

2nd Regional Expert Group Meeting on Foot and Mouth Disease

31 October – 1 November 2019

Bangkok, Thailand

The 2nd REG meeting noted:

Purpose of serological tests for FMD

1. Serological tests for FMD are performed to support four main purposes
 - a. To certify individual animals prior to import or export (i.e. for trade)
 - b. To confirm suspected cases of FMD
 - c. To substantiate absence of infection
 - d. To demonstrate the efficacy of vaccination
 - e. To identify the historical infection
2. For substantiating freedom from infection, different approaches are required according to whether the population has been vaccinated or not and if vaccination has been used, whether this has been applied as an emergency application or as part of an ongoing programme of vaccination.
3. The test used and the interpretation of test results will depend on the purpose of the serological assay. The validation of the selected procedure must take into account the purpose. The validation of the selected procedure must take into account the 'fitness-of-purpose' of the test before interpreting the results. For example, test cut-off may be set at a different threshold for herd-based serosurveillance than for certifying freedom from infection for individual animals for the purposes of international trade.

Types of serological tests for FMD

4. There are two types of serological tests for FMD
 - a. Tests that detect antibodies to viral structural proteins (SP)
 - b. Tests that detect antibodies to viral non-structural proteins (NSP)

Non-structural protein antibody detection tests (NSP tests)

5. The NSP tests can be used to identify past or present infection with any of the seven serotypes of FMDV, whether or not the animal has been vaccinated. Therefore the test can be used to confirm suspected cases, to identify the historical infection of FMD and to evaluate prevalence of infection or to substantiate freedom from infection on a population basis.

6. The overall test system sensitivity and specificity must be considered when designing the serosurveillance program using NSP ELISA.
7. There is experimental evidence that some cattle, vaccinated and subsequently challenged with live virus and confirmed persistently infected, may not be detected in some anti-NSP tests, causing false-negative results.
8. Also, lack of vaccine purity may affect diagnostic specificity as the presence of NSPs in some vaccine preparations may result in misclassification of animals that have been repeatedly vaccinated.

Structural protein antibody detection tests (SP tests)

9. The SP tests such as VN test, solid-phase competitive ELISA (SPCE) and liquid-phase blocking ELISA (LPBE) are serotype specific and detect antibodies elicited by vaccination and infection.
10. These tests are sensitive, providing that the virus or antigen used in the test is closely matched to the strain circulating in the field.
11. These tests are used to certify animals prior to movement, including for international trade purposes and are appropriate for confirming previous or ongoing infection in nonvaccinated animals as well as for monitoring the immunity conferred by vaccination in the field.
12. These tests could be used to determine the serotype from the historical infection subject to the confirmation from other tests especially by VNT.

Virus neutralization test

13. VN test is strain specific and there is little or no cross-reactivity; however, it requires infectious virus and cell culture. VN tests are more variable and have poorer repeatability than ELISAs.
14. Interpretation of tests can vary between laboratories, in regard to the negative/positive cut-off threshold. Laboratories should establish their own criteria by reference to standard reagents that can be obtained from the OIE Reference Laboratory such as The Pirbright Institute.
15. Cut-off titres for evaluating immunological protection afforded by vaccination have to be established from experience of potency test results with the relevant vaccine and target species.

Solid-phase competitive ELISA (SPCE)

16. The ELISAs use serotype-specific antibodies, are quicker to perform than VNT and are not dependent on tissue culture systems and the use of live viruses. Low titre

false-positive reactions can be expected in a small proportion of the sera in either ELISA formats.

17. Laboratories should validate the assay in terms of the cut-off value above which sera should be considered positive in relation to (i) the particular serotypes and strains of virus under investigation (ii) the purpose of testing (iii) the population under test, using the methods described in chapter 1.1.6 Principles and methods of validation of diagnostic assays for infectious disease” of OIE Terrestrial Manual.

Liquid phase blocking ELISA

18. The ELISAs use serotype-specific antibodies, are quicker to perform than VNT and are not dependent on tissue culture systems and the use of live viruses. Low titre false-positive reactions can be expected in a small proportion of the sera in either ELISA formats.
19. Cut-off titres for evaluating immunological protection afforded by vaccination have to be established from experience of potency test results with the relevant vaccine and target species.
20. This ELISA is also used for vaccine matching in certain laboratories such as RRL-Pakchong.

Discrepancy between LPBE & VNT

21. LPBE and VNT target different epitopes to detect structural protein antibodies measured by LPBE and VNT target different epitopes. VN tests only detects neutralising antibodies (surface exposed at defined sites). LPBE detects a wider-range of epitopes and cannot distinguish between external and internal epitopes.
22. FMDV capsids are easily degraded. Host polyclonal responses are directed at antigenic sites (contribute to neutralising responses), other surface “binding” epitopes and epitopes exposed after capsid degradation. Serotypic determinants are on the outside of the capsid, while many internal epitopes are shared between serotypes. These internal epitopes detected by the LPBE could potential explain the cross-reactivity we see.

Importance of serological validation

23. Validations of serological tests and reagents are highly important. Specificity is measured using negative cohort sera which are from animals without vaccination or infection. Sensitivity is obtained using experimentally infected or vaccinated animals of known serotype (preferably monovalent).



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24. Validations within reference laboratories must also take place.

Conclusions, Recommendations and Next steps

Conclusions:

1. The REG agreed that VNT is more specific than SP ELISA methods with less cross-serotypic reaction, and thus the preferred method for studies to identify true positive samples or individuals (high specificity is required).
2. The REG agreed that cross-reactivity between serotypes in SP ELISA is common and the results require robust technical skills to interpret. However, for countries which do not have capacity to conduct VNT, validated SP ELISA tests (LPBE or SPCE) can be used for specific study purposes.
3. The REG agreed that virological surveillance is preferred over serosurveillance to assess the prevalence of different FMDV serotypes and lineages in endemic countries. Serosurveillance study should be based on NSP ELISA, and if needed, also SP ELISA. Cross-serotypic reaction of SP ELISA may result in false positive results.
4. The REG agreed that the correlation between SP antibody levels and protection can only be defined by *in vivo* challenge studies; such correlation differs by the vaccine used for immunisation and the SP ELISA or VNT used for testing.

Recommendations:

To measure/examine immune responses against the SP or NSP of FMDV for different diagnostic purposes:

- To study antibody levels in individual animals or herds for post-vaccination monitoring(PVM), the REG recommends using SP ELISA (LPBE or SPCE) or VNT. Antigen should be selected with support from the vaccine and ELISA reagents producers.
- To study prevalence of FMDV infection (serosurveillance), the REG recommends using NSP ELISA together with SP ELISA and/or VNT.
- To identify FMDV infected animals and the serotype of infected virus, the REG recommends testing the animal by NSP ELISA together with SP ELISA (LPBE or SPCE). Given the cross-reactivity between different FMDV serotypes is common in SP ELISA (LPBE or SPCE), the REG recommends interpreting the serotype prevalence data

from such tests with caution. If positive in SP ELISA, confirmatory testing by VNT is required.

- For vaccine-matching study, the REG recommends conducting VNT or LPBE at capable OIE reference labs only.

To improve verification of reagents quality and assay performance :

- To verify performance of a new batch of reagents to replace existing reagents for a validated diagnostic assay, the REG recommends optimising the performance of new reagents with old reagents and control samples;
- To verify performance of new assays, the REG recommends using IQC samples and reference sera samples;
- To monitor performance of serological assays (VNT, SP and NSP ELISAs), the REG recommends using monovalent reference serum panel;
- For inconclusive results from serological assays, the REG recommends developing a systematic approach to further verify the results.

Next steps:

1. The OIE and FAO will circulate the draft conclusions and recommendations of the 2nd REG meeting to the REG members for their comment and endorsement before 30 November 2019;
2. The OIE and FAO will circulate the draft recommended list of reference sera (Annex 1), draft protocol of assay verification (Annex 2) , and draft protocol to manage inconclusive results (Annex 3) to the REG group for further comments/edits before 30 November 2019;
3. The REG members will provide feedback to the OIE and FAO before 15 December 2019;
4. The OIE and FAO will review and compile the comments from the REG, with support from leading experts (Dr Anna Ludi, Dr Yanmin Li and Dr Nagendra Singanallur), and circulate the updated version before 20 December 2019;
5. The finalized protocols will be shared with the SEACFMD LabNet and FAO LabTag members by the OIE and FAO, respectively.

Annex 1: Monovalent Reference Serum for serological assays (VNT, LPBE, SPCE and NSP ELISA)

Optimal monovalent serum for reference panel:

1. Positive Serum:
 - a. Experimental serum from vaccinated, vaccinated/infected and/or infected sera
 - b. Monovalent serum (1 serotype/strain)*
*this is a gap; vaccine companies in the region supply bivalent/trivalent serum only
2. Negative Serum
 - a. FMD free country without vaccination
3. Cattle and pig serum*
*this is dependent on the purpose of the testing and could be expanded
4. Panel should include NSP negative and NSP positive serum

Monovalent serum available to the region for reference serum panel

WRL has serum that can be supplied to the region. The following serum was recommended but alternative serum can be made available. Please note that this serum is for cattle only.

Serotype O	Serotype A	Serotype Asia 1
O1 Manisa	A22 IRQ	Asia 1 Shamir
O 3039	A/MAY/97	
O/SKR*	A24**	Negative Cattle Serum

*volume needs to be confirmed

**this would need to be obtained from S. America. Dr Anna Ludi will lead the discussion with Biogenesis. This will take time and therefore this is a long-term aim.

Except for the negative serum, two individual animal sera will be provided for each of the sera types listed above. Fifty ml will be provided for each serum.

For each type of sera, the following will be provided:

1. LPBE, SPCE, VNT and PrioCHECK results from WRL
2. If available, one NSP positive and one NSP negative sera
3. If available, high and mid-range sera determined by VNT

The following will also be used as control serum in the laboratory

Thailand	Japan	China
O-3039 (or O MYA-98) A/MAY/97 Asia 1 Shamir	O1 Manisa O-3039 A22 IRQ	Needs to be confirmed

Logistics

1. All serum will be sent as part of the 2020 PTS. Please return all necessary paperwork as soon as possible. NOTE – Japan doesn't participate in the PTS and shipping costs will need to be covered by alternative means

2. It is recommended that laboratories report back with initial results at the next regional laboratory meeting

Storage

It is recommended that all sera are stored at either -20°C or -80°C. If samples are kept at less than -20°C there is a possibility of obtaining false-positives especially when using the NSP ELISA.

Therefore, if the samples are stored at this temperature for longer than 24hrs they must be heat inactivated at 56°C for 30min before use. It is essential that all sera are aliquoted upon receiving it.

Remaining GAP

Monovalent pig serum is essential for the region; however, there is none available. This needs to be produced and maybe an activity that EuFMD/IAEA could assist with. Regional laboratories should however also consider alternative means of obtaining these sera.

Additional comments:

- Inconclusive results can also include samples that give unexpected results. For example, if we were to find SAT 2 in Asia. Therefore all results should be reviewed by someone familiar with the current situation of FMD. If inconclusive tests are found, then alternative methods should be used to verify the results. Additional samples may also need to be collected. The samples could also be sent to an OIE/FAO reference laboratory.
- If weak positive serum is needed than the positive serum provided by WRLFMD can be diluted to achieve this sample type. This would need to be done for all tests as there will be different dilutions needed for each – this is because each test measures something different.

Annex 2: Assay verification

Aims:

1. Verification for performance of existing assays with new batch of reagents
2. Verification for new batches of commercial diagnostic kits or new kits introduced in the market

Verification for performance of new batch of reagents for existing assays

1. Optimise the performance of new reagents with old reagents and control samples.
2. Perform checker board titrations of the antibodies and antigen to optimise assay performance and minimise cross reactions (Ideally the cross reaction must be <10 percent).
3. Titration of antigen to establish linearity of antigen dilution.
4. Strengthen assay performance by multiple testing of antigen (batch testing at different time points say every 3 months), inter-personnel comparison or day-to-day comparison of results.
5. Monitor the performance of IQC standards under the new set of reagents and compare with the existing reagents.
6. Use of 1-5 reference sera (different levels of specific antibodies as high, moderate and low titres along with negative samples) to establish equivalence.
7. When using commercial conjugates, it must be titrated for optimal dilution every time a new vial from same make or a new make is used, and equivalence must be established with existing conjugate.

Verification for new batches of commercial diagnostic kits or new kits introduced in the market

1. Monitor the performance of IQC standards under the new set of reagents and compare with the existing reagents.
2. Use of 1-5 reference sera (different levels of specific antibodies as high, moderate and low titres along with negative samples) to establish equivalence.

Additional comment:

1. Follow the 'Westgard Rules' while monitoring the IQC results.
2. When IQCs are exhausted, establish the equivalence of the fresh batch of IQC with at least 5-10 runs before IQCs are changed to the fresh batch.
3. Test the specificity and cross-reactions of Skim Milk Powders used in the blocking steps, if used.



Annex 3: Management and reporting of inconclusive results

Inconclusive results are obtained in the following test methods:

1. Serological assays for detecting antibodies against structural proteins of FMDV (SP).
 - a. Liquid Phase Blocking ELISA (LPBE)
 - b. Solid Phase Competition ELISA (SPCE) and
 - c. Virus Neutralisation Test (VNT)
2. Serological assays for detecting antibodies against non-structural proteins of FMDV (NSP): NSP- Ab ELISA

Outcomes from discussion:

1. LPBE (Titration): In case of inconclusive results, repeat the assay. If it is still inconclusive then perform VNT if available. If VNT is not available, the sample can be sent to reference laboratory or tested by SPCE
2. SPCE (Titration, Screening; P/N): In case of inconclusive results, repeat the assay. If it is still inconclusive then perform VNT if available. The sample can also be tested using another set of test (serotype specific) or kit. The sample can be sent to reference laboratory for confirmation by VNT.
3. VNT (Titration; Screening; identification of exposure): In case of inconclusive results, repeat the assay. If it is still inconclusive then request for resampling from the field or perform NSP-Ab ELISA.
4. NSP-Ab ELISA (for identification of exposure): Repeat the test in case of an inconclusive result or test with another kit or assay. If it is still inconclusive then request for resampling from the field including probang and serum samples after a week. Probang sample can be tested by RT-qPCR. The sample can also be sent to a reference laboratory for confirmation with VNT and NSP-Ab ELISA.

Assuring quality of VNT in Reference or National Laboratories

1. Testing the susceptibility of cells used in VNT for susceptibility to FMD (at least once in 3 months or as appropriate from the lab)
 - a. Using a well characterized reference virus pools and check CPE at 24 & 48 hrs post infection
 - b. Establish susceptibility of cells at different passage levels and set a maximum passage levels for each cell type used.
 - c. Set up a 3-tier cell culture system with master stocks (MB), and working stocks (WB1 and WB2).
2. Establish the titre of the reference control sera (Usually a vaccinated or convalescent bovine serum)
 - a. Include the controls in every plate of every run.
 - b. Monitor titre and establish moving averages (running mean)
3. Virus monitoring titre
 - a. Virus control in every plate of every run; monitor and establish moving averages (running mean) of the titres
 - b. Back titration of virus in every assay to confirm the virus dose (32-320 TCID₅₀/well or 1.5-2.5 Log₁₀ TCID₅₀/well)
4. Contamination in cell



- a. Check for Mycoplasma contamination at least every 6 months using a conventional PCR.
- b. Cell control in every plate of every run.
- c. Observe for any physical change in cell growth: discoloration of media, contamination etc on uninfected cell controls.