isolation to achieve a more complete epidemiological picture (Callens and De Clercq, 1999; Callens et al., 1998; Hofner et al., 1993; Laor et al., 1992; Marquardt et al., 1995; Rodriguez et al., 1994).

Realtime RT-PCR

Realtime RT-PCR has been widely used in FMDV diagnosis because it offers improved analytical sensitivity and a simpler testing format, i.e., electrophoresis is not required. The first universal FMDV realtime RT-PCR used primers and probes specific to a highly conserved region within a polypeptide gene (P3) and achieved an analytical sensitivity for all FMDV serotypes estimated at 1×10^2 $TCID_{50}$ (Meyer et al., 1991). Carillo et al. (2005) compared whole genome sequences of 113 FMDV isolates and found that the 5′UTR and 3D (RNA-dependent

249 RNA polymerase gene) regions shared a high degree of nucleotide identity among FMDV
250 isolates, i.e., 83% (5'UTR) and 91% (3D) homology. Further studies showed that primers and
251 probes based on 5'UTR or 3D were analytically specific, i.e., no false positives were observed
252 when testing specimens containing swine vesicular disease virus (SVDV), vesicular stomatitis
253 virus (VSV), or vesicular exanthema of swine virus (VESV) (Callahan et al., 2002; Ferris et al.,
254 2006a,b; Reid et al., 2002; Shaw et al., 2007). Although OIE currently recommends the use of
255 "universal" primers and probes targeting conserved sequences within the 5′ UTR or 3D regions,
256 serotype specific assays have also been created (Bachanek-Bankowska et al., 2016; Reid et al.,
257 2014).
258
259 Several studies have evaluated the diagnostic performance of 5′UTR and 3D FMD RT-PCRs.
260 Using a variety of specimens containing viruses representing O, A, and Asia-1 serotypes plus
261 serum and vesicular samples from FMDV-negative animals, Reid et al. (2014) reported no false
262 positive results and detection rates of 91% and 96% for 3D and 5′UTR rRT-PCRs, respectively.
263 Hindson et al. (2008) evaluated 5′UTR, 3D, or both rRT-PCRs using vesicular epithelium
264 samples containing FMDV (serotypes O, C, Asia-1, SAT1, SAT2, SAT3), SVDV, or VESV.
265 The diagnostic sensitivity of the 5′UTR and 3D rRT-PCRs was 87% and 97%, respectively.
266 Combining the two methods resulted in a diagnostic sensitivity of 98%. King et al. (2006)
267 compared the diagnostic sensitivity of the 5′UTR and 3D FMDV rRT-PCRs using 394 FMDV
268 clinical specimens (serum, vesicular epithelium). Approximately 94% (367 of 392) samples
269 were positive on one of the two rRT-PCRs, with 88.1% (347 of 394) positive on both assays.
270 Sequence analyses showed that all false negative results were the result of nucleotide
271 substitutions within the region targeted by the primers or probes (King et al., 2006). Therefore,

laboratories may need to provide for both 3D and 5′UTR RT-PCR testing to reduce the likelihood of false negative results caused by nucleotide changes in the 3D or 5'UTR target areas (Moniwa et al., 2007).

2.2 Antibody detection

FMDV antibody detection methods are routinely used for several purposes, e.g., in import/export to certify that animals and/or animals from which by-products were derived are free from FMDV infection, to demonstrate previous FMDV infection or vaccination, or to evaluate antigenic matching of vaccines.

2.2.1 Indirect complement fixation test

The indirect complement fixation test was the first *in vitro* test developed for the detection of FMDV-specific antibody (Rice and Brooksby, 1953). The assay was further developed to detect FMDV antibodies from multiple FMDV serotypes (Nordberg and Schjerning-Thiesen, 1956; Sakaki et al., 1977; Sakaki et al., 1978). At present, use of the indirect complement fixation test is recommended by OIE only if FMDV ELISA testing is not available (OIE, 2012).

2.2.2 Serum-virus neutralization test

The FMDV serum-virus neutralization test (SVN) is a serotype-specific assay for the detection of neutralizing antibodies elicited by vaccination and/or infection (Golding et al., 1976). Post-vaccination sero-surveys for FMDV are a major indicator in the assessment of preventive vaccination programs (Sobrino et al., 2001). The existence of circulating neutralizing antibody is associated primarily with resolution of viremia (Pacheco et al., 2010). The test may be performed on various cell lines, although Moonen et al. (2000) found that BHK or IBRS-2 cells

provided better results than PK-2 cells. The test is more specific than the indirect complement fixation test and is recommended for international trade by OIE, but the slow throughput (72 hours to perform the test) is incompatible with rapid response and/or routine commerce. In addition, the assay's requirement for infectious virus mandates that testing be performed in a high-level biocontainment facility; often a difficult and expensive hurdle to clear.

2.2.3 Enzyme-linked immunosorbent assay (ELISA)

Elzein and Crowther (1978) developed the first indirect FMDV antibody ELISA. Subsequently, various FMDV ELISAs have been developed for the detection of antibodies and/or serotyping of viruses (Hamblin et al., 1984; Ouldridge et al., 1982; Ouldridge et al., 1984; Pattnaik and Venkataramanan, 1989; Rai and Lahiri, 1981; Roeder and Le Blanc Smith, 1987). ELISAs are highly repeatable, cost-effective, and compatible with a variety of sample types, e.g., milk, probang, and oral fluid specimens (Longjam et al., 2011; Senthilkumaran et al., 2017; Blackwell et al., 1981; Burrows, 1968; de Leeuw et al., 1978).

2.2.3.1 Structural protein ELISAs

FMDV structural protein ELISAs are serotype-specific tests designed to detect antibodies elicited by vaccination and/or infection. Several blocking or competitive ELISAs have been developed based on serotype-specific polyclonal or monoclonal antibodies against the capsid proteins (VP1, VP2, and VP3), 146S or 12S subunit epitopes (Cartwright et al., 1980; Roeder and Le Blanc Smith, 1987; Sáiz et al., 1994). These assays provide faster throughput than SVN and avoid the need for tissue culture and live FMDV.

2.2.3.2 Non-structural protein ELISAs

Several FMDV recombinant NSPs, e.g. 3ABC, 3AB, 3A, 3B, 3C, 2A, 2B, and 2C have been used as target antigens in FMDV blocking and indirect ELISAs. Among these, antibodies against the 3ABC polyprotein are the most sensitive indicator of FMDV replication (Grubman, 2005; Henderson, 2005). Brocchi et al. (2006) compared four commercial NSP ELISAs and the OIE index screening assay using serum samples (n = 3551) from vaccinated and unvaccinated cattle, pigs, and sheep exposed to FMDV (Table 1). Diagnostic specificity was adequate for all tests (97 to 98%) and all tests displayed excellent diagnostic sensitivity (100%) when testing samples from recently exposed, unvaccinated animals. However, detection rates were much lower when testing vaccinated/exposed animals. As discussed previously, NSP antibody ELISAs can play a key role in verifying the status of countries considered FMD-free with vaccination.

3.0 Sampling and sample types

3.1 Serum

Transmission of FMDV can occur via respiratory, oral, or percutaneous exposure (Alexandersen et al., 2003a). The initial replication of virus usually occurs at the site of entry followed by spread to regional lymph nodes through the circulatory system (Henderson, 1948). Viremia appears as soon as 24 hours post-exposure (Alexandersen et al., 2002a, 2003a,b; Cottral and Bachrach, 1968; Kitching et al., 2002a; Murphy et al., 2010). Viremia typically lasts 4 to 5 days in ruminants and 2 to 10 days in pigs, although the level of viremia is usually higher in pigs than in ruminants (Alexandersen et al., 2001, 2002b,c2003 a,b; Alexandersen and Donalsdon, 2002; Hughes et al., 2002; Murphy et al., 2010; Stenfeldt et al., 2016).

Serum specimens are useful for detection of FMDV during viremia, i.e., serum samples collected ≤ 7 days post-infection can be used for FMDV detection by virus isolation, rRT-PCR, and antigen capture ELISA, with later samples useful for antibody detection. In cattle and pigs, Alexandersen et al. (2002) reported the appearance of ELISA-detectable FMDV serum antibody by 5 days post inoculation (DPI) and neutralizing antibodies ≤ 2 days later (Alexandersen et al., 2002a, 2003a). In sheep, ELISA-detectable serum antibody appeared by 9 DPI and neutralizing antibody between 6 and 10 DPI (Armstrong et al., 2005). Coincident with the first detection of antibody is the progressive clearance of virus from circulation and a reduction of virus in most tissues, with the exception of the pharyngeal region of ruminants (Alexandersen et al., 2003b; McCullough et al., 1992). Paired serum samples collected 7 to 14 days apart may be used to diagnose FMDV on the basis of rising antibody levels in response to infection. Serum antibody remains at high levels for several months post-infection and is detectable for years, with the exception that FMDV specific antibody may be detected for only a few months in young pigs (Alexandersen et al., 2003a). The use of filter papers for antibody detection or FTA cards for nucleic acid detection has been reported as a method to achieve diagnosis without the need to refrigerate or freeze serum samples (OIE, 2008).

3.2 Vesicular epithelium and fluid

During viremia, FMDV is distributed to secondary replication sites, i.e., tongue epithelium, nasal mucosa, salivary glands, coronary band epithelium, myocardium, kidney, spleen, and liver (Alexandersen et al., 2001, 2003a). Viral amplification occurs mainly in cornified stratified squamous epithelium, e.g. feet, teats, dental pad, gum, tongue, and lip, resulting in the formation

364 of liquid-filled vesicles (Alexandersen et al., 2001; Arzt et al., 2011a,b; Oleksiewicz et al., 2001).
365 FMDV replication in pharyngeal epithelial and lymphoid tissues of cattle, sheep, and goats
366 occurs in both the acute and persistent phases of disease (Alexandersen et al., 2001, 2003a).
367
368 Depending on the route of introduction, vesicles become visible 1 to 3 days after exposure
369 (Alexandersen et al., 2001, 2003a; Arzt et al., 2011a; Murphy et al., 2010). However, subclinical
370 infection is common in small ruminants, e.g. sheep and goats (Cardassis et al., 1966; Gibson and
371 Donaldson, 1986; Kitching et al., 2002; McVicar and Sutmoller, 1972; Pay, 1988). If present,
372 vesicles are generally on the feet of small ruminants, e.g. sheep and goats (Cardassis et al., 1966;
373 Gibson and Donaldson, 1986; Littlejohn, 1970; Pay, 1988). If oral lesions are present in small
374 ruminants, they commonly occur on the dental pad, rather than tongue as occurs in cattle
375 (Geering, 1967). Vesicular fluid from unruptured vesicles on the dental pad, gum, tongue, lip, or
376 feet of clinically affected animals is an ideal specimen for FMDV identification because it
377 contains a high concentration of virus (there no reports of antibody detection in vesicular fluid)
378 (Alexandersen et al., 2001). However, vesicular fluid is generally only present in 1 to 2 day-old
379 lesions before they have ruptured. Alternatively, vesicular epithelium from ruptured lesions can
380 be collected. FMDV can be detected in these samples up to 10-14 days (Alexandersen et al.,
381 2003). These samples are stored in glycerine containing 0.04 M phosphate buffer 382 saline
382 (PBS, pH 7.6) (Ferris and Dawson, 1988). This specimen can be crushed with sterile sand or
383 beads and then mixed with laboratory medium to make a 10% suspension for diagnostic analysis
384 by virus isolation, rRT-PCR, and/or antigen-capture ELISA (Alexandersen and Donaldson,
385 2002; Oliver et al., 1988; Reid et al., 2001, 2002; Sakamoto et al., 2002). Presently, in a
386 clinically suspect case, FMDV RNA can be detected directly from dry vesicular material by

387 homogenized with RNA extraction kit's lysis buffer followed by rRT-PCR (Howson et al., 2017;
388 2018). Collection of vesicular fluid and epithelium are most appropriate in the acute stage of
389 infection. Both specimens are the sample of choice for FMDV detection using RT-PCR,
390 antigen-capture ELISA, and antigen-lateral flow device (OIE, 2017).
391
392 **3.3 Buccal samples**
393 FMDV replicates in pharyngeal epithelial tissues and may be detected in esophageal-
394 oropharyngeal fluid by 24 hours post-exposure (Salt, 1993). In ruminants, FMDV replication in
395 pharyngeal epithelial tissues is protracted, i.e., the virus may be isolated from esophageal-
396 oropharyngeal fluid samples for up to 9 months in sheep and 3.5 years in cattle (Arzt et al.,
397 2011a,b; Juleff et al., 2008; McVicar and Sutmoller, 1969; Straver et al., 1970; Zhang and
398 Kitching, 2001). In swine, infectious FMDV is present in most buccal samples for < 28 days
399 (oral fluid, nasal swab, esophageal-oropharyngeal fluid, tissues of the pharynx, tonsil, tongue,
400 epiglottis, larynx, soft palate, nasopharynx, lung), although FMDV RNA was still detected in the
401 tonsils of the soft palate at 28 DPI (Arzt et al., 2011b; Stenfeldt et al., 2016; Zhang and
402 Bashiruddin, 2009).
403
404 Probang sampling was first described as a method to collect esophageal-oropharyngeal fluid
405 from ruminants by Sutmoller and Gaggero (1965). The sample is collected by inserting a small
406 metal cup ("probang cup") on a long shaft through the mouth and into the pharyngeal region,
407 thereby allowing the esophageal-oropharyngeal secretions to pool in the cup. Different sizes of
408 probang cups are used, depending on the ruminant species. Probang sampling from pigs has
409 only been reported under research conditions (Parida et al., 2007; Stenfeldt et al., 2013).

Although esophageal-oropharyngeal fluid samples are the only method that offers a realistic chance of detecting FMDV in late-stage infection and in persistently infected ruminants, probang sampling is labor-intensive (involves several persons), requires technical skill, and necessitates animal restraint during the collection process (Kitching, 2002; Kitching and Alexandersen, 2002; Kitching and Hughes, 2002). Stenfieldt et al. (2013) reported that farmers were reluctant to allow probang sampling because of concerns that the collection process might harm their animals.

Oral fluid samples from pigs and cattle have been used to detect FMDV antibody and/or nucleic acid (Alexandersen et al., 2003b; Callens et al., 1998; Grau et al., 2015; Mouchantat et al., 2014; Parida et al., 2006; Parida et al., 2007; Senthilkumaran et al., 2017; Stenfeldt et al., 2013; Vosloo et al., 2015). Oral fluid samples can be collected from individual animals using various absorbent materials or from groups housed in the same space (pens or corrals) by allowing them to chew on rope suspended in the pen (Alexandersen et al., 2003b; Kittawornrat et al., 2010; Mouchantat et al., 2014; Prickett et al., 2008; Senthilkumaran et al., 2017; Stenfeldt et al., 2013; Vosloo et al., 2015). Oral fluid collection is simple, non-invasive, rapid, and cost-effective; for which reasons it has been widely applied to livestock surveillance, especially swine (Prickett and Zimmerman, 2010). FMDV can be detected in oral fluid samples by RT-PCR for up to 15 DPI in cattle, 8 DPI in sheep, and more than 27 DPI in pigs (Alexandersen et al., 2003b; Parida et al., 2007).

Conventional inactivated FMDV vaccines induce only a systemic antibody response whereas viral replication in infected animals produces both systemic and mucosal immune responses

(McCullough et al., 1992). Therefore, FMDV infection results in antibody-positive oral fluid or esophageal-oropharyngeal fluid samples, but vaccinated animals remain antibody negative (DIVA) (Kitching, 2002b; Parida et al., 2006). Virus neutralization assays and IgA-specific ELISAs for esophageal-oropharyngeal or oral fluid samples have been developed to detect FMDV infected animals in vaccinated populations (Amadori et al., 2000; Archetti et al., 1995; Biswas et al., 2008; Eblé et al., 2007; Mohan et al., 2008; Pacheco et al., 2010; Parida et al., 2006; Salt et al., 1996; Stenfeldt et al., 2016). Using an experimental ELISA based on a 3ABC polyprotein, FMDV-specific IgA was detected in oral fluids from pigs by 14 DPI (Senthilkumaran et al., 2017). Earlier workers reported that FMDV-specific IgA could be detected in esophageal-oropharyngeal or oral fluid samples for up to 182 DPI in cattle and 112 DPI in pigs (Eblé et al., 2007; Mohan et al., 2008).

3.4 Mammary secretions

In 1968, Burrows reported that FMDV appeared in the milk of cattle exposed to infected animals an average of 2.2 days before clinical signs. Subsequent experiments showed extensive viral replication in bovine mammary gland parenchyma beginning 8 to 32 hours post exposure (Alexandersen et al., 2003b; Burrows, 1971). FMDV can also be detected in pig, sheep, and goat milk coincident with the appearance of viremia, but higher viral titers are present in sheep milk vs serum, suggesting either FMDV replication in small ruminant mammary gland tissue and/or the concentration of virus in milk (Arzt et al., 2011a,b; Burrows et al., 1968; McVicar et al., 1977). Blackwell et al. (1981) reported that FMDV could be shed in cattle mammary secretions for up to 14 DPI and was detectable in pasteurized whole milk, skim milk, cream, and cellular components in mammary secretions. Using rRT-PCR, FMDV nucleotide can be detected in

456 cattle milk for up to 23 days. These data justify the testing of bulk tank milk samples by RT-
457 PCR for the early detection of FMDV in dairy herds (Reid et al., 2006). Modeling the
458 concentration of FMDV in bulk milk as a function of the number of cows shedding virus at any
459 point in time, Thurmond and Perez (2006) predicted that FMDV nucleic acids could be detected
460 in bulk tank milk samples between 2.5 and 6.5 days post-exposure, depending on the within-herd
461 transmission rate. Further, it was predicted that nucleic acid could be detected in bulk tank milk
462 before 10% of the cows showed clinical signs.
463
464 Individual and bulk tank milk samples have also been tested for FMDV-specific antibody, either
465 for detection or for monitoring the response to vaccination (Armstrong and Mathew, 2001; Fayed
466 et al., 2013; Rémond et al., 2002; Thurmond and Perez, 2006). Serum antibody is concentrated
467 into mammary secretions by active transport mediated by neonatal Fc receptors on the
468 basolateral surface of the mammary epithelial cells. As a result, mammary secretion collected
469 from FMDV-infected cattle can contain higher levels of antibody than serum (Stone and DeLay,
470 1960). FMDV neutralizing antibody can be detected in mammary secretions within 7 days after
471 exposure in cattle (Stone and Delay, 1960). ELISA-detectable FMDV antibody can be detected
472 in mammary secretions for up to 12-months post-vaccination in cattle, 24 weeks post-vaccination
473 in pigs, and 83 days post-vaccination in sheep (Armstrong, 1997; Blackwell et al., 1982;
474 Burrows, 1968; de Leeuw et al., 1978; Francis and Black, 1983; Kim et al., 2017).
475
476 **3.5 Nasal and upper respiratory tract secretions**
477 Respiratory tract mucosa is the initial site of FMDV replication and the virus is present in both
478 upper and lower respiratory tract secretions during the acute phase of infection (Alexandersen et

al., 2003a,b; Donaldson and Ferris, 1980; Korn, 1957). The specimens can be used in preclinical diagnosis because FMDV RNA may be detected in nasal swabs from one day before clinical signs through 10 to 14 days after the appearance of serum antibodies (Alexandersen et al., 2003a,b; Callahan et al., 2002; Marquardt et al., 1995). In pigs, FMDV RNA can be detected in nasal swabs from 6 hours through 7 DPIs, i.e., up to 2 days after the appearance of serum antibody (Alexandersen et al., 2003a).

3.6 Aerosols

Airborne infectious FMDV can be resuspended from any FMDV source that can become aerosolized, e.g., from secretions or excretions produced in respiratory, oral, and/or pedal epithelia (Brown et al., 1992; Burrows et al., 1981; Sorensen et al., 2000; Sutmoller et al., 1976). Re-analysis of epidemiological and meteorological data collected during the 1982–1983 epidemic in Denmark suggested that FMDV was aerosolized and transmitted over a distance of 70 km (Christensen et al., 2005). Infectious FMDV can be detected in respiratory exhalations 1 to 6 days post-exposure in cattle (Alexandersen et al., 2003a). FMDV RNA can be detected in respiratory exhalations 6 hours to 4 days post-exposure in pigs (Alexandersen et al., 2001; Oleksiewicz et al., 2001). Notably, pigs aerosolize more virus than ruminants, i.e., $1 \times 10^{6.1}$ median tissue culture infective dose ($TCID_{50}$) per day in pigs (Sellers, 1971) compared to $1 \times 10^{4.3}$ $TCID_{50}$/day in cattle and sheep (McVicar and Sutmoller, 1976), because the virus replicates more extensively in swine respiratory mucosa (Alexandersen and Donaldson, 2002; Alexandersen et al., 2002a,b,c; Arzt et al., 2011a; Oleksiewicz et al., 2001). In sheep, FMDV was detectable in respirations 17 hours to 13 days post-exposure, i.e., FMDV is shed in aerosol 1 to 2 days before the appearance of clinical signs (Alexandersen et al., 2002b; Burrows, 1968;

Sellers et al., 1969). Experimentally, cattle and sheep can be infected by airborne exposure to as little as 1 x 10^1 $TCID_{50}$, whereas pigs require more than 1 x 10^3 $TCID_{50}$ (Alexandersen and Donaldson, 2002; Alexandersen et al., 2002a; Donaldson and Alexandersen, 2001; Stenfeldt et al., 2016).

Air samples for FMDV detection have been collected using a variety of sampling devices. Pacheco et al. (2017) reported that air samples containing FMDV RNA can be collected by pulling air in a room containing FMDV infected cattle through Fluoropore membrane filter (1.0 µm) or polyester filter disc (1.0 µm) using an air pump (4.6 to 144 L/min air flow capacity) for 24 hours. The filters were then cutted into 433 nm^3 pieces, then disrupted by glass beads and tissue mixer system. FMDV RNA could be extracted from the pieces of filter using column RNA extraction kit using procedure described elsewhere (Pacheco et al., 2012). Exhaled air containing FMDV RNA from infected cattle can be collected individually using a microchip-based hand-held air sampling device (Ilochip A/S, Denmark). FMDV RNA can be harvested by washing the chip chamber with 25 µl of 0.1% (v/v) TritonX-100 solution (Sigma-Aldrich) following by RNA extraction (Oem et al., 2005).

Aerosol sampling has primarily been a research tool for understanding and modeling the transmission of FMDV over distances, but theoretically, on-farm air sampling could be used for pre-clinical non-invasive FMDV surveillance. Such a system would need to account for the fact that viral aerosols are highly dynamic, non-uniform, and subject to atmospheric and climactic conditions (Verreault et al., 2008).

3.7 Other sample types

Information concerning the shedding and/or detection of FMDV in urine and/or feces from FMDV-susceptible species is sparse, but shedding of FMDV in cattle urine and feces between 2 and 6 DPI has been reported (Garland, 1974; Bachrach, 1968). FMDV may be resistant in the environment, depending on the virus strain and the ambient conditions, and has been detected by virus isolation for up to 39 days in cattle urine and 14 days in feces (Alexandersen et al., 2003a; Bachrach, 1968; Cottral, 1969; Donaldson et al., 1987; McColl et al., 1995). In general, urine and feces have not been considered suitable diagnostic specimens because they contain little virus and are likely to be mixed with environmental contaminants and other body fluids (Alexandersen et al., 2003a; Parker, 1971). However, in the context of molecular diagnostics, these sample types may deserve further evaluation in terms of their suitability for environmental surveillance and monitoring.

Conclusions

FMDV remains an important pathogen of livestock more than 120 years after it was first identified because it is highly contagious, genetically and antigenically diverse, infectious for a wide variety of species, able to establish subclinically infected carriers in some species, and widely geographically distributed (Brito et al., 2017). The "burden of disease" imposed by FMDV is economically astonishing. Globally, Knight-Jones et al. (2013), estimated the annual costs from production losses and vaccination at €5.3 to €17 billion EURO ($6.5 to $21 billion USD) in FMDV-endemic areas. In FMDV-free areas, they estimated the annual costs of FMDV outbreaks at ≥ €1.2 billion EURO ($1.5 billion USD).

With good reason, the World Animal Health Organization (OIE) and the Food and Agriculture Organization (FAO) have proposed the global eradication of FMD by the year 2030 (Rodriguez and Gay, 2011). This objective creates the needs for alternative control methods, i.e., vaccines that provide broad-range protective immunity and diagnostic methods that can differentiate the vaccinated from infected animals. Nevertheless, eradication is not feasible without the inclusion of accurate, cost-effective surveillance.

Historically, FMDV surveillance has been typically based on individual animal serum, vesicular fluid, or epithelial samples. Although current methods are still necessary for FMDV diagnoses, individual animal sampling/testing is impractical and expensive for surveillance in countries endemic with the disease. In an outbreak scenario, it would be feasible for individual sampling to occur. However, FMDV and/or antibody are also present in other body secretions, e.g., buccal and nasal secretions, respiratory exhalations (aerosols), mammary secretions, urine, feces, and environmental samples (Table 2). Alternative specimens can be used to support control and/or elimination programs by enabling herd-level sampling for FMDV surveillance at a lower cost and with less effort. Future research should focus on the development of diagnostic assays able to exploit the detection opportunities offered by alternative specimens because without these tools the goal of FMDV eradication is unlikely to succeed.

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Table 1. Detection of FMDV infection in cattle using non-structural protein-based ELISAs (modified from Brocchi et al., 2006)[a]

	Days post-exposure	n	Percent positive (95% confidence interval[b])				
			3ABC ELISA[c]	3ABC ELISA[d]	3ABC ELISA[e]	3ABC ELISA[f]	3B ELISA[g]
1. Non-vaccinated cattle exposed to infection (n = 54)	7 - 14	5	100 (48, 100)	100 (48, 100)	100 (48, 100)	100 (48, 100)	100 (48, 100)
	15 - 27	27	100 (87, 100)	100 (87, 100)	100 (87, 100)	100 (87, 100)	100 (87, 100)
	28 - 100	26	100 (87, 100)	100 (87, 100)	96 (80, 100)	92 (75, 100)	100 (87, 100)
2. Vaccinated cattle exposed to infection (n = 285)	7 - 14	180 -181	49 (41, 56)	49 (41, 56)	41 (34, 49)	50 (43, 58)	32 (26, 40)
	15 - 27	131	60 (51, 69)	53 (45, 62)	50 (42, 59)	53 (44, 61)	38 (30, 47)
	28 - 100	107 - 108	69 (60, 78)	64 (54, 73)	58 (49, 68)	50 (40, 61)	56 (46, 65)
	>100	47	72 (57, 84)	75 (60, 86)	57 (42, 72)	38 (25, 54)	47 (32, 62)

[a]Cattle serum samples obtained from experimental and known-status field animals

[b]95% confidence intervals calculated from proportional data given in Brocchi et al., 2006.

[c] NCPanaftosa-screening (Panaftosa, Pan American Health Organization, Rio de Janeiro, Brazil).

[d]Ceditest® FMDV-NS (Cedi diagnostics B.V., Lelystad, The Netherlands. Currently produced and marketed as Priocheck® FMDV-NS by Thermo Fisher Scientific Prionics Lelystad B.v., Lelystad, The Netherlands).

[e] SVANOVIR™ FMDV 3ABC-Ab ELISA (Svanova, Upsala, Sweden).

[f] CHEKIT-FMD-3ABC (Bommeli Diagnostics/Idexx, Bern, Switzerland).

[g]UBI® FMDV NS ELISA (United Biomedical Inc., New York, USA).

Table 2. Temporal range for the detection of FMDV and/or viral components in alternative specimens

Species	Assay	Specimen	DPI[a]	References
Cattle	rRT-PCR	Serum	1 - 6	Alexandersen et al., 2003, Stenfeldt et al., 2013
		Probang sample	1 - 553	Alexandersen et al., 2002; Moonen et al., 2004; Stenfeldt et al., 2013; Subramanian et al., 2012
		Buccal sample[b]	1 - 15	Alexandersen et al., 2003; Stenfeldt et al., 2013
		Nasal swab	3 - 18	Subramanian et al., 2012
		Feces	4 - 8	de Rueda et al., 2015
	Virus isolation	Serum	1 - 8	Burrows, 1968, Blackwell et al., 1982
		Respiratory exhalation	1 - 4	Alexandersen et al., 2003
		Probang sample	1 - 469	Blackwell et al., 1982; Burrows, 1968; de Leeuw et al., 1978; Moonen et al., 2004; Subramanian et al., 2012
		Nasal swab	3 - 5	Subramanian et al., 2012
		Milk	1 - 13	Blackwell et al., 1982; Burrows, 1968; de Leeuw et al., 1978
Swine	Ag-ELISA	Buccal sample[b]	1 - 7	Morioka et al., 2014; Senthilkumaran et al., 2017
	rRT-PCR	Serum	1 - 11	Alexandersen et al., 2003; Doel et al., 2009; Senthilkumaran et al., 2017; Stenfeldt et al., 2013
		Buccal sample[b]	1 - 27	Alexandersen et al., 2003; Grau et al., 2015; Mouchantat et al., 2014; Parida et al., 2007; Senthilkumaran et al., 2017; Stenfeldt et al., 2013; Vosloo et al., 2015
		Respiratory exhalation	1 - 5	Doel et al., 2009; Parida et al., 2007
		Pharyngeal swab	1 - 15	Mouchantat et al., 2014
		Probang sample	1 - 27	Parida et al., 2007; Stenfeldt et al., 2013
		Nasal swab	1 - 14	Alexandersen et al., 2003; Parida et al., 2007; Senthilkumaran et al., 2017
		Feces	3 - 11	Fukai et al., 2015
	Virus isolation	Serum	1 - 4	Alexandersen et al., 2003
		Buccal sample[b]	1 - 5	Parida et al., 2007; Senthilkumaran et al., 2017
		Respiratory exhalation	1 - 5	Alexandersen et al., 2003; Parida et al., 2007
		Pharyngeal fluid	2 - 10	Burrows, 1968
		Nasal swab	2 - 5	Parida et al., 2007
		Feces	3 - 4	Fukai et al., 2015
		Rectal swab	1 - 7	Burrows, 1968

[a]Day post inoculation (DPI) represents the minimum and maximum detection points reported.
[b]Buccal samples including samples collected with cotton swabs, cotton rope, or rope-in-a-bait collection devices