Selection and use of reference panels: a case study highlighting current gaps in the materials available for foot and mouth disease

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Summary

The World Organisation for Animal Health (OIE) *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* describes a diverse array of assays that can be used to detect, characterise and monitor the presence of infectious agents of farmed livestock. These methods have been developed in different laboratories, at different times, and often include tests or kits provided by the commercial sector. Reference panels are essential tools that can be used during assay development and in validation exercises to compare the performance of these varied (and sometimes competing) diagnostic technologies. World Organisation for Animal Health Reference Laboratories already provide approved international standard reagents to help calibrate diagnostic tests for a range of diseases, but there remain important gaps in their availability for comparative purposes and the calibration of test results across different laboratories. Using foot and mouth disease (FMD) as an example, this review highlights four specific areas where new reference reagents are required. These are to: reduce bias in estimates of the diagnostic sensitivity and inter-serotypic specificity of tests used to detect diverse strains of FMD virus (FMDV), provide bio-safe positive controls for new point-of-care test formats that can be deployed outside high containment, harmonise FMDV antigens for post-vaccination serology, and address inter-laboratory differences in serological assays used to measure virus-specific FMD antibody responses. Since there are often limited resources to prepare and distribute these materials, sustainable progress in this arena will only be achievable if there is consensus and coordination of these activities among OIE Reference Laboratories.

Keywords

Assay validation – Case study – Diagnostic testing – Foot and mouth disease – Foot and mouth disease vaccine – Gaps – Inter-laboratory calibration – Reagent – Reference panel.

Introduction

Accurate and robust diagnostic tests provide essential evidence to support international initiatives to detect, monitor and control livestock diseases, and to support safe trade in animals and animal products. A diverse landscape of different test technologies is recognised by the World Organisation for Animal Health (OIE). Each assay included in the OIE *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* (OIE *Terrestrial Manual*) has been validated and shown to be fit for its specific purposes (1).

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Reference panels play an important role during:

a) assay development

b) method validation and adoption

c) continuous assessment of the performance of established tests, including batch-to-batch consistency.

Examples of these materials include well-characterised viral isolates, viral or engineered nucleic acid, and sera collected from host species with a well-defined provenance. These reagents can be used to define the minimum requirements for test sensitivity and specificity, ensuring that assays are validated to similar standards. This makes it possible for laboratories to establish their own secondary standards and allows scientists to compare the performance of different tests and different batches of reagents or kits when they are used in different laboratories. The simple definition provided by the OIE describes reference materials as: 'substances whose properties are sufficiently homogeneous and well established to be used for the calibration of an apparatus, the assessment of a measurement method, or for assigning values to materials', and the important contribution of these materials to the development and validation of new assays is highlighted in Chapter 2.2.6. (2).

Although these points are widely recognised, laboratories and producers of diagnostic kits may often experience challenges in accessing appropriate reference panels, and there are important gaps in the availability of these materials for most diseases. For example, the current list of OIE-approved international standard reagents only covers a limited number of diseases and tests (3).

This review focuses on foot and mouth disease (FMD) to highlight specific areas where new reference panels are required to improve the routine use of diagnostic tests to detect FMD virus (FMDV) and FMDV-specific antibodies (including tests used to monitor post-vaccination responses in different hosts). It is important that these reagents are regularly updated from our increasing knowledge of FMD epidemiology throughout the world to reflect the genetic and antigenic diversity of established and emerging viral lineages (and immune responses against these viruses and vaccines generated in susceptible host species). In this way, they can be designed to address the specific purposes of validated assays, as well as new diagnostic tests that might be developed in the future.

Diagnostic challenges for foot and mouth disease

Foot and mouth disease is a transcontinental disease which reduces the productivity of animals and has subsequent impacts upon rural livelihoods and the trade in livestock and animal products. The disease is caused by FMDV (family: *Picornaviridae*; genus: *Aphthovirus*), which is endemic in Africa, many countries in Asia and parts of South America. Foot and mouth disease virus is an RNA virus that exists as six co-circulating serotypes (the seventh, serotype C, is most likely extinct), and the high evolutionary rate of FMDV constantly gives rise to new viral variants that have the potential to threaten livestock industries in different parts of the world.

For the purposes of this review, FMDV is used as a case study, to highlight the challenges of developing robust and reliable diagnostic assays to detect a virus that exhibits a high degree of genetic and antigenic variability. Recommended laboratory assays that can be used for routine FMD testing and provide mutually recognised results to facilitate international trade are described in the OIE *Terrestrial Manual* (Chapter 3.1.8.) (4). These standardised diagnostic tests are used to detect and characterise FMDV to support the clinical diagnosis of field outbreaks and to measure FMDV-specific antibodies (for diagnosis, population surveillance or to monitor vaccine-specific responses) (5).

The inherent genetic and antigenic diversity of FMDV influences the performance of these diagnostic assays. These factors motivate reference and other FMD laboratories to undertake surveillance to monitor the circulation of viruses in the field and to regularly perform local validation exercises to ensure that tests can detect contemporary viral strains. In order to help with the validation of new tests, well-characterised virus isolates, representative clinical material and sera are made available by laboratories within the OIE and Food and Agriculture Organization of the United Nations (FAO) FMD Reference Laboratory Network (https://www.foot-and-mouth.org).

Despite these endeavours, there is still a large degree of bias in the reagents that are supplied, and it is sometimes difficult to access key reagents for testing (such as vaccine antigens and antisera from commercial sources). The authors highlight four areas where new reference panels and reagents for FMD diagnostic tests could be established, to improve confidence in assay performance and to help calibrate the test results that are generated between different laboratories.

Reference panels to define the diagnostic sensitivity of tests used to detect diverse isolates of foot and mouth disease virus

The tests to detect FMDV described in the OIE *Terrestrial Manual* include antigen enzyme-linked immunosorbent

assay (Ag–ELISA), a serotype-specific indirect ELISA that detects the antigen in a clinical sample (6, 7); real-time reverse transcription polymerase chain reaction (RT–PCR) (8, 9); and lateral-flow devices (10, 11). However, a formal assessment of the diagnostic sensitivity of these tests is complicated by the genetic and antigenic variability that exists in naturally occurring field isolates of FMDV. In the case of FMDV, biosafety regulations that place restrictions on the distribution of live viruses and derivative materials to different laboratories, and national rules that prevent the supply or importation of certain viruses, can result in the limited availability of FMD strains. Thus, many laboratories (as well as commercial companies) only have a very restricted range of archived strains available to be used to define test performance, and it is inevitable that assay validation exercises are biased by the extent of FMDV isolates and materials that are locally available. Consequently, published values for the diagnostic sensitivity and specificity of tests should always be carefully examined to consider whether the reported values are unduly influenced by gaps in the coverage of the viruses included in the validation panel. These challenges are not unique to FMDV and the recent emergence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has highlighted the importance of reference reagents so that diagnostic tests can be rapidly validated and calibrated (12).

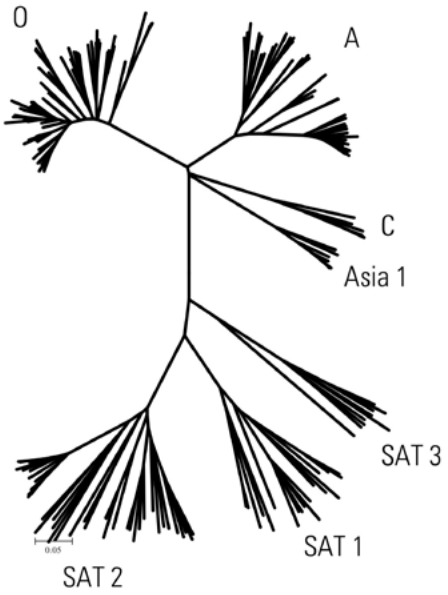


Fig. 1
Phylogenetic representation of foot and mouth disease virus reference antigens selected for assay validation purposes
The neighbour-joining phylogenetic tree is drawn to represent the genetic diversity of viral protein 1 (VP1) sequences for each of the candidate reference antigens (9). Further details of these viruses can be requested from the Pirbright Institute, United Kingdom

One solution to this problem is to prepare a common reference virus panel that is representative of the antigenic/genetic diversity of FMDV and to make this widely available to diagnostic laboratories. To address this issue, the OIE Reference Laboratory for FMD at Pirbright (United Kingdom) has recently described a set of isolates that could be used for this purpose (13). At present, the prototype panel consists of 203 isolates, covering all seven FMDV serotypes: O (n = 71), A (n = 42), C (n = 5), Southern African Territories (SAT) 1 (n = 20), SAT 2 (n = 44), SAT 3 (n = 9) and Asia 1 (n = 12). These isolates were selected on the basis of phylogenetic analyses (Fig. 1), to most efficiently cover the global diversity and geographically distinct evolutionary lineages (topotypes) of FMDV. Each virus isolate has been propagated in baby hamster kidney 21 (BHK-21) cells and the homogeneous nature of these materials is confirmed by sequence analyses and by antigen-detection ELISA. Representative subsets of this panel have been recently used in the validation of two antigen-detection ELISAs and a real-time RT–PCR (data in preparation).

While a subset of this panel is currently available from the European Virus Archive – Global (EvaG) consortium (www.european-virus-archive.com/), further work is required to scale up production of suitable batches of these materials for shipment to other laboratories. It is expected that these reference FMDVs could be provided as innocuity-tested binary ethyleneimine (BEI)-inactivated antigens, so that they are suitable for use in validating immunoassays and molecular tests that are not dependent on live FMDV. This work could encompass the definition of intra-serotype sensitivity and inter-serotype specificity of newly developed real-time RT–PCR assays that can detect and discriminate among different FMDV lineages (14, 15).

Live replication-competent virus strains could also be supplied for those laboratories with suitable high-containment facilities but, in view of the limits placed by the International Air Transport Association (IATA) on the total volume of infectious biological material that can be included in a shipment, a smaller sub-panel may be more appropriate for these purposes. These reference reagents should provide a more standardised approach to evaluating and comparing the performance of diagnostic assays and kits, although regular review by the international reference laboratories will be required to ensure that material in these panels is kept updated and remains representative of contemporary circulating viruses.

Reference control materials for molecular tests

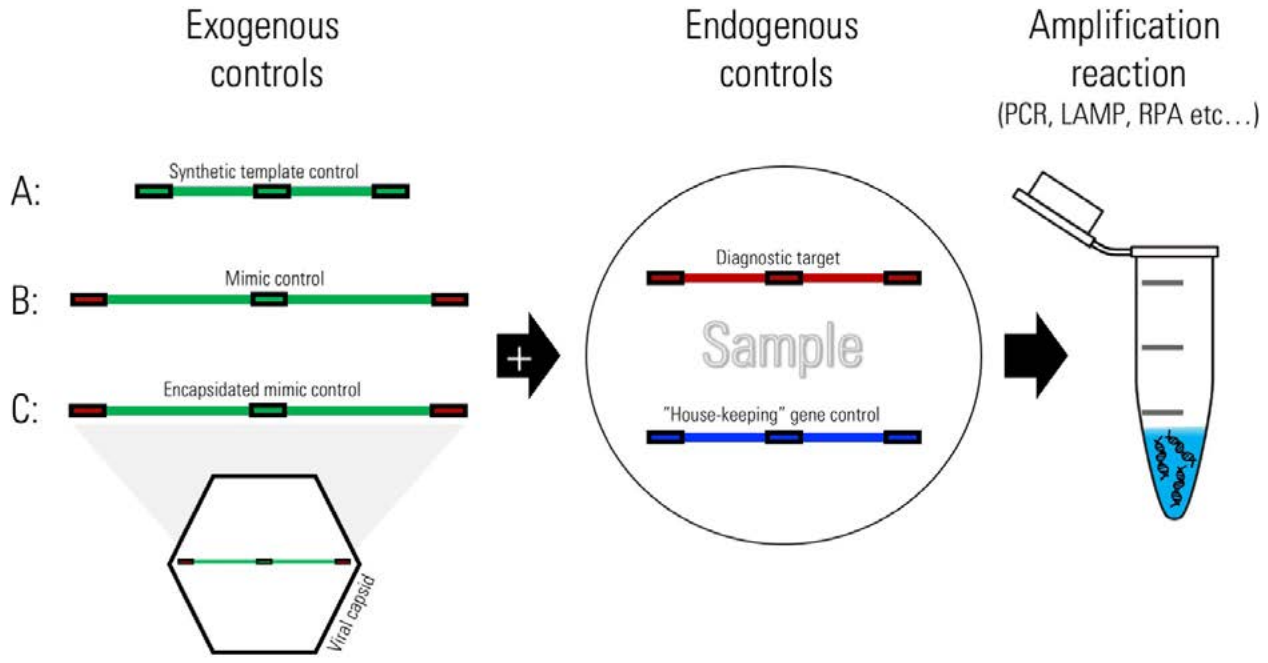
Molecular tests such as real-time RT–PCRs are widely used as front-line tests by national and international

FMD Reference Laboratories (8, 9). Polymerase chain reaction tests are dependent upon temperature-sensitive enzymes. Such enzymes can be inhibited by tissue factors, causing false-negative results (16). Therefore, it is usually recommended that testers use internal controls (reviewed by Yan *et al.* [17]) to confirm that there has been no inhibition of the amplification reaction. When used to validate test results, internal controls should ideally have a shelf life that is appropriate for long-term storage (>12 months) and not readily degrade. Internal controls are broadly classified as: *(a)* exogenous nucleic acid template controls or *(b)* endogenous host 'house-keeping' gene controls (Fig. 2).

Exogenous internal controls represent artificial sequences, not expected to be naturally present in a sample, that are added and co-amplified with the viral target. Exogenous internal controls can be added to the sample before processing to allow the efficiency of the assay (including the potential loss of nucleic acid during extraction) to be monitored. Generic exogenous internal controls are already available for molecular diagnostics and this approach has been adopted for laboratory-based, real-time RT–PCR assays for FMDV (18, 19).

In practice, exogenous controls have two weaknesses. First, they do not provide any way of verifying the quality of the nucleic acid template in a sample. This is particularly important for samples that might be received from the field where they could be degraded, due to delays in shipping and inadequate temperature control (i.e. disruption of the cold chain). Second, since exogenous controls require a second set of primers to amplify the exogenous control target, they do not directly confirm that the RT–PCR components specific for the viral target are working correctly.

Endogenous internal controls (such as glyceraldehyde-3-phosphate [GADPH]; beta-actin [β-actin]; 18S ribosomal RNA; glutamate decarboxylase [GAD], and β2-microglobulin) have the advantage that they can be used to confirm that samples have been correctly collected and contain intact template RNA (20). However, they also require a second reaction to amplify the target sequences.



LAMP: loop-mediated isothermal amplification
PCR: polymerase chain reaction
RPA: recombinase polymerase amplification

Fig. 2

An overview of different strategies that can be used to incorporate internal controls into molecular diagnostic tests (polymerase chain reaction, loop-mediated isothermal amplification and recombinase polymerase amplification)

This figure highlights specific sequences that are targeted by real-time reverse transcription polymerase chain reaction (PCR) tests. The boxes denote the location of polymerase chain reaction oligonucleotides and probe detection sites. Similar approaches can be adopted for other molecular assay technologies

Mimic sequences comprise target sequences that can be amplified by virus-specific PCR, and a heterologous probe sequence that is typically detected using a probe conjugated to a second fluorophore that is distinct from the virus-specific assay

Duplex and triplex real-time RT–PCR assays that use the 3D and 5' untranslated region (UTR) FMDV targets outlined in the OIE *Terrestrial Manual*, and that include β-actin internal control targets (21) or β-actin internal control targets and synthetic RNA exogenous internal control targets (22), respectively, have been developed. Mimic internal controls (*in vitro* transcripts, plasmids or chimeric viruses [23]) can be engineered to contain homologous target 'viral' sequences. These can be amplified, using the same primer pairs as the diagnostic test, and also include artificial sequences so that the control can be easily differentiated from wild-type viruses using specific probes, such as those used in real-time RT–PCR assays.

To speed up the clinical diagnosis of suspected FMD cases, mobile molecular test formats employing RT–PCR, RT-loop-mediated isothermal amplification (LAMP), and recombinase polymerase amplification (RPA) are being developed and are undergoing field evaluation (24). These portable tests have high analytical sensitivity and can rapidly confirm the FMDV status of samples collected in remote settings (25). For confidence in the data generated by field tests, it is essential that these assays incorporate appropriate internal controls that are non-infectious and safe, as well as maintained in a stable and easy-to-store format. These materials are especially important in circumstances where the tests are performed by 'non-diagnosticians', away from laboratories and without established quality assurance systems. For FMD and other livestock diseases, the key requirements for such reference controls are:

a) a long shelf-life and stability suitable for use in environments across a wide range of ambient temperatures

b) high biosafety with no hazards to the test operator or potential to cause disease

c) a different genetic background from the wild-type viral strains, allowing potential cross-contamination to be easily recognised.

One approach is to use encapsidated 'armoured' RNA controls (26) to improve the stability of the labile RNA template. For FMDV, positive and negative controls using a novel approach have been prepared by engineering FMDV diagnostic sequences into cowpea mosaic virus (CPMV; family: *Secoviridae*; genus: *Comovirus*) capsids. The capsid structure of CPMV resembles picornaviruses such as FMDV but has been shown to be thermostable and ribonuclease resistant (27, 28).

Low-cost control reagents are particularly helpful for laboratories that are beginning to establish molecular methods for routine testing. In view of the continued uptake of these technologies by laboratories, and the importance of their role, more emphasis should be placed on the development and supply of the universal controls used in these tests. This should include recommendations to standardise suitable internal controls used in routine tests, as well as controls used to validate the results from 'pen-side' molecular assays.

Reference antigen panels for the assessment of foot and mouth disease vaccine performance

In common with diagnostic tests, FMD vaccines also need to cover the different serotypes and field strains circulating in various parts of the world. These strains often exhibit considerable antigenic heterogenicity. In contrast to other veterinary diseases, in which common vaccine antigens are sometimes used, the situation for FMD vaccines is complex, involving a wide range of manufacturers supplying FMD vaccines that contain different antigens, at different potencies and payloads (29). Exceptions are South America and India where the composition of FMD vaccines is prescribed and locally controlled. Foot and mouth disease vaccines should be carefully selected for their ability to elicit heterologous immune responses that protect against the specific virus lineages that threaten the target host species. The OIE *Manual* outlines how *in vitro* vaccine-matching methods and *in vivo* potency studies can be used to assess the two interconnected intrinsic factors that define whether FMD vaccines will be effective (30):

a) cross-reactivity of the antigens included in the vaccine

b) potency of the formulated product.

Together with the other essential steps that are used to monitor the quality of FMD vaccines (including tests for safety, sterility, innocuity, purity and identity), data from potency studies (designed around the *in vivo* 50% protective dose [PD_{50}] or protection against generalised podal infection [PGP]) provide evidence for formal registration of FMD vaccines. One-way antigenic relationship tests (r_1 vaccine-matching tests), using defined reference antisera from vaccinated animals, are used by FMD reference and other laboratories, as well as vaccine producers, to monitor the cross-reactive potential of vaccine antigens against identified virus threats. This current two-tier approach of vaccine matching and potency testing has limitations (30), and relies upon access to well-characterised reference antisera and 'live' master seed vaccine viruses, which are required to perform these tests. In addition, it is important to recognise that these methods usually focus on the assessment of single monovalent vaccine antigens and do not easily accommodate multivalent vaccines, which are often supplied by producers to cover different serotypes or strains. Although the performance of vaccines containing

multiple components can be studied in animal potency models, the scope of work that can be undertaken is usually constrained by ethical considerations, the need to perform these tests in high-containment facilities and finances. As a consequence, empirical data to demonstrate that vaccine products generate adequate heterologous responses are often lacking, although evidence to show homologous vaccine performance is usually available.

To reduce doubts about FMD vaccine performance, the OIE/FAO FMD Laboratory Network has proposed a modified approach to assess heterologous responses of FMD vaccines. Rather than using monovalent antisera from vaccinated animals, sera collected from animals vaccinated with the final formulated product can be tested against common FMDV antigen panels representing the viral risks in the region that would be targeted by the vaccine. As part of an OIE Twinning project with the Pan African Veterinary Vaccine Centre of the African Union (AU–PANVAC, Ethiopia), work is under way to establish reference antigen panels. These panels would be used to evaluate the serological responses of FMDV vaccines in the context of their suitability for use in eastern African countries. This particular panel is tailored to cover the genetic diversity within the FMDV lineages that circulate in eastern African countries and includes 16 FMD viruses from the following contemporary viral lineages:

- O/EA-2
- O/EA-3
- O/EA-4
- A/AFRICA/G-I
- A/AFRICA/G-IV
- SAT1/I
- SAT2/IV
- SAT2/VII.

This approach has an obvious advantage in that there is no requirement to access the master seed vaccine strains from commercial companies, and the work can be driven by the customer, independently of the vaccine producer, using sera collected from pilot studies in the customer's vaccinated animal populations. It can be anticipated that this 'proof-of-concept' study will support the use of this method in other FMDV-endemic settings (Fig. 3), and could even accommodate multiple virus antigens across different FMD endemic pools if FMD-vaccinated animals are imported into new regions. Furthermore, the use of a panel of standardised

Pool	Region	Key viral lineages (2020)
1	Southeast/East Asia	O/SEA/Mya-98, O/ME-SA/PanAsia, O/ME-SA/Ind-2001, O/CATHAY, A/ASIA/Sea-97
2*	South Asia	O/ME-SA/Ind-2001, A/ASIA/G-VII, Asia-1
3	West EurAsia	O/ME-SA/PanAsia-2, O/ME-SA/Ind-2001, A/ASIA/Iran-05, A/ASIA/G-VII, Asia-1/Sindh-08
4	East Africa	O/EA-2, O/EA-3, O/EA-4, A/AFRICA/G-I, A/AFRICA/G-IV, A/AFRICA/G-VII, SAT 1/I, SAT 2/IV, SAT 2/VII
5	West Africa	O/WA, O/EA-3, A/AFRICA/G-IV, A/AFRICA/G-VI, SAT 1/X, SAT 2/VII
6	Southern Africa	SAT 1 (topotypes I, II and III), SAT 2 (topotypes I, II and III), SAT 3 (topotypes I, II and III)
7*	South America	O/EURO-SA, A/EURO-SA

Fig. 3

Key foot and mouth disease virus lineages circulating in endemic pools and their identification as candidates for use as standardised antigens in serological tests tailored to cover risk

*Foot and mouth vaccine strains used in India and South America are locally prescribed

FMDV antigens could be adopted to provide a common metric to assess post-vaccination serological responses. For example, a recent study in Mongolia (31) used FMD viruses that were tailored to the viruses circulating in the country, or to those posing a high risk, to compare post-vaccination responses in cattle, sheep and Bactrian camels, using either oil- or aqueous-adjuvanted FMD vaccines.

Reference sera to calibrate assays used to measure vaccine-induced responses

The virus neutralisation test (VNT) described in the OIE *Manual* is the gold standard method to assess the performance of FMD vaccines, and in serosurveys to monitor the prevalence of different FMDV serotypes in host populations. In response to the publication of the OIE/FAO guidelines on *Foot and mouth disease vaccination and post-vaccination monitoring* (32), the demand for post-vaccination monitoring (PVM) by FMD Reference Laboratories has increased.

The uptake of PVM is a very positive development. However, there are still several technical and analytical gaps that need to be addressed to provide the most reliable information to vaccine users and suppliers. Antibodies measured by VNT provide a better correlation to protection from infection than structural protein (SP) ELISAs: the commonly used, liquid-phase blocking ELISA (33), which provides a titre readout, and the solid-phase competition ELISA, with a percentage inhibition result (34, 35).

Nonetheless, there is a high degree of random within-session and across-day error associated with the VNT, due to the inherent variability of cell-culture methods, even when the tests are performed in the same laboratory and by the same operator.

These errors are further confounded by important differences in the cells and local protocols used by laboratories, with at least four different cell systems (IB-RS-2 pig kidney, BHK-21, lamb kidney or pig kidney cells) identified in the OIE *Terrestrial Manual* for this purpose. Together, these factors contribute to variability in the precise FMDV-specific titres measured by different laboratories, making it difficult to reliably compare results generated from different studies or with different vaccines, including vaccine matching (36). These problems have been recognised for more than 20 years (37) and the increasing adoption of harmonised technical methods and uniform reagents has not produced any tangible progress. While reference serum panels are available to calibrate non-structural protein (NSP)-specific antibody responses (38), and a panel is available from the Pan American Center for Foot-and-Mouth Disease and Veterinary Public Health in Brazil (Panaftosa) (37), equivalent reagents for VNT and SP–ELISAs are currently lacking.

There is no universally recognised antibody titre that defines a 'protective' response in vaccinated animals. Since potency studies are expensive and need to be ethically justified, they are only performed for relatively small numbers of vaccine/field-strain combinations. Furthermore, although there is generally good correlation between FMDV-specific antibody titres and protection, the controlled conditions and route of infection used for these studies do not necessarily parallel the severity of challenge experienced by animals in the field. Work continues to collect sera from potency studies and field trials to more accurately define the relationship between antibody titre/concentration and the clinical outcome from vaccination studies. Despite these current and ongoing research activities, there are still immediate gaps in the availability of reference serum panels that can be used to calibrate test results, so that results from different laboratories can be compared. The suggested criteria for sera to be included in the first iteration of this reference sera panel include the following:

– collection after administration of a monovalent vaccine into FMDV-naïve individual animals (between 21 and 28 days after a single dose of vaccine);

– the sera should be suitable for use in low-containment laboratories that have not adopted Biosecurity Level 3/Veterinary Biosecurity Level 3 working protocols. Therefore, the vaccine used should contain inactivated antigen and the sera are expected to be non-infectious and free of any adventitious FMDV, according to approved and internationally recognised biosafety protocols;

– the documented origin of the sera, including the commercial supplier of the vaccine and host species;

– an adequate homologous titre of the selected individual sera, which should be greater than 1.8 $\log_{10}$ measured by VNT (according to the protocol used by the World Reference Laboratory for Foot and Mouth Disease, Pirbright, or equivalent).

As a starting point, large bulk volumes (>500 ml) of sera collected from cattle, vaccinated with a small set of commercial vaccines, are now available from the OIE/FAO FMD Reference Laboratory Network partners (Table I) and can be requested from the authors for this purpose. Members of the Network also request that commercial companies provide similar sera, which have been collected during vaccine potency or batch-testing studies. Although the highest priority is to access post-vaccination sera from potency challenge studies (PD_{50} or PGP study designs) where the clinical outcome, either 'protected' or 'not protected' has been documented, post-vaccination sera from other studies, including those where multivalent vaccines have been used in the field, as

Table I
Reference sera currently available to calibrate post-vaccination responses to foot and mouth disease

FMDV serotype	Vaccine strain	Laboratory providing material	Species	FMD status	Days post vaccination (dpv)	Commercial source of antigen	No. of sera available
O	O1 Manisa	Pirbright, UK	Cattle	Vaccinated only	21 dpv	Boehringer Ingelheim	5
	O1 Manisa	Sciensano, Belgium	Cattle	Vaccinated only	21 dpv*	Boehringer Ingelheim	1
	O-3039	Pirbright, UK	Cattle	Vaccinated only	21 dpv	Boehringer Ingelheim	5
	O1 BFS	Pirbright, UK	Cattle	Vaccinated only	21 dpv	Boehringer Ingelheim	5
A	A IRN 05	Pirbright, UK	Cattle	Vaccinated only	21 dpv	Boehringer Ingelheim	5
	A IRAN 96	Sciensano, Belgium	Cattle	Vaccinated only	21 dpv*	Boehringer Ingelheim	1
	A IRN 87	Pirbright, UK	Cattle	Vaccinated only	21 dpv	Boehringer Ingelheim	5
	A22 IRQ/24/64	Pirbright, UK	Cattle	Vaccinated only	21 dpv	Boehringer Ingelheim	5
	A22 IRQ/24/64	Sciensano, Belgium	Cattle	Vaccinated only	21 dpv*	Boehringer Ingelheim	1
	A MAY 97	Pirbright, UK	Cattle	Vaccinated only	21 dpv*	Boehringer Ingelheim	5
Asia 1	Asia 1 Shamir	Pirbright, UK	Cattle	Vaccinated only	21 dpv	Boehringer Ingelheim	5
	Asia 1 Shamir	Sciensano, Belgium	Cattle	Vaccinated only	21 dpv*	Boehringer Ingelheim	1
SAT 1	SAT-105 RHO 12/78	Pirbright, UK	Cattle	Vaccinated only	21 dpv	Boehringer Ingelheim	5
	SAT-105 RHO 12/78	Sciensano, Belgium	Cattle	Vaccinated only	21 dpv*	Boehringer Ingelheim	1
SAT 2	SAT 251 ZIM 7/83	Pirbright, UK	Cattle	Vaccinated only	21 dpv	Boehringer Ingelheim	5
	SAT 2 Eritrea 3218	Pirbright, UK	Cattle	Vaccinated only	21 dpv	Boehringer Ingelheim	5
	SAT 2 Eritrea 3218	Sciensano, Belgium	Cattle	Vaccinated only	21 dpv*	Boehringer Ingelheim	1
SAT 3	SAT 309 ZIM 2/83	Pirbright, UK	Cattle	Vaccinated only	21 dpv	Boehringer Ingelheim	5
	SAT 309 ZIM 2/83	Sciensano, Belgium	Cattle	Vaccinated only	21 dpv*	Boehringer Ingelheim	1

* Similar sera are available from cattle vaccinated twice with the same vaccines (i.e. after booster vaccination at days 21–28)
FMDV: foot and mouth disease virus
UK: United Kingdom

well as sera collected after booster vaccination, would be very helpful. It is intended that these well-characterised sera should be adopted as primary standards by FMD Reference Laboratories, as proposed by Mackay *et al.* (37), and used to calibrate local secondary standards produced by each laboratory for routine day-to-day use.

Conclusions

Reference panels play an important role in the development, optimisation, validation and adoption of tests by veterinary diagnostic laboratories. To ensure test reliability and reproducibility, many of the tests described in the OIE *Terrestrial Manual* are performed under formal quality assurance schemes, such as Standard 17205 of the International Organization for Standardization/ International Electrotechnical Commission (ISO/IEC) (39).

Although, in the past, many of the older established methods were adopted for use without extensive validation, it is inevitable that the performance of new tests is now examined in greater detail before they can be shown to be fit for purpose. Recognising that no diagnostic test, not even the gold standard method, is perfect, Chapter 1.1.6. of the OIE *Terrestrial Manual* describes the different stages of assay development to validate new tests for routine diagnostic purposes and the use of reference panels to compare assay performance (40). This process is well established for FMD, and external funding has provided opportunities to design and implement studies to compare the performance of different tests beyond their use in single laboratories (41, 42, 43). The formal assessment of test reproducibility in different laboratories is defined by proficiency-testing schemes (44; also described elsewhere in this issue). It is worth noting that, once these proficiency-testing exercises have been completed (and decoded), samples provided in the panels often represent pragmatic alternatives for local validation. This may be necessary in cases where specific reference materials are not readily available, but such samples do not replace the requirement for international reference standards.

This short review highlights some important gaps for FMDV. There are specific biosafety rules that raise the costs associated with the preparation, archiving and shipment of reference panel materials. Identifying suitable reference panels for assay validation is a generic challenge across all livestock diseases, and these activities require funding support from international donors, as well as coordination between international reference laboratory networks, to generate these reagents and ensure their sustainability.

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Sélection et utilisation des panels de référence : à partir de l'exemple de la fièvre aphteuse, étude soulignant les lacunes actuelles en la matière

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Résumé

Le *Manuel des tests de diagnostic et des vaccins pour les animaux terrestres* de l'Organisation mondiale de la santé animale (OIE) décrit une vaste panoplie d'essais utilisables pour la détection, la caractérisation et la surveillance des agents pathogènes affectant les animaux d'élevage. Ces méthodes ont été mises au point par des laboratoires différents à diverses périodes et intègrent souvent des tests ou des kits fournis par le secteur privé. Les panels de référence sont des outils essentiels aussi bien lors de la conception d'un essai que lors d'exercices de validation, leur but étant alors de comparer les performances de technologies diagnostiques variées (et parfois concurrentes). Les Laboratoires de référence de l'OIE fournissent des réactifs de référence internationaux validés afin d'aider à calibrer les tests de diagnostic pour un certain nombre de maladies animales ; toutefois, on constate que nombre de ces réactifs ne sont pas disponibles pour la comparaison et le calibrage inter-laboratoires des résultats de tests. À partir de l'exemple de la fièvre aphteuse, les auteurs soulignent quatre domaines spécifiques pour lesquels il conviendrait de disposer de nouveaux réactifs de référence. Il s'agit des réactifs nécessaires pour : (1) réduire les biais dans l'estimation de la sensibilité diagnostique et de la spécificité pour différents sérotypes des tests utilisés pour détecter diverses souches du virus de la fièvre aphteuse ; (2) fournir des contrôles positifs sûrs au plan biologique pour les nouveaux formats de tests utilisables sur le lieu d'intervention et non plus dans des laboratoires de confinement à haute sécurité ; (3) harmoniser les antigènes du virus de la fièvre aphteuse pour la sérologie post-vaccinale ; (4) résoudre le problème des différences obtenues entre laboratoires lors d'essais sérologiques visant à mesurer la réponse en anticorps spécifiques du virus de la fièvre aphteuse.

Compte tenu des ressources souvent limitées consacrées à la préparation et à la distribution de ces réactifs, des progrès durables ne seront obtenus que s'il existe un consensus en la matière et une coordination de ces activités parmi les Laboratoires de référence de l'OIE.

Mots-clés
Calibrage inter-laboratoires – Étude de cas – Fièvre aphteuse – Lacunes – Panel de référence – Réactif – Test diagnostique – Vaccin contre la fièvre aphteuse – Validation d'un essai.

Selección y uso de paneles de referencia: estudio de las carencias de los paneles disponibles actualmente a partir del ejemplo de la fiebre aftosa

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Resumen
En el *Manual de pruebas de diagnóstico y vacunas para los animales terrestres* de la Organización Mundial de Sanidad Animal (OIE) se describe todo un conjunto de ensayos que se pueden emplear para detectar y caracterizar agentes infecciosos del ganado doméstico y hacer así controles sistemáticos de su eventual presencia. Estos métodos, concebidos en distintos laboratorios en distintos momentos, suelen acompañarse de pruebas o estuches analíticos que proporcionan empresas privadas. Los paneles de referencia son una herramienta esencial, que se puede emplear durante la concepción de ensayos y en los procesos de validación para comparar el funcionamiento de estas diferentes técnicas de diagnóstico, que a veces compiten unas con otras. Los Laboratorios de Referencia de la OIE ya facilitan reactivos de referencia internacional aprobados que ayudan a calibrar las pruebas de diagnóstico de una serie de enfermedades, pero todavía hay importantes carencias por lo que respecta a la posibilidad de procurárselos con fines de comparación y a la calibración de los resultados que obtienen diferentes laboratorios. Sirviéndose del ejemplo de la fiebre aftosa, los autores destacan cuatro aspectos específicos para los que hacen falta nuevos reactivos de referencia. Se trata de los siguientes: reducir el sesgo a la hora de calcular la sensibilidad de diagnóstico y la especificidad interserotípica de las pruebas empleadas para detectar diversas cepas del virus de la fiebre aftosa; proporcionar controles positivos que ofrezcan seguridad biológica para nuevos modalidades de ensayo utilizables en el lugar de consulta, esto es, en condiciones que no sean de alta contención; armonizar los antígenos víricos para la práctica de análisis serológicos tras la vacunación; y solventar las diferencias entre laboratorios por lo que respecta a los ensayos serológicos empleados para medir la respuesta de anticuerpos específicos contra el virus de la fiebre aftosa. Dado que suele haber escasos recursos para preparar y distribuir este tipo de material, solo será posible avanzar duraderamente en la materia si los Laboratorios de Referencia de la OIE consensúan y coordinan estas actividades.

Palabras clave
Calibración entre laboratorios – Carencias – Estudio monográfico – Fiebre aftosa – Panel de referencia – Pruebas de diagnóstico – Reactivo – Vacuna contra la fiebre aftosa – Validación de ensayos.

References

1. World Organisation for Animal Health (OIE) (2019). – Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 8th Ed. OIE, Paris, France. Available at: www.oie.int/en/what-we-do/standards/codes-and-manuals/terrestrial-manual-online-access/ (accessed on 24 May 2021).

2. World Organisation for Animal Health (OIE) (2019). – Chapter 2.2.6. Selection and use of reference samples and panels. *In* Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 8th Ed. OIE, Paris, France. Available at: www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.02.06_REFERENCE_SAMPLES.pdf (accessed on 13 January 2021).

3. World Organisation for Animal Health (OIE) (2021). – OIE-approved international standard reagents. OIE, Paris, France. Available at: www.oie.int/en/what-we-offer/veterinary-products/reference-reagents/ (accessed on 24 May 2021).

4. World Organisation for Animal Health (OIE) (2019). – Chapter 3.1.8. Foot and mouth disease (infection with foot and mouth disease virus). *In* Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 8th Ed. OIE, Paris, France. Available at: www.oie.int/fileadmin/Home/eng/Health_standards/tahm/3.01.08_FMD.pdf (accessed on 13 January 2021).

5. Ryan E., Gloster J. […] & Paton D.J. (2008). – Clinical and laboratory investigations of the foot and mouth disease epidemic in the United Kingdom in 2007. *Vet. Rec.*, **163** (5), 139–147. doi:10.1136/vr.163.5.139.

6. Ferris N.P. & Dawson M. (1988). – Routine application of enzyme-linked immunosorbent assay in comparison with complement fixation for the diagnosis of foot-and-mouth and swine vesicular disease. *Vet. Microbiol.*, **16** (3), 201–209. doi:10.1016/0378-1135(88)90024-7.

7. Roeder P.L. & Le Blanc Smith P.M. (1987). – Detection and typing of foot-and-mouth disease virus by enzyme-linked immunosorbent assay: a sensitive, rapid and reliable technique for primary diagnosis. *Res. Vet. Sci.*, **43** (2), 225–232. doi:10.1016/S0034-5288(18)30778-1.

8. Callahan J.D., Brown F., Osorio F.A., Sur J.H., Kramer E., Long G.W., Lubroth J., Ellis S.J., Shoulars K.S., Gaffney K.L., Rock D.L. & Nelson W.M. (2002). – Use of a portable real-time reverse transcriptase–polymerase chain reaction assay for rapid detection of foot-and-mouth disease virus. *JAVMA*, **220** (11), 1636–1642. doi:10.2460/javma.2002.220.1636.

9. Reid S.M., Ebert K., Bachanek-Bankowska K., Batten C., Sanders A., Wright C., Shaw A.E., Ryan E.D., Hutchings G.H., Ferris N.P., Paton D.J. & King D.P. (2009). – Performance of real-time reverse transcription polymerase chain reaction for the detection of foot-and-mouth disease virus during field outbreaks in the United Kingdom in 2007. *J. Vet. Diagn. Invest.*, **21** (3), 321–330. doi:10.1177/104063870902100303.

10. Ferris N.P., Hutchings G.H., Reid S.M., King D.P., Ebert K., Paton D.J., Brocchi E., Nordengrahn A. & Merza M. (2009). – Development and laboratory validation of a lateral flow device for the detection of foot-and-mouth disease virus in clinical samples. *J. Virol. Meth.*, **155** (1), 10–17. doi:10.1016/j.jviromet.2008.09.009.

11. Oem J.K., Ferris N.P., Lee K.N., Joo Y.S., Hyun B.H. & Park J.H. (2009). – Simple and rapid lateral-flow assay for the detection of foot-and-mouth disease virus. *Clin. Vac. Immunol.*, **16** (11), 1660–1664. doi:10.1128/CVI.00213-09.

12. Matheeussen V., Loens K., Lammens C., Vilken T., Koopmans M., Goossens H., Ieven M. & RECOVER Consortium (2020). – Preparedness of European diagnostic microbiology labs for detection of SARS-CoV-2, March 2020. *J. Clin. Virol.*, **128**, 104432. doi:10.1016/j.jcv.2020.104432.

13. Morris A., Mioulet V., Wood B., Henry L., Gray A., Thapa B., Wadsworth J., Knowles N.J. & King D.P. (2016). – Development of a reference foot-and-mouth disease virus antigen panel for the consistent validation of diagnostic assays. *In* Proc. Open Session of the Research Group of the Standing Technical Committee of the European Commission for the Control of Foot-and-Mouth Disease, Cascais, Portugal. Available at: http://www.fao.org/3/a-bs510e.pdf (accessed on 27 January 2021).

14. Reid S.M., Mioulet V., Knowles N.J., Shirazi N., Belsham G.J. & King D.P. (2014). – Development of tailored real-time RT–PCR assays for the detection and differentiation of serotype O, A, Asia-1 foot-and-mouth disease virus lineages circulating in the Middle East. *J. Virol. Meth.*, **207**, 146–153. doi:10.1016/j.jviromet.2014.07.002.

15. Bachanek-Bankowska K., Mero H.R., Wadsworth J., Mioulet V., Sallu R., Belsham G.J., Kasanga C.J., Knowles N.J. & 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. Meth.*, **237**, 114–120. doi:10.1016/j.jviromet.2016.08.002.

16. Schrader C., Schielke A., Ellerbroek L. & Johne R. (2012). – PCR inhibitors – occurrence, properties and removal. *J. Appl. Microbiol.*, **113** (5), 1014–1026. doi:10.1111/j.1365-2672.2012.05384.x.

17. Yan L., Toohey-Kurth K.L., Crossley B.M., Bai J., Glaser A.L., Tallmadge R.L. & Goodman L.B. (2019). – Inhibition monitoring in veterinary molecular testing. *J. Vet. Diagn. Invest.*, **32** (6), 758–766. doi:10.1177/1040638719889315.

18. Hofmann M.A. (2003). – Construction of an infectious chimeric classical swine fever virus containing the 5'UTR of bovine viral diarrhea virus, and its application as a universal internal positive control in real-time RT–PCR. *J. Virol. Meth.*, **114** (1), 77–90. doi:10.1016/j.jviromet.2003.09.004.

19. Armson B., Mioulet V., Doel C., Madi M., Parida S., Lemire K.A., Holder D.J., Das A., McIntosh M.T. & King D.P. (2018). – Detection of foot-and-mouth disease virus in milk samples by real-time reverse transcription polymerase chain reaction: optimisation and evaluation of a high-throughput screening method with potential for disease surveillance. *Vet. Microbiol.*, **223**, 189–194. doi:10.1016/j.vetmic.2018.07.024.

20. Guan J., Chan M., Grenier C., Brooks B.W., Spencer J.L., Kranendonk C., Copps J. & Clavijo A. (2010). – Degradation of foot-and-mouth disease virus during composting of infected pig carcasses. *Can. J. Vet. Res.*, **74** (1), 40–44. Available at: www.ncbi.nlm.nih.gov/pmc/articles/PMC2801310/ (accessed on 5 January 2021).

21. Gorna K., Relmy A., Romey A., Zientara S., Blaise-Boisseau S. & Bakkali-Kassimi L. (2016). – Establishment and validation of two duplex one-step real-time RT–PCR assays for diagnosis of foot-and-mouth disease. *J. Virol. Meth.*, **235**, 168–175. doi:10.1016/j.jviromet.2016.03.020.

22. Vandenbussche F., Lefebvre D.J., De Leeuw I., Van Borm S. & De Clercq K. (2017). – Laboratory validation of two real-time RT–PCR methods with 5'-tailed primers for an enhanced detection of foot-and-mouth disease virus. *J. Virol. Meth.*, **246**, 90–94. doi:10.1016/j.jviromet.2017.04.014.

23. Ballagi-Pordány A. & Belák S. (1996). – The use of mimics as internal standards to avoid false negatives in diagnostic PCR. *Mol. Cell Probes*, **10** (3), 159–164. doi:10.1006/mcpr.1996.0022.

24. Howson E.L., Armson B. […] & Fowler V.L. (2017). – Evaluation of two lyophilized molecular assays to rapidly detect foot-and-mouth disease virus directly from clinical samples in field settings. *Transbound. Emerg. Dis.*, **64** (3), 861–871. doi:10.1111/tbed.12451.

25. Howson E.L.A., Soldan A., Webster K., Beer M., Zientara S., Belák S., Sánchez-Vizcaino J.M., Van Borm S., King D.P. & Fowler V.L. (2017). – Technological advances in veterinary diagnostics: opportunities to deploy rapid decentralized tests to detect pathogens affecting livestock. *In* Biological threat reduction (T. Beckham, ed.). *Rev. Sci. Tech. Off. Int. Epiz.*, **36** (2), 479–498. doi:10.20506/rst.36.2.2668.

26. WalkerPeach C.R., Winkler M., DuBois D.B. & Pasloske B.L. (1999). – Ribonuclease-resistant RNA controls (armored RNA) for reverse transcription–PCR, branched DNA, and genotyping assays for hepatitis C virus. *Clin. Chem.*, **45** (12), 2079–2085. doi:10.1093/clinchem/45.12.2079.

27. King D.P., Montague N., Ebert K., Reid S.M., Dukes J.P., Schädlich L., Belsham G.J. & Lomonossoff G.P. (2007). – Development of a novel recombinant encapsidated RNA particle: evaluation as an internal control for diagnostic RT–PCR. *J. Virol. Meth.*, **146** (1–2), 218–225. doi:10.1016/j.jviromet.2007.07.002.

28. Madi M., Mioulet V., King D.P., Lomonossoff G.P. & Montague N.P. (2015). – Development of a non-infectious encapsidated positive control RNA for molecular assays to detect foot-and-mouth disease virus. *J. Virol. Meth.*, **220**, 27–34. doi:10.1016/j.jviromet.2015.04.002.

29. World Organisation for Animal Health (OIE) & Food and Agriculture Organization of the United Nations (FAO) Reference Laboratory Network for Foot-and-Mouth Disease (2021). – FMD vaccine producers. FAO, Rome, Italy. Available at: www.foot-and-mouth.org/fmd-vaccine-producers (accessed on 13 January 2021).

30. Paton D.J., Reeve R., Capozzo A.V. & Ludi A. (2019). – Estimating the protection afforded by foot-and-mouth disease vaccines in the laboratory. *Vaccine*, **37** (37), 5515–5524. doi:10.1016/j.vaccine.2019.07.102.

31. Ulziibat G., Maygmarsuren O., Khishgee B., Basan G., Sandag B., Ruuragc S., Limon G., Wilsden G., Browning C., King D.P., Ludi A. & Lyons N.A. (2020). – Immunogenicity of imported foot-and-mouth disease vaccines in different species in Mongolia. *Vaccine*, **38** (7), 1708–1714. doi:10.1016/j.vaccine.2019.12.053.

32. Ferrari G., Paton D., Duffy S., Bartels C. & Knight-Jones T. (2016). – Foot and mouth disease vaccination and post-vaccination monitoring: guidelines. Food and Agriculture Organization of the United Nations (FAO), Rome, Italy; & World Organisation for Animal Health (OIE), Paris, France; 75 pp. Available at: www.fao.org/3/a-i5975e.pdf (accessed on 13 January 2021).

33. Hamblin C., Barnett I.T.R. & Hedger R.S. (1986). – A new enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies against foot-and-mouth disease virus. I. Development and method of ELISA. *J. Immunol. Meth.*, **93** (1), 115–121. doi:10.1016/0022-1759(86)90441-2.

34. Mackay D.K., Bulut A.N., Rendle T., Davidson F. & Ferris N.P. (2001). – A solid-phase competition ELISA for measuring antibody to foot-and-mouth disease virus. *J. Virol. Meth.*, **97** (1–2), 33–48. doi:10.1016/s0166-0934(01)00333-0.

35. Paiba G.A., Anderson J., Paton D.J., Soldan A.W., Alexandersen S., Corteyn M., Wilsden G., Hamblin P., Mackay D.K. & Donaldson A.I. (2004). – Validation of a foot-and-mouth disease antibody screening solid-phase competition ELISA (SPCE). *J. Virol. Meth.*, **115** (2), 145–158. doi:10.1016/j.jviromet.2003.09.016.

36. Willems T., De Vleeschauwer A. […] & De Clercq K. (2020). – FMD vaccine matching: inter laboratory study for improved understanding of r_1 values. *J. Virol. Meth.*, **276**, 113786. doi:10.1016/j.jviromet.2019.113786.

37. Mackay D.K.J., Rendle T. & Kitching R.P. (1998). – FAO Collaborative Study Phase XV: standardisation of FMD antibody detection. Report of the Session of the Research Group of the European Commission for the Control of Foot-and-Mouth Disease. Available at: www.fao.org/3/ca9417en/ca9417en.pdf (accessed on 5 January 2021).

38. Parida S., Fleming L., Gibson D., Hamblin P.A., Grazioli S., Brocchi E. & Paton D.J. (2007). – Bovine serum panel for evaluating foot-and-mouth disease virus nonstructural protein antibody tests. *J. Vet. Diagn. Invest.*, **19** (5), 539–544. doi:10.1177/104063870701900513.

39. International Organization for Standardization/International Electrotechnical Commission (ISO/IEC) (2017). – ISO/IEC 17025: General requirements for the competence of testing and calibration laboratories. ISO, Geneva, Switzerland, 30 pp. Available at: imed.ir/userfiles/files/11/ISO-IEC%20 17025-2017.pdf (accessed on 7 January 2021).

40. World Organisation for Animal Health (OIE) (2019). – Chapter 1.1.6. Principles and methods of validation of diagnostic assays for infectious diseases. *In* Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 8th Ed. OIE, Paris, France. Available at: www.oie.int/fileadmin/Home/eng/Health_standards/tahm/1.01.06_VALIDATION.pdf (accessed on 13 January 2021).

41. Browning C.F.J., Di-Nardo A., Henry L., Pollard T., Hendry L., Romey A., Relmy A., Eblé P., Brocchi E., Grazioli S., King D.P. & Ludi A.B. (2020). – Inter-laboratory comparison of 2 ELISA kits used for foot-and-mouth disease virus nonstructural protein serology. *J. Vet. Diagn. Invest.*, **32** (6), 933–937. doi:10.1177/1040638720962070.

42. Brocchi E., Bergmann I.E. […] & De Clercq K. (2006). – Comparative evaluation of six ELISAs for the detection of antibodies to the non-structural proteins of foot-and-mouth disease virus. *Vaccine*, **24** (47–48), 6966–6979. doi:10.1016/j.vaccine.2006.04.050.

43. King D.P., Ferris N.P., Shaw A.E., Reid S.M., Hutchings G.H., Giuffre A.C., Robida J.M., Callahan J.D., Nelson W.M. & Beckham T.R. (2006). – Detection of foot-and-mouth disease virus: comparative diagnostic sensitivity of two independent real-time reverse transcription–polymerase chain reaction assays. *J. Vet. Diagn. Invest.*, **18** (1), 93–97. doi:10.1177/104063870601800114.

44. Ferris N.P., King D.P. […] & De Clercq K. (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. doi:10.1016/j.vetmic.2006.06.001.