IAEA-TECDOC-1546

# The Use of Non-structural Proteins of Foot and Mouth Disease Virus (FMDV) to Differentiate Between Vaccinated and Infected Animals

*Publication Prepared under the Framework of an RCA Project with the Technical Support of the Joint FAO/IAEA Programme of Nuclear Techniques in Food and Agriculture*

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# FOREWORD

The Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture has a long history of coordinating isotope aided research projects for improving animal productivity in developing countries. Foot and mouth disease (FMD) remains a tremendous problem in developing countries and is a constant threat to developed countries. Tests to determine the immune status of animals form the basis of understanding the control of the disease. Vaccination is widely employed and has to be on a continuous basis. The antibodies produced against the FMD virus (FMDV) after infection are the same as those produced on vaccination. However, tests have been devised to use non-structural proteins (NSP) of FMDV since it is only on infection that antibodies are produced against such proteins. Thus, through their specific detection, it is possible to determine whether animals are infected in the face of vaccination. This is important since any contact with replicating virus in cattle, sheep and goats may result in a non-clinical situation where virus is carried by the affected animal without symptoms, and may be a threat to others. There is great suspicion over animals where virus has multiplied and so their identification is paramount and essential where countries are trying to demonstrate virus freedom.

There have been many developments in this field and the IAEA sought to try and validate methods in this coordinated research project (CRP). Validation *per se* is always addressed by the IAEA and they have been instrumental in improving guidelines for test certification through the OIE. Although FMD tests had been devised they were not fully examined in a large geographical spread, nor were they compared directly. During the CRP many variations of tests were produced and this complicated the validation process.

The resulting TECDOC reflects the relative instability of developments but value adds to the latest opinions on the use of NSP tests in the control of FMD. Several commercial kits are now available as well as IAEA developed reagents. The roles of NSP tests are well established for analysis of populations in the face of vaccination to assess whether there is virus multiplying; the control of NSP in vaccines; the monitoring of a population under threat; the examination of animals in quarantine and the examination of carrier animals.

The IAEA has been a prominent supporter of the use NSP in tests at an international level and the results will be of great interest to a wide arena of developed and developing country scientists. Such tests are now in routine use in many laboratories around the world. The IAEA officer responsible for compiling this publication was J.R. Crowther of the Joint FAO/IAEA Division.

## EDITORIAL NOTE

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# SUMMARY

The Coordinated Research Project (CRP) was initiated at a time when there was a high level of activity to find a test, or tests, able to differentiate between foot and mouth virus (FMDV) infected and non infected vaccinated animals, as well as identify infection in non vaccinated animals in a single test. The need arises from the possibility that some animals will show no clinical signs of disease but will have had replication of virus. Such animals may carry the FMD virus for many years and possibly represent a threat to others if introduced. The use on non-structural proteins (NSP) allows detection of antibodies that are mostly only produced during infection where virus has replicated. Several systems were being investigated at the onset. Eventually, four main commercial systems emerged plus a South American system. None had been tested widely in different FMD affected countries. None had been extensively compared or validated against large numbers of sera (except the non-commercially available S. American test). The objective of the CRP was to validate the tests in parallel with sera from national stockpiles as well as new sera collected in well run statistical surveys. This process was complicated since tests changed in format requiring repeat studies. This situation settled down during the last two years of the CRP whereby suppliers (partly as a result of IAEA data) managed to achieve better protocols in terms of antigens used and the stability of the Enzyme Linked Immunosorbent Assay (ELISA) systems exploited. Comparative data also became available from other sources such as the EU, leading to a better understanding and agreement as the specific uses of such tests in various control areas for FMD. The CRP has been part of an international effort. Presently, such tests are at the heart of confirming whether a country is free from FMD virus (replication) even where there is no observed clinical disease. This is vital to trade issues in live animals and animal products. What is apparent from this work and other data is that the criteria for selecting cut-offs as to whether animals are positive or negative have to be re-assessed. This has a direct bearing on diagnostic sensitivity and many of the arguments seen about the 'best' test revolve around this issue. Another finding is that the tests are very useful at the herd level, but due to the measured diagnostic uncertainly, are not so useful at the single animal level in tune with most serological testing. Single ELISA systems using NSP for screening are powerful tools for assessing infection in a herd since they only require a single test, but are best backed up by confirmatory tests such as the immunoblotting system as exemplified in South America. There has not been much exploitation of whole herd by this method or simplified versions; for example, the dipstick pen side test, to allow checking of animals when moving in relatively low numbers. The use of accurate highly mobile testing by many individuals (veterinarians) would be very useful, and allow more immediate action where animal proved positive for past infection or possibly carrier animals. The first paper is an extensive review by the Technical Officer of the background science; use and implications of testing for antibodies against non-structural proteins in efforts to control foot and mouth disease. An overview of the impact of their CRP is given in Conclusions by the Technical Officer at the end of this publication.

# REVIEW OF DEVELOPMENTS IN THE DETECTION OF ANTIBODIES TO NON-STRUCTURAL PROTEINS OF FOOT AND MOUTH DISEASE VIRUS

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**Abstract**
This paper reviews the backround science for the use of non-structural proteins of foot and mouth disease in the differentiation of vaccinated and infected livestock. It puts the tests developed into the context of fitness for purpose and desciribes the needs for tests for different epidemiological niches.

## 1.     BACKGROUND SCIENCE

Foot and mouth disease (FMD) recognition involves clinical observation of disease signs and laboratory confirmation from samples. Direct detection of foot and mouth disease virus (FMDV) relies on the direct detection of whole virus, virus antigens or virus RNA from samples or after passage and amplification in tissue culture. The Enzyme Linked Immunosorbent assay (ELISA) has provided a very successful method for confirming disease and differentiating FMDV at the serotype level (FMDV has seven serotypes O, A, C, SAT1, SAT2, SAT3 and Asia1). Methods based on the Polymerase Chain Reaction (PCR) have been developed to detect FMD RNA in samples and primer sets and protocols can be used routinely to verify FMD presence at the serotype level and to assess samples for FMDV in general. Sequencing of PCR products also allows rapid characterisation of strains to allow molecular epidemiological studies.

There are various needs for assessing animal populations by serological techniques related to the epidemiological scenarios possible with FMD. The basic complications in the study of FMD are summarized in Table I. Disease is not always easily observed clinically (e.g. sheep and goats). Vaccines are used which elicit different antibody profiles in terms of quality and quantity. Differentiating vaccinated from infected animals is a problem and this has implications in control programmes where vaccines are used either in mass vaccination campaigns or as strategic interventions in a small population. A complicating factor is the identification of so called 'carrier' animals where virus can persist a long time after an outbreak in the same species or in close proximity in another species. Carriers are produced only after infection where there is some multiplication of the virus, but not all infected animals show clinical signs, so remain undocumented unless identified by other means, such as laboratory testing. Carriers are deemed to be a threat to non-immune livestock and can be demonstrated in cattle, sheep, goats and buffaloes. The risk of disease transmission from carriers is not yet established and this leads to sometimes drastic measures of slaughter by veterinary authorities where an animal is shown to have been in contact with infected animals and/or can be shown to have various antibodies against FMD. The public debate about distinguishing vaccinated from infected animals by serological tests to identify uninfected animals and the fate of such animals was very heated. Questions about vaccination to live policies being most humane were reasonable in the absence of any good data to show the risk they possess.

## 1.1. Scientific basis of antibody production against NSP

As shown in Fig. 1, when FMD infects a cell in tissue culture or an animal, it replicates. The FMDV linear RNA is released and is used as a template to produce FMDV proteins. The proteins are those virus coat (capsid) structural proteins (SP) and non-structural proteins (NSP) that are needed to produce assembly of live infectious FMDV from the structural proteins. For every linear RNA molecule there is the same process where equal molar amounts of SP and NSP are produced. The proteins produced are all potential antigens when interacting with the immune system of an animal on infection or act as immunizing proteins when injected into an animal. The process of multiplication is the same in all infected animal cells and in tissue culture but there are differences affecting the antigenicity of the proteins in these two situations that have a bearing on serological tests. A further consideration is in the production of vaccines, which result from infected tissue culture.

As in infection in animals, FMDV replicates and produces NSP and SP. Vaccines are then formulated by inactivation of the FMDV in tissue culture, some level of purification of the inactivated virus from cellular and NSP components residual in the cells and addition of adjuvants to increase the antigenicity (ability to produce antibodies) of the relatively small amount of protein in a vaccine.

The differences manufacturing methods in purification means that there is a chance that cellular and NSP proteins contaminate vaccines and therefore as act with adjuvant to elicit antibodies. In the most modern vaccine production, it is claimed that the preparations are purified to high levels and that NSP contamination is below levels where antibodies are produced. In more conventional vaccines where purification steps are minimal, there is a great increase in the chance that NSP proteins are present to possibly complicate assays depending on vaccines not producing anti-NSP antibodies. This last point will be considered later when discussing assay performance. Table II indicates some quantitative and qualitative factors concerning production of antibodies against NSP.

A further complication after production is that all proteins are subject to processing and breakdown into polypeptides and peptides, which themselves offer different epitopes (antigenic sites) to the immune system. Such epitopes are likely to be linear as compared to the initially produced NSP complex, where conformational epitopes are presented. On infection of an animal the mass of NSP produced is very high and therefore the subsequent level of processed NSP high also. Antibodies in an infected animal would be expected to react with a wide range of the possible linear and conformational epitopes of NSP proteins. In a vaccine, any contaminating NSP might be expected to be of a more processed form (linear epitopes) due to the vaccine manufacturing process itself, following the infection of cells, so that any antibodies produced on vaccination might reflect this.

The spectrum of antibodies produced against both situations is thus expected to be different (discussed later in terms of confirmation of immune status of livestock using western blotting techniques and use of different kits to examine post-conventional vaccine NSP antibodies). It is worth considering the production of antibodies against SP of FMDV, since the differentiation of livestock by tests requires differentiation of anti-SP and anti-NSP antibodies.

Fig. 2 shows the basic structure of FMD and Fig. 3 shows the genome of FMD and the proteins associated with the genome. Table III shows the possible epitopes presented on infection and likely antibody responses. The tables indicate that there is both a quantitative

and qualitative difference in antigens presented on infection and vaccination and that resulting antibodies reflect these differences and tests devised to distinguish infection from vaccination only have to take these into account.

TABLE I. SITUATIONS DEVELOPING WITH FMD AND CONSEQUENCES TO TESTS

| Observation | Consequence to tests |
|---|---|
| (1) Infects a range of livestock including cattle, pigs, sheep, goats, camelids, and buffalo. | Multi species detection systems are needed. |
| (2) Infection can be mild with few signs. | Reliance on serology to diagnose past infection in surveys (accuracy and diagnostic specificity/sensitivity need to be highly validated). |
| (3) After infection antibody levels rise from 7–28 days then go down with half-life of antibodies to non-detectable levels (3-15 m) depending on maximum titre achieved. Antibodies are produced against structural and NSP (See Fig. 1). | Need to address time in equation of test data and sample (not always easy to determine).<br><br>Levels of antibodies are rarely titrated. |
| (4) Post-infected animals (cattle, sheep, goats, and buffalo) may become carriers and produce antibodies (against NSP) over a long period. Pigs are not deemed to have carrier state. | Time of infection is needed to assess relevance of test results. Infectious status is required. Clinical picture may not be available; e.g. contact sheep and goats may not have been examined or have shown appreciable signs. |
| (5) Post-infected animals may become carriers and produce no antibodies over long term. | Clinical picture is not available. Sheep and goats may not be examined or have shown appreciable signs. Such animals appear as in (1) with clinical presentation and documentation or are regarded as sero negative where there is no evidence of FMD clinically or tests done to establish infection status in an outbreak. |
| 6. Vaccinated animals (i).<br>Highly purified vaccines are used where virus particles are separated from tissue culture NSP. Antibodies are produced against structural proteins only. (See Fig. 1). | Antibodies against NSP detected in some animals after vaccination (debatable point). |
| 7. Vaccinated animals (ii)<br>Not so well purified vaccines are used.<br>A percentage of animals produce antibodies structural and NSP. | Antibodies against NSP detected in some animals after vaccination. |

Outside cell          Inside cell

Infectious virus

Virus infects cell. Virus RNA is read as complete sequence to produce proteins.

RNA

Virus structural proteins          Non structural proteins which aid structural protein assembly

Non structural proteins left in cell

Infectious virus

Infectious virus produced which leaves cell
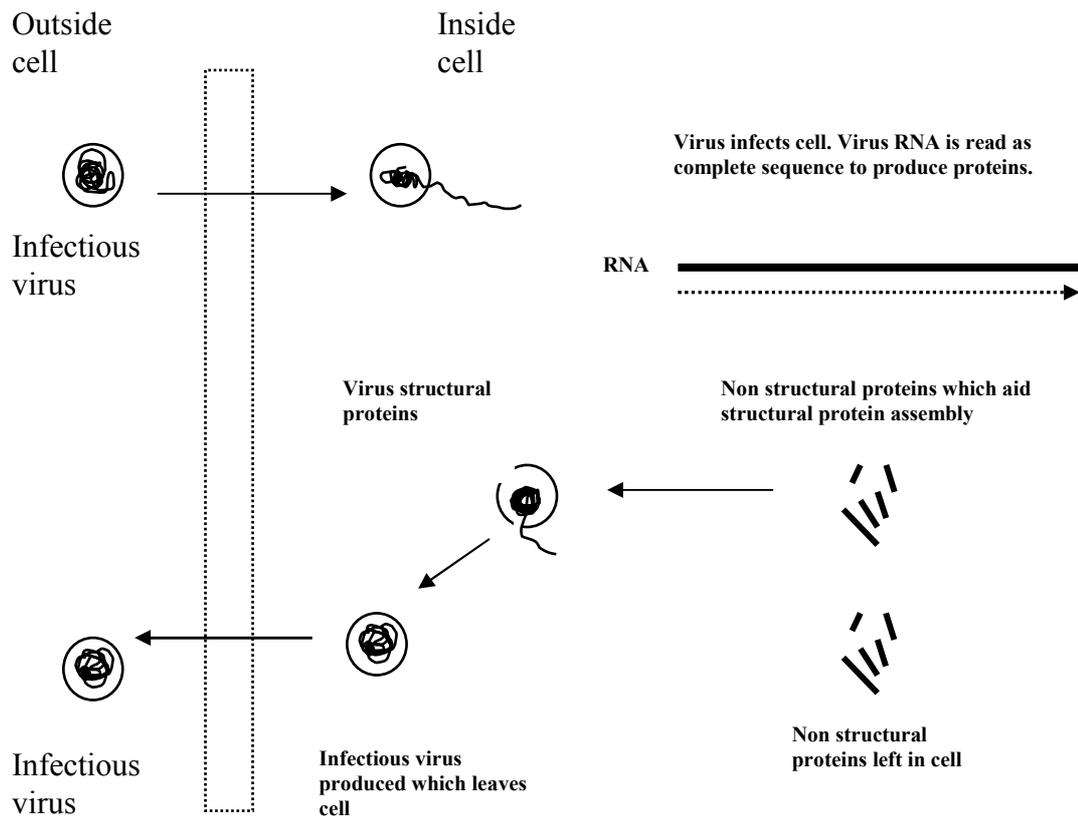
*FIG. 1. Sequence of events in FMD infected cell.*

TABLE II. NSP PRODUCTION IN DIFFERENT SITUATIONS

| NSP source | Mass | Time course | Antibody response |
|---|---|---|---|
| (1) Replication in animal | Very high | 2–10d | +++ |
| (2) Replication in cell | High | Hours | |
| (3) In purified vaccine | Very low | Single dose | - |
| | | Multiple | -/+ |
| | | | |
| (4) In conventional vaccine | Variable and low | Single | +/- (percentage animals?) |
| | High | Multiple | +/- /? (Increased percentage animals?) |
| (5) Processed / denatured NSP in infection | High | 2–10d | +++ Against polypeptides and peptides |
| (6) Processed / denatured NSP in tissue culture for vaccine production | Low | Hours | + /- Against polypeptides and peptides, not necessarily the same as for post infection |

**146S particle of FMD**

Virus capsid proteins

VP1

VP2

VP3

12S subunit

*FIG. 2. Basic structure of FMD.*



VP

Poly c

Capsid

Proteases

Genome linked VPg

RNA polymerase (VIAA)

L | 1A | 1B | 1C | 1D | 2A | 2B | 2C | 3A | 3B | 3C | 3D

Non structural proteins

1    2    3    4    5    6    7    8

Kilobases
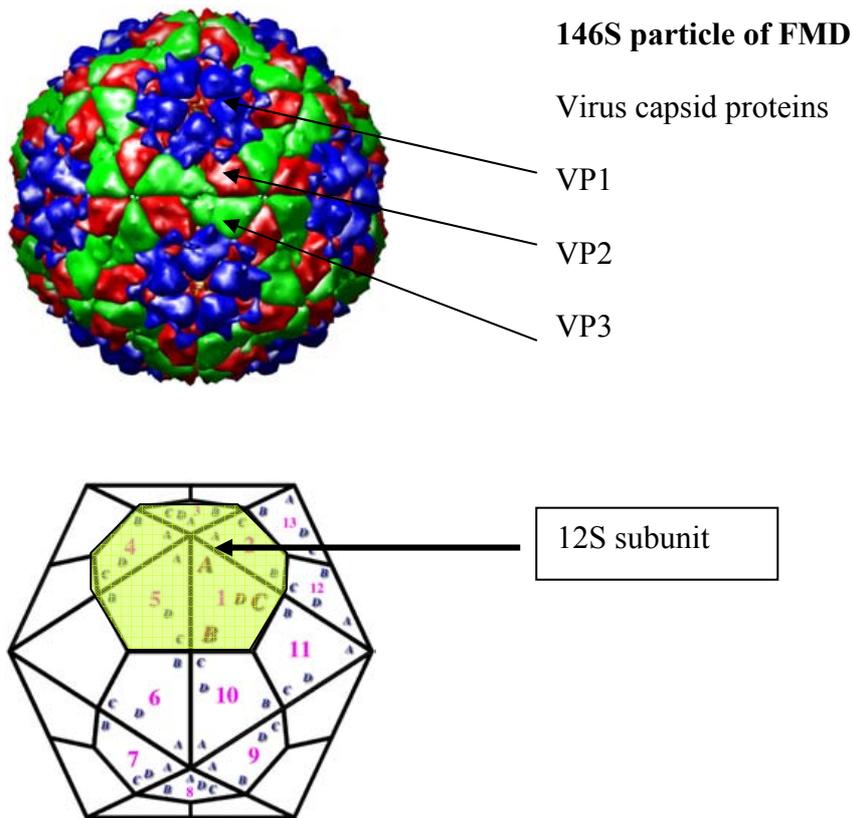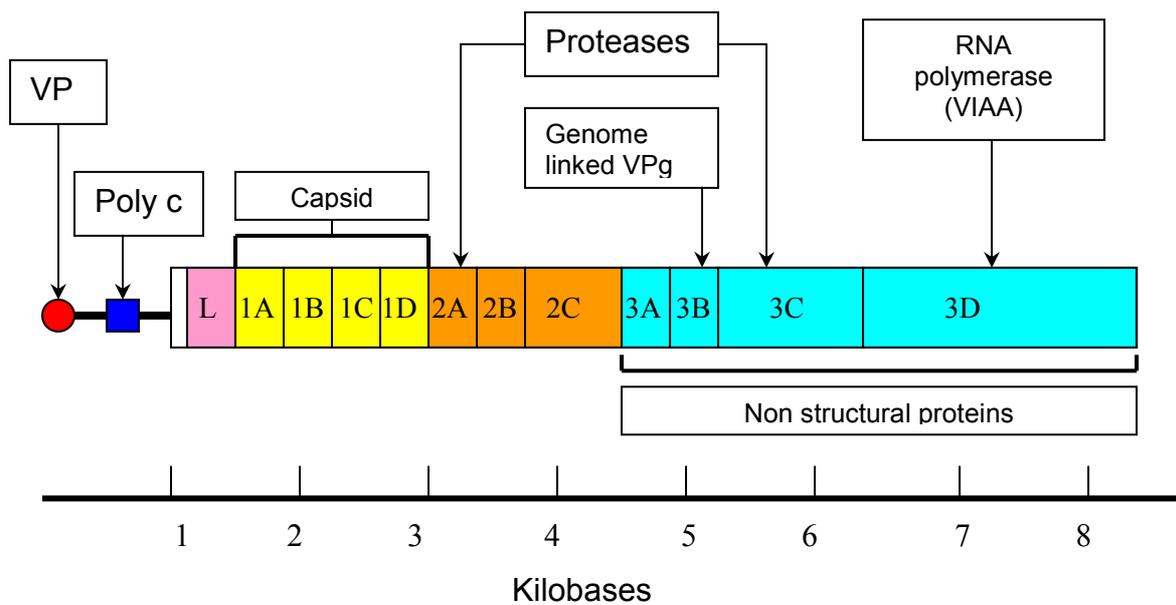
*FIG. 3. Genome of FMD and proteins produced on replication.*

## 2. NSP TEST DEVELOPMENT

### 2.1. Europe

Serological tests such as the Liquid Phase Blocking ELISA (LPBE) measure antibodies produced against the structural capsid proteins of FMDV and are serotype specific. Antibodies against the capsid proteins are produced in both infected and vaccinated animals and thus it is not possible to distinguish between them using LPBE. As already indicated, this differentiation is important when looking for evidence of infection during serological surveys and as a follow-up to ring vaccination programmes. After an animal is infected, a number of NSPs are produced during the replication cycle in the cells. Some of these have been shown to be antigenic, particularly 2C, 3A, 3D and the polyprotein, 3ABC. Note these definitions refer to specific proteins of the NSP complex. They have been characterized by molecular methods and are individual proteins all with a specific function. Some of the abbreviations are used to indicate which particular protein of the NSP is used in tests, e.g. 3ABC or 3A. As indicated earlier, even though the same proteins are produced in the manufacture of vaccines, the quantity and stability of the proteins in the final formulation is low or non-existent. Thus, antibodies against NSP are unlikely in the majority of vaccinated animals. Various assays have been developed to try and distinguish vaccinated from infected animals using this principle. One great advantage of the so-called, NSP assays is that they can be used for all seven serotypes of FMDV, since the NSPs are common to all serotypes.

In the UK, FMD NSP proteins Lb, 2C, 3A, 3D and 3ABC were expressed as fusion proteins in *E. coli* The proteins were attached to glutathione-S-transferase (GST) using the pGEX system and apart from 3ABC, were purified through glutathione-sepharose beads. The 3ABC was found to be insoluble and required extraction from the *E. coli* with first $1M$ then $7M$ urea. Although it was not possible to express 2B as a fusion protein, a peptide was synthesised corresponding to this protein and used as antigen in ELISA. The results were inconclusive and further work with the 2B peptide was not continued. Assays using 3D (VIAA-the virus infection associated antigen) as antigen were developed to investigate non-structural antibodies in infected and vaccinated animals but it was found that they could not be used to distinguish between the two categories. Although 3D is a very good indicator of infection, antibodies to 3D can be found not only in vaccinated animals but also in a small number of naïve cattle. An indirect ELISA, using fusion proteins Lb, 2C, 3A, 3D and 3ABC as well as GST on its own, was developed and used to test a representative number of sera. The sera were from non infected, post-infected and post vaccinated animals whose history was well documented. The samples also represented all seven serotypes of FMDV. By examining a statistically significant number of negative and positive sera, it was possible to establish the test/positive ratio above which sera could be considered positive. Using this indirect or profile ELISA, antibody response and duration of antibody were studied in two sets of cattle that had been experimentally infected with FMDV type $0_1$BFS. Further validation of the test was done on field sera from Albania following an outbreak in May 1996 and on multiply vaccinated sera from Italy collected 5–6 m after last vaccination. Of the NSPs, 3ABC was shown to be the most reliable in distinguishing vaccinated from infected animals. In collaborative work between UK and Italy, the GST-3A fusion protein was used to make monoclonal antibodies to both 3A and GST. The Italians developed a monoclonal antibody trapping ELISA (MAT-ELISA) using the anti-3A MAb, 2C2, and antigen MS2-3ABC. Further evaluation was made in the UK on a bank of reference sera from non infected, post-infected and post vaccinated animals. The Italy MAT-ELISA was later modified in the

UK by replacing the antigen, MS2-3ABC, with GST-3ABC, one of the antigens in the profile ELISA and by using a commercial anti-species conjugate.


TABLE III. ANTIGENS PRESENTED AND ANTIBODY PRODUCTION

|  | In animal | Mass |  | Mass | P. Inf | Post vacc |
|---|---|---|---|---|---|---|
| Capsid 146S | External conformational epitopes of VP1, VP2 and VP3. Induce neutralising antibodies | +++ | External conformational epitopes of VP1, VP2 and VP3. Induce neutralising antibodies | + | +++ | ++ |
|  | Linear epitopes VP1, VP2 and VP3 before processing | ++ | Linear epitopes VP1, VP2 and VP3 before processing | + | ++ | ++ |
|  | Linear epitopes VP1, VP2 and VP3 after processing | ++ | Linear epitopes VP1, VP2 and VP3 after processing (differences to infection) | + | ++ | + |
| 12S | External conformational epitopes of VP1, VP2, VP3 | ++ | Not so high since 146 S enhanced | +/- | ++ | +/- |
|  | Internal conformational epitopes VP1, 2, 3 | ++ | Breakdown products | +/- | ++ | + |
|  | Linear epitopes of VP1, VP2, VP3 before processing | ++ | Lower concentration | +/- | ++ | + |
|  | Linear epitopes VP1, VP2 and VP3 after processing | ++ | Lower concentration | +/- | ++ | + |
| VIAA | 3D associated with capsid conformational and linear epitopes | ++ | 3D associated with capsid conformational and linear epitopes reduced in mass | + | +++ | ++ |
| Non-structural proteins | 3A, 3B, 3C, 3D Conformational epitopes against combinations of proteins | +++ | Depends on vaccine purification schedule | -? | +++ | +/-? |
|  | 3A, 3B, 3C, 3D Linear epitopes on processing on individual proteins | ++ | Depends on vaccine purification schedule | -? | ++ | +/-? |

Evaluation of the UK adaption was made on bovine, ovine and porcine experimental samples; bovine field sera from Albania, Philippines, Zimbabwe and Botswana and ovine/caprine sera from Albania, Morocco, Macedonia, Serbia and Saudi Arabia. Sera from multi vaccinated cattle were also tested in a well-documented series of samples from Uruguay where antibody to 3ABC was found in animals which had >16 vaccinations. Similar results have been found in multi vaccinated cattle in Saudi Arabia during a comparison study on NSP antibody response in milks and sera.

Several conclusions were made from the evaluation and application of the GST-3ABC MAT-ELISA.

- Where animals were positive for structural antibody, detection of antibody to 3ABC in these animals was the single most reliable indicator of FMDV infection.
- Although a positive 3ABC result meant that the animal had been in contact with the virus, a negative result did not mean that infection could be ruled out.
- Antibody response studies showed that measurable levels of antibody to NSPs are seen several days after antibody to structural proteins.
- Results from a single animal, whether positive or negative, must be viewed with caution and the 3ABC MAT-ELISA cannot be recommended for single-sample testing.
- The measurement of antibody to 3ABC on a herd or group basis can be used to detect previous infection in a vaccinated population. Evaluation of the 3ABC MAT-ELISA has demonstrated its potential as a diagnostic tool particularly as an indicator of virus activity in vaccinated or naïve populations.

The use of *E. coli* expressed 3ABC in an Indirect ELISA format using anti-species conjugates for cattle, sheep and goats or for pigs is the basis of commercial kits from various sources.

A competition ELISA was developed in Denmark (Lindholm) using guinea pig hyperimmune sera and a baculovirus expressed 3A and 3B NS proteins. Later this was developed with monoclonal antibody and is now marketed as a kit.

## 2.2. South America

The earliest developments of the indirect ELISA using *E. coli* expressed 3ABC were in South America. The ELISA was developed in tandem with an Enzyme linked immuno electrotransfer blot assay (EITB) involving the individual NSP 3D, 2C, 3ABC, 3A and 3B. The EITB relies on providing strips coated with the respective antigens. Addition of sera and development with anti-species conjugates then reveals antibodies to the proteins in any sample. Results indicated that it was possible to discriminate between vaccinated and infected animals and that there was a high reproducibility between the two tests as well as agreement with the European assays. The sensitivity of the South American assays has been set slightly higher to allow for needs of national use. The use of the combination of ELISA and EITB assays can also be adapted to different requirements depending on import-export or epidemiological surveillance usage. The EITB does provide a profile of antibodies produced and therefore gives more information than the ELISAs used. For epidemiological surveillance the screening of sera by ELISA then the confirmation of positive results by EITB was recommended.

For import/export, both assays were recommended for all samples. Such a method offers high sensitivity and specificity and is needed in the later stages of proving that FMD is no longer circulating in a country. This system is not commercialised but is provided from a central reference laboratory in Brazil for national laboratories in South America. This format has been used on a very large scale and is the OIE prescribed and Index test for NSP.

### 2.3.  Australia

Since there is no FMD in Australia they rely on an ELISA based on the detection of antibodies against 3D (VIA) antigen. This assay detects post infection or vaccination antibodies without discrimination. More recently a Competition ELISA using *baculo* expressed NSP and chicken antibodies made against *E. coli* expressed 3ABC has been successfully developed for cattle with the IAEA.

### 2.4.  United States of America

Developments in the use of NS proteins in ELISA. A commercial company has used chemically synthesised specific peptides (3A and 3B) to produce an indirect ELISA for cattle, sheep and goats as well as pigs. The pig test was most successful in Taiwan and was the first test to be used on large scale.

### 3.  BASIC ASSAY FORMATS

### 3.1.  Indirect ELISA

The scheme for this test is shown in Fig. 4. The format using 3ABC as antigen is available commercially through two sources. At the time of writing they are Bommeli Diagnostics/IDEXX, (USA) and Svanova (Sweden). The format is greatly used in South America where it is supplied centrally from a reference laboratory in Brazil.

The validation exercises indicate that all the kits are similar, although there are differences when specific sera are examined e.g. in some early and late sera taken after infection and also where certain species are involved e.g. buffalo. Differences arise in the exact purification of the *E. coli* expressed antigen used and the conjugate specificity for detecting bound animal antibodies. All the sources of the I-ELISA use 2 kits, one for cattle, sheep and goats and one for pigs.

One commercial company (UBI, USA) uses synthetic NSP 3B peptide as antigen. This also shows some variation in detecting antibodies from certain animals due to the more limited antigenic repertoire presented to antibodies. For example, care must be taken if evaluating antisera against SAT 2 and 3. This test may have advantages when studying animals vaccinated with conventional less purified vaccines (discussed later).

Some commercial companies also supply companion tests to determine overall antibody, thus a negative animal by NSP and positive by companion, can be assured as being infected.

TABLE IV. SUMMARY OF THE PRESENT STATE OF PLAY WITH REGARD TO KITS

| Format | Antigen | Producer/Supplier |
|---|---|---|
| Indirect ELISA cattle | 3ABC *E. coli* expressed | Bommeli Diagnostics- IDEXX CHEKIT-FMD-3ABC bo-ov |
| Indirect ELISA pig | 3ABC *E. coli* expressed | Bommeli Diagnostics IDEXX CHEKIT-FMD-3ABC po |
| Indirect ELISA | 3ABC *E. coli* expressed | Svanova, Sweden |
| Indirect ELISA pigs | 3B | United Biomedical Inc. (UBI) USA. |
| Competitive ELISA pigs | 3B | United Biomedical Inc. (UBI) USA. |
| Indirect ELISA pigs | 3A | United Biomedical Inc. (UBI) USA. |
| Indirect ELISA cattle, sheep, goats | 3B | United Biomedical Inc. (UBI) USA. |
| Competition ELISA cattle, sheep, goats | 3B | United Biomedical Inc. (UBI) USA. |
| Indirect ELISA cattle, sheep, goats. | 3A | United Biomedical Inc. (UBI) USA. |
| Indirect ELISA cattle | 3ABC *E. coli* expressed | PANAFTOSA Brazil  (NON commercial) |
| Western blotting | NS proteins | PANAFTOSA Brazil (NON commercial) |
| C-ELISA | Capture MAb and enzyme labelled MAb for competition, baculo 3ABC antigen. | CEDI diagnostics |
| Pen side. | Chromatographic strip | BioSign. Princeton BioMeditech Corporation (PBC) NJ, USA. |

## 3.2.    Indirect ELISA scheme



**Test serum**

**-Enz**

**Anti-species conjugate**

**Colour**

**3ABC non structural Proteins (E.coli) or peptide (UBI)**
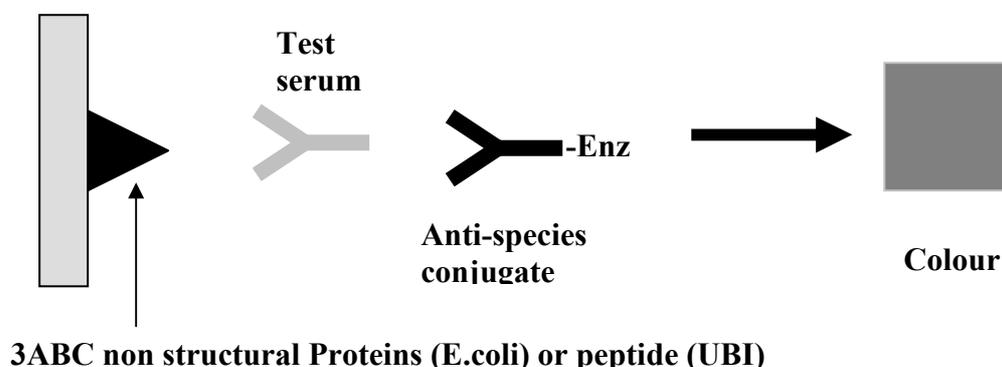
*FIG. 4. Indirect ELISA; 3ABC directly on wells.*

## 3.3. Competition ELISA scheme

**Pre titrated system**



Colour = negative for antibody

**Competition by test serum antibodies (Test)**



Test serum

No colour = Positive for antibody
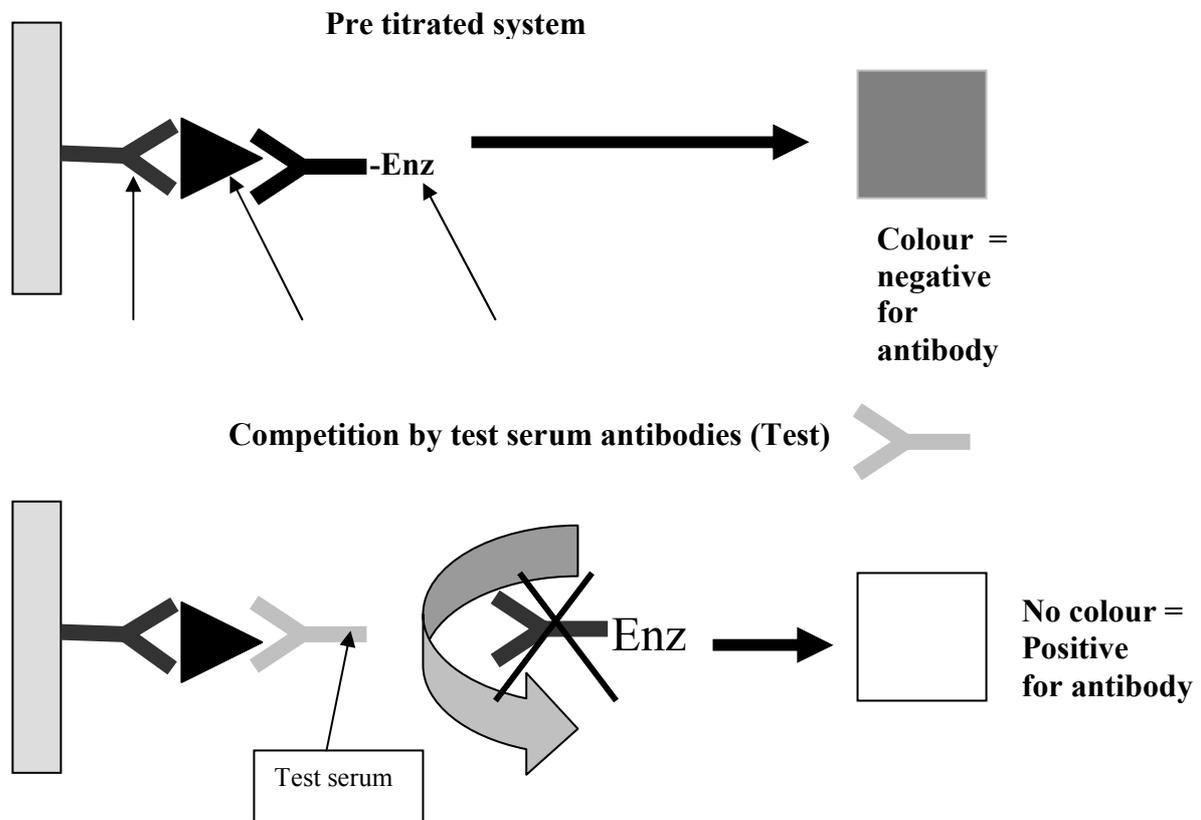
*FIG. 5. Competition NSP ELISA.*

TABLE V. ENZYME CONJUGATES USED IN I-ELISA

| Supplier | Anti-species enzyme conjugate |
|---|---|
| Bommeli | Monoclonal anti bovine |
| Svanova | Goat anti bovine polyclonal |
| PANAFTOSA | Believed to be anti polyclonal bovine? |
| UBI | Protein A and G? |

The competitive assay illustrated in Fig. 5 from CEDI is a single monoclonal antibody (MAb) labelled with enzyme, so that anti-species considerations do not matter. However, the binding affinity of the MAb and the antigen used do matter to the diagnostic specificity criteria and great care is needed to make the same specific activity enzyme labelled MAb for each batch. The antibodies required to compete for the MAb may also not be present since the animal has not 'seen' such a baculo expressed antigen. It is unlikely that the exact mechanism of competition has been worked out for this system, and very likely that most antibodies compete by steric interference on the MAb-presented 3ABC, more than a specific blocking of common sites.

4.     VALIDATION STUDIES

The pressure to develop and obstacles in developing tests to differentiate vaccinated from infected livestock stems from many sources. These include:

- Outbreaks in the United Kingdom. Arguments about definitive identification of animals after infection of carriers (persistent virus).
- Public awareness effects, disbelief in proof of science, vaccination arguments.
- EU needs, vaccine to live policies?
- The OIE mandate to use NSP assays as tests to declare freedom from infection before validation of methods was established.
- The OIE prescription of the South American NSP test (non commercial).
- Resistance to commercial organisations by International standards bodies. (The commercial sector is the only one that can reliably produce and distribute kits).
- No understood system of registration of kits. Bio terrorism effects.
  Since around 1998 there have been many developments as already indicated. Some of the major problems for validation stem from:

(1)  A very high rate of change in kit formulation.
(2)  Poorly defined populations of sera examined with poor documentation of sources.
(3)  Lack of suitable populations of sera.
(4)  Lack of reference sera.
(5)  Lack of coordination (competitive instincts) and harmonisation exercises.
(6)  Lack of experimental sera or sera available only in specific laboratories unwilling to release it.
(7)  Poor support to harmonise experimental data by Institutions.
(8)  Quality control and external quality assurance not made.
(9)  Institutional/commercial links.
(10) Poor ideas about validation as a process, in fact no clear process available e.g. registration by OIE.

Most of the factors centre on validation criteria. The OIE have now adopted 'fitness for purpose' criteria for the registration of tests and have set up a registration process. It is useful to examine these criteria and then examine where we are with respect to ND SP testing and in fact all tests applicable to testing livestock.

## 4.1.  OIE developments

During the 71st General Session of the OIE in May 2003, the International Committee adopted Resolution No. XXIX. This Resolution endorses the principle of validation and certification of diagnostic assays (test methods) for infectious animal diseases by the OIE and gives a mandate to the Director General of the OIE to set up the specific standard procedures to be used before the final decision for the validation and certification of the diagnostic assay is taken by the OIE International Committee.

The Resolution establishes that 'fitness for purpose' should be used as a criterion for validation.

- Demonstrate population 'freedom' from infection (prevalence apparently zero).
- 'Free' with and/or without vaccination.

- Historical 'freedom'.
- Re-establishment of 'freedom' following outbreaks.
- Demonstrate freedom from infection or agent in individual animals or products for trade purposes.
- Demonstrate efficiency of eradication policies.
- Confirmatory diagnosis of clinical cases.
- Estimate prevalence of infection to facilitate risk analysis (surveys, classification of herd health status, and implementation of disease control measures).
- Determine immune status in individual animals or populations (post-vaccination).

The Resolution states that the Director General of the OIE should make provisions to establish a registry of assays with levels of validation specified. He is given the mandate to review the procedures involved in the timely approval of assays and is authorised to recover if necessary any costs incurred in the process of validation, certification and registry of such assays. Resolution No. XXIX establishes that OIE Reference Laboratories should be intimately involved with the validation procedures and that they should establish serum/sample reference collections to be used for validation in line with their mandates. The aim of the procedure for diagnostic kits is to produce a register of recognised assays for OIE Member Countries and for test manufacturers. The OIE Member Countries need assays that are known to be validated according to OIE criteria in order to improve the quality of assays to ensure that the test can be used to correctly establish animal disease status and to enhance the confidence in assays. This process of producing a register of recognised assays will provide greater transparency and clarity of the validation process and also a means for recognizing manufacturers who produce validated and certified tests in a kit format. In order to render the process transparent, all results of the test validation procedure by the OIE will be included in a detailed form on the OIE web site. Validation is a continuous process. At various stages of development a test will be used and data obtained. It is this data that determines the validation status. The demonstration of data to support test claims is the purpose of this section. Four stages in validation are proposed and OIE acceptance of the application will be linked to a determination of the validation status based on the four stages. The stages rely on quantity of work done; the number of people and laboratories where the test is used; the quality of data and measures taken to routinely examine the test in use, both directly by users and indirectly through exercises designed to measure repeatability and reproducibility over time.

Table VI reviews the fitness for purpose criteria against which validation data should be supplied. Table VII reviews the areas in which tests can be demonstrated as being appropriate through validation studies. These can be used to assess here we are in these terms with NSP tests for FMD. Since the scheme for test registration with OIE has not started at the time of writing this paper, no one has submitted validation data for fitness for purpose. This is attempted in Table VIII places NSP tests in each category. It can be seen that the testing for NSP antibodies is relevant to all fitness for purpose criteria but that some applications have to be in combination with other tests. Fitness for purpose also has to take into account the system evolved for their use therefore, appropriate survey designs have to be developed. The wrong testing strategies may invalidate the use and hence, purpose of the test.

Examples of the use and validation of NSP assays will be examined later.

TABLE VI. FITNESS FOR PURPOSE AREAS

| Fitness for purpose criteria |
|---|
| (1)     "Free" with vaccination |
| (2)     Historical "freedom" |
| (3)     Re-establishment of "freedom" after outbreaks |
| (4)     Demonstrate freedom from infection or agent in individual animals or products for trade purposes |
| (5)     Eradication of infection from defined populations |
| (6)     Confirmatory diagnosis of clinical cases |
| (7)     Estimate prevalence of infection to facilitate risk analysis (surveys) |
| (8)     Identifying infected animals or groups toward implementing disease control measures |
| (9)     Classify herd health status |
| (10)    Determine immune status in individual animals or populations (post-vaccination) |
| (11)    Others |

TABLE VII. VALIDATION STAGES

| Stage 1 validation requires: | |
|---|---|
| 1.1. Calibration | Some calibration of a test against standards (in house at least). Inclusion of some reference standards (in house at least). |
| 1.2. Repeatability data | A minimum of 3 in-house samples representing activity within linear range of assay. Within run tests (quadruplicates preferred). Between run tests (a minimum of 20 runs total, 2 or more operators preferably on separate days, where runs are independent. Between serial repeatability, ideally three production batches. Data should include mean, SD, upper and lower control (UCL and LCL) on unprocessed and processed data. |
| 1.3. Analytical specificity data | Cross-reactivity, near-neighbour data. Document cross-reactivity by comparing samples from animals infected with organisms with similar. Clinical presentations and organisms that are genetically closely related. Type/group specificity data. Documentation affirming serotype or group specificity. |
| 1.4. Analytical sensitivity data | Specify standard of comparison (i.e. currently accepted test method). Comparison may include: end point titrations; earliest time of detection post-exposure. Duration of detection post-exposure (if applicable). |
| **Stage 2 validation requires: Stage 1 criteria plus:** | |
| 2.1. Negative reference animals/samples. (Negative refers to lack of exposure to or infection with the agent in question) | Complete description age, sex, breed, etc. Immunological status. Relatedness to intended target population. Selection criteria including historical, epidemiological and/or clinical data. Pathognomonic and/or surrogate tests used to define status of animals or prevalence within population. Sampling plan and procedures. |
| 2.2. Positive reference animals | (Note: negative refers to known exposure to or infection with the agent in question). Complete description Age, sex, breed, etc. Immunological status. Relatedness to intended target population. Selection criteria including historical, epidemiological and/or clinical data. Pathognomonic and/or surrogate tests used to define status of animals or prevalence within population. Sampling plan and procedures. |
| 2.3. Experimental animals | Complete description. Age, sex, breed, etc. Immunological status. Relatedness to intended target population. Exposure. Inoculum, source, dose, etc. Type of exposure – inoculation, aerosol, contact, etc. Sampling plan and procedures. |
| 2.4. Threshold determination | Complete description of method used: empirical, ROC, mean ± SD, etc. Descriptive statistics, frequency distribution diagrams, etc. |
| 2.5. Performance estimates | Irrespective of the method chosen, the standard method(s) of comparison should be run in parallel on all samples, i.e., the test methods in current use. |
| 2.6. Diagnostic sensitivity and specificity estimates– | Conventional method using reference animals. Assuming a minimum sensitivity and specificity of 75% with an allowable |

| with defined reference animals | error of ± 5% in the estimate at a level of confidence of 95%, number of reference animals required is 300 for each population.<br>Individual animals must be selected from negative and positive reference populations. Include 2x2 table, calculations for diagnostic sensitivity and specificity including error and confidence. Include same calculations for other tests if being compared to the test in question. |
|---|---|

| 2.7. Diagnostic sensitivity and specificity estimates– without defined reference animals | Complete description of model used. Bayesian inference, latent class analysis, etc.<br>Describe rationale, priors, supporting data. Population selection criteria, including prevalence estimates. Other test methods in evaluated should also include the standard method of comparison. Using best available priors, choose test populations with appropriate prevalences and select animals in sufficient numbers to generate estimates of sensitivity and specificity with an allowable error of ± 5% at a level of confidence of 95%. |
|---|---|
| 2.8. Agreement between tests | Complete description of test methods in comparison. Presumptive vs confirmatory tests. Relatedness of analytes. Potential biases.<br>Complete description of samples tested. Source of samples may include experimental animals sequentially sampled over time. May also include animals or herds defined by reactivity in confirmatory tests or multiple presumptive tests and sampled over a period of time. Describe measures of agreement and explanations for results not in agreement. |
| **Stage 3 validation requires: Stages 1 and 2 plus:** | |
| 3.1. Laboratory identification | Selection criteria for candidate laboratories. Location, i.e. country. Status, i.e. regional, national, provincial/ state. Level of expertise, familiarity with technology. Accreditation status. Number of laboratories included. A minimum of 3 laboratories should also include OIE Reference Laboratory, if possible. |
| 3.2. Evaluation panel | Description of test panel. Selection criteria, number of samples minimum of 20). Sample volume, allowable number of repeats. Panel composition, i.e. number of replicates, range of analyte concentrations/ reactivities. Sample processing requirements, i.e. extractions, spiking, serial dilutions, preservatives, and sterilization. Coding of unknown (blind) samples. Frequency of testing. |
| 3.3. Reproducibility | Description of type of data/ interpretation. Qualitative (categorical). Quantitative or semi quantitative data. Single dilution vs titration. Description of type of analysis. Pre-determined limits, consensus, Youden plots. Descriptive statistics. Include mean, SD, range of results.<br>Should include controls, as well as, blind samples. Number and proportion of accepted/rejected runs should be included. |
| **Stage 4 requires: Stages 1, 2 and 3 plus:** | |
| 4.1. Laboratories | List laboratories where this test method is in current use. Location, i.e. country. Status, i.e. regional, national, provincial/ state. Accreditation status. |
| 4.2. Test Applications | For each laboratory. Indicate purpose of test. Integration with other tests Status test, i.e. official test, supplementary, etc. Throughput, i.e. daily, monthly, annual. Turn-around-times. |
| 4.3. International reference standards | List type and availability of international reference reagents. Source. Negative, weak/ strong positive reference reagents. Other key biologicals, e.g. antigens, antibodies, etc. |
| 4.4. Inter-laboratory testing programmes | Describe programmes involving inter-laboratory comparisons utilizing this test method. National, international. Describe eligibility and number of laboratories participating. |
| 4.5. International recognition | List internationally recognized reference laboratory responsible for this test method and/or biologicals. Listed international standards containing this test method. Listed international programmes employing this test method. |

## 4.2.    Fitness for purpose criteria

The fitness for purpose criteria detailing the scenarios where tests can be used to solve problems, are shown in Table VIII.

TABLE VIII. FITNESS FOR PURPOSE CRITERIA

| Fitness for purpose | Tests | Information available on validation? | Factors involved |
|---|---|---|---|
| (1) "Free" with vaccination | All NSP tests | +++ | Survey design critical. Establishing vaccine effect. False positive rate? |
| (2) Historical "freedom" | All NSP tests | ++ | False positive rate needed. |
| (3) Re-establishment of "freedom" after outbreaks | All NSP tests | +++ | Survey design critical. |
| (4) Demonstrate freedom from infection or agent in individual animals or products for trade purposes | All NSP in combination | + | Identifying infected herds where there are no clinical signs. |
| (5) Eradication of infection from defined populations | NSP tests in combination | + | Identifying infected herds where there are no clinical signs |
| (6) Confirmatory diagnosis of clinical cases | NSP in combination | + | Later in infection correlating infection with rise in anti NSP antibodies. |
| (7) Estimate prevalence of infection to facilitate risk analysis (surveys) | NSP tests | ++ | Identifying infected herds where there are no clinical signs. Carriers. |
| (8) Identifying infected animals or groups toward implementing disease control measures | NSP in combination | + | Carrier identification Identifying infected herds where there are no clinical signs |
| (9) Classify herd health status | NSP tests in combination | + | Identifying infected herds where there are no clinical signs. |
| (10) Determine immune status in individual animals or populations (post-vaccination) | NSP tests with combination and reservations | + | Full histories of animals needed. Individual animals if whole herds (small) tested. Quarantine use. Movement control use. |

## 5.    COMPLICATIONS OF FMD

An examination of the likely scenarios in requiring diagnostic tests to evaluate the immune/disease status of animals indicates the complexities in FMD. The complications include:

(1) Many cloven-footed species can be infected by FMD

(2) The progression of clinical and sub-clinical disease in the different species is different in terms of severity and time course

(3) The immunological responses varies in individual animals terms of:

    (a) Quantity (sensitivity) of antibodies produced

    (b) Quality (specificity) of antibodies produced

    (c) Time that antibodies of any quantity and quality are produced

These factors in individual animals are greatly complicated at the herd level where there is a possible mixture of events and even more complicated where there is progressive spread of disease into other populations that may or may not mix with the original infected focus. Tests have to address these factors and are influenced by the species-specificity of assays; the variable avidity of immune responses and the existence of carrier states for virus that may or may not be associated with production of antibody.

This is compounded by the known and unknown movement of animals in any outbreak or endemic situation and further complicated by the use of vaccines in one or more of the species, since not all vaccines are the same and some less well purified vaccines may induce antibodies against NSP. Testing animals when there is a known history of movement and disease signs is ideal but often this information is not known. Sampling becomes a critical factor in assessing what NSP test results mean. This lack of information also affects validation studies that require that populations representative of the field situation be studied. It is possible theoretically to assign immune status criteria to animals in an attempt to examine the possibilities for the use of assays. This helps design appropriate sampling frames and measure statistically defined confidence factors. Table IX indicates various disease states of animals and possible consequences.

TABLE IX. POSSIBLE DISEASE STATUS OF ANIMALS

| Status | Status | Clinical Signs | Carrier possibility? | Clinical Signs | Carrier possibility |
|--------|--------|----------------|----------------------|----------------|---------------------|
|  |  |  |  |  |  |
| Non immune |  |  |  |  |  |
| COW | Un | - | - | NA | NA |
| PIG | Un | - | - | NA | NA |
| SHEEP | Un | - | - | NA | NA |
| Non immune |  |  |  |  |  |
| COW | Inf | - | +/- | + | +/- |
| PIG | Inf | - | - | + | - |
| SHEEP | Inf | - | +/- | + | +/- |
| Post vac1 |  |  |  |  |  |
| COW | Un | - | - | NA | NA |
| PIG | Un | - | - | NA | NA |
| SHEEP | Un | - | - | NA | NA |
| Post vac1 |  |  |  |  |  |
| COW | Inf | - | +/- | + | +/- |
| PIG | Inf | - | - | + | - |
| SHEEP | Inf | - | +/- | + | +/- |
| Post vac 2 + |  |  |  |  |  |
| COW | Un | - | - | NA | NA |
| PIG | Un | - | - | NA | NA |

| Status | Status | Clinical Signs | Carrier possibility? | Clinical Signs | Carrier possibility |
|--------|--------|----------------|---------------------|----------------|---------------------|
| SHEEP | Un | - | - | NA | NA |
| Post vac 2 + | | | | | |
| COW | Inf | - | +/- | + | +/- |
| PIG | Inf | - | - | + | - |
| SHEEP | Inf | - | +/- | + | +/- |

Un = uninfected; Inf = infected; - = No clinical signs or Not a carrier; + = Clinical Signs or a carrier
NA = Not applicable

## 5.1. Antibody responses in animals following infection by FMDV
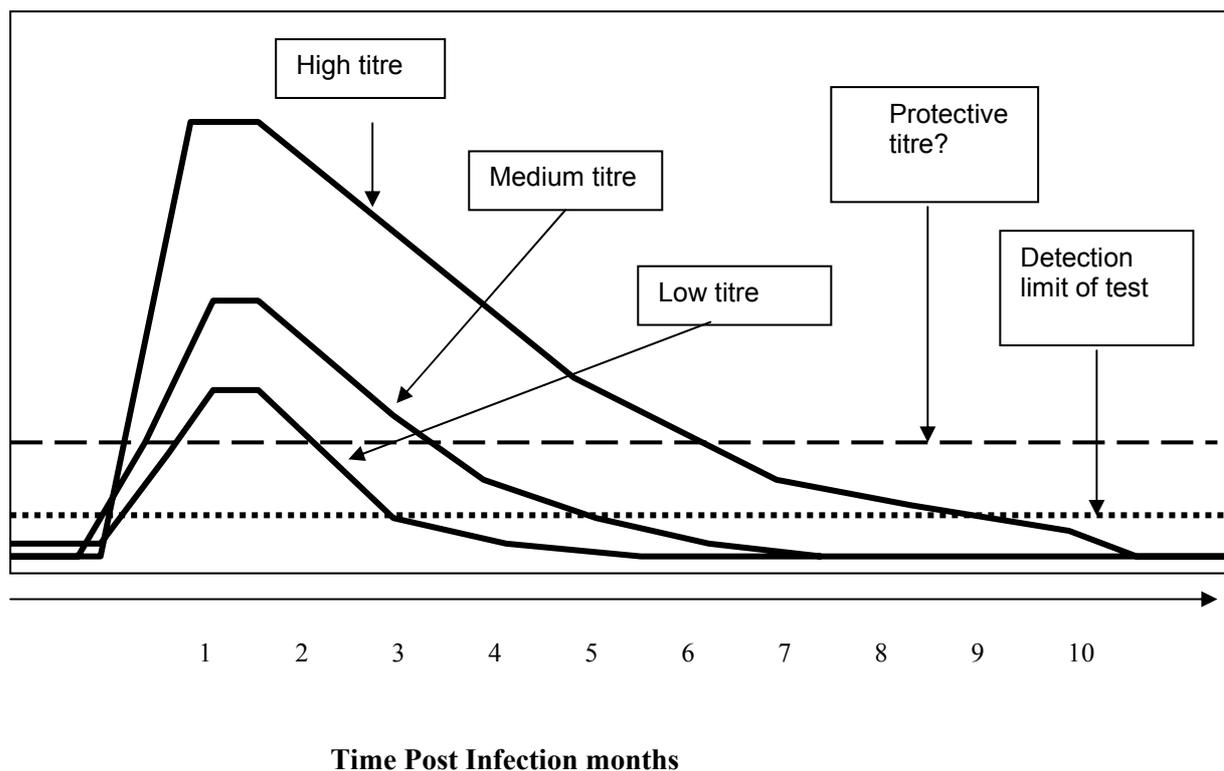


*FIG. 6A. Post infected typical of anti 146S and anti NSP (mainly non carrier studies).*

Typically antibodies are detectable after 5–7 d, rise to a peak at between 21 and 28 d then decline at a similar half-life. The maximum titre therefore, imposes the duration where antibodies can be detected. High titres mean that antibodies can be detected up to 8 m in this example; low titres to approximately 3 m and intermediate to 4-5 m. Similar profiles for anti structural and NSP are obtained. Protective titres are not easy to predict and will be significantly less than detectable antibody titres (here demonstrated by upper horizontal line). It must be remembered that rarely are any antibody titres measured and that most tests determine positivity and negativity based on validation criteria at a single dilution. The above examples are regarded as non-carriers. The antibody spectrum will also vary so that the specificities of the antibodies vary in time (quality) as well as the quantity. This also affects test characteristics.
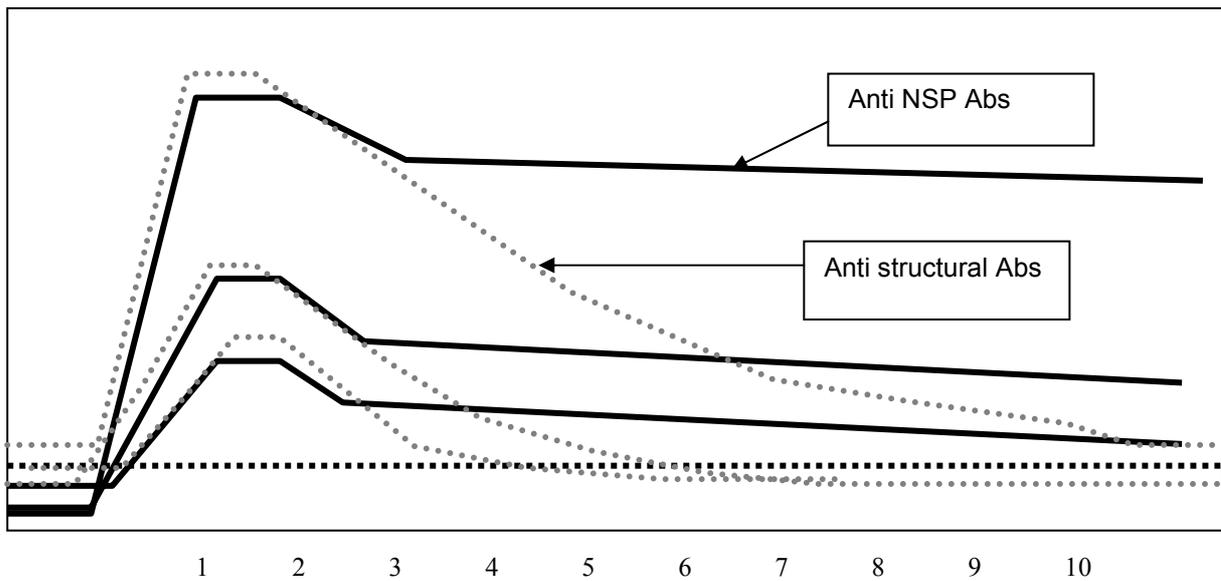
*FIG. 6B. Anti NSP antibodies in some carrier animals are maintained.*

In some animals the anti-structural antibodies decline but the anti NSP antibodies are maintained. Where this extends over approximately 45 d and remains fairly constant there is good evidence that such animals are carriers. NOT all animals shown to be carriers produce any antibody against NSP.

The next figures show that the sampling time obviously affects the level of antibodies detectable and hence the diagnostic potential of any test. Sampling therefore becomes a very important feature of explaining results and making conclusions about disease status of herds, populations comprising many herds and a country as a whole. All features of epidemiology have to be examined to make conclusions in tandem with the diagnostic sensitivity and diagnostic specificity features of a test. This in turn determines the fitness for purpose of a test and the data required from suitable representative populations for validating such tests.
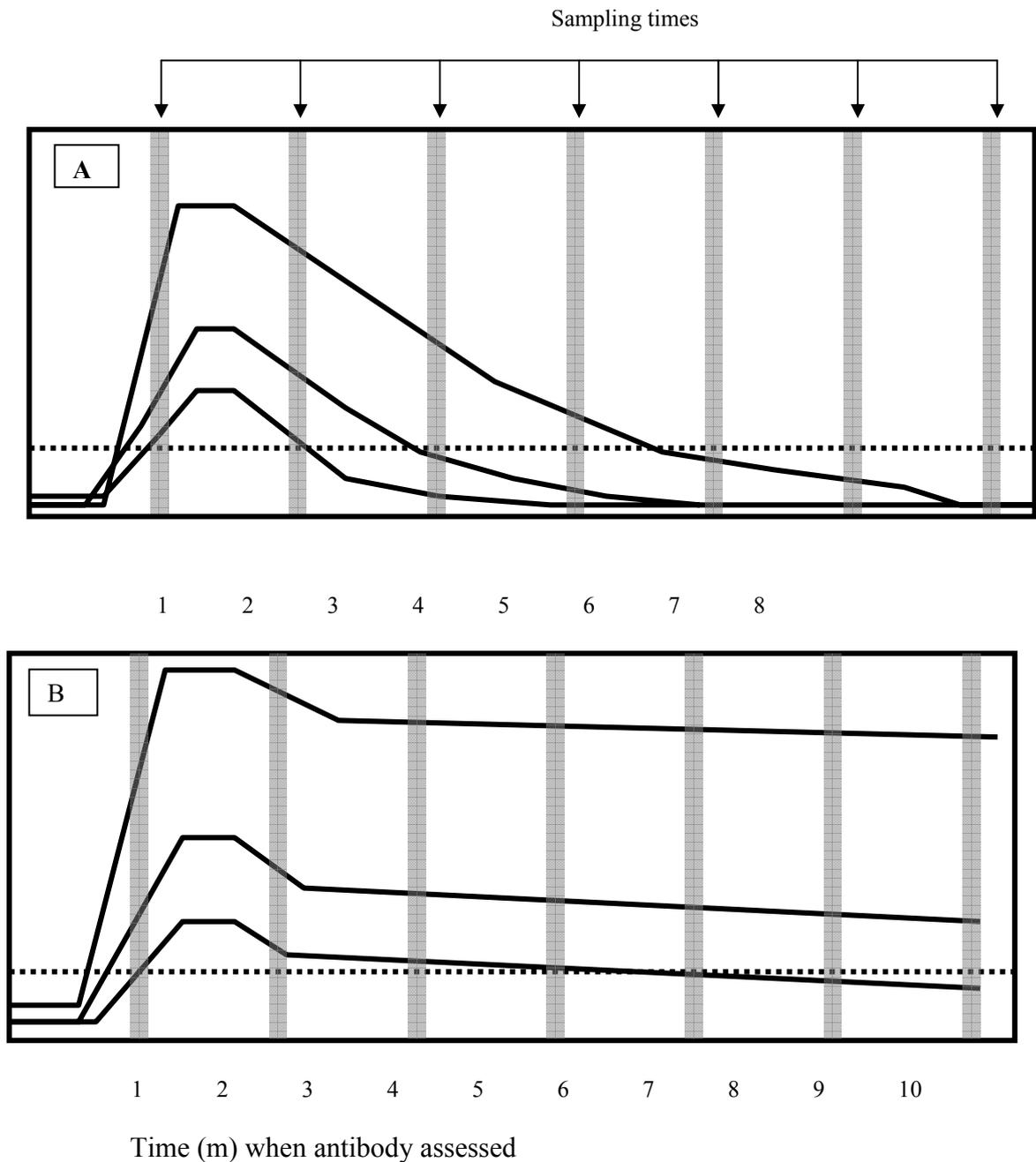
Sampling times

FIG. 7. Measuring antibodies at different times after infection.

Obviously the time of sampling affects the interpretation of data, as does the knowledge on the history of animals. In Fig. 7B following an outbreak, animals maintaining NSP can be deemed carriers. This would be easy to confirm where there were a large percentage of the animals in an outbreak infected and hence a higher chance that carriers are produced. The prevalence of disease thus is a vital component in understanding results. There is little work on this. Titres are not measured so that ratios of anti SP to NSP cannot be made. It established whether titres fluctuate (possibly corresponding to infectious cycle events). The examples in Figs 6 and 7 illustrate simple statements about development of antibodies following infection. In herds the situation can be greatly complicated because disease may not start at the same time; herds may mix and pass on infection after which they divide. The population studied then, is often not fully understood. A complicating factor is that sampling

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has to be made and that the origin of samples may not be well documented. This leads to the merging of data that can greatly complicate conclusions. The fitness for purpose criteria reflect a perfect scenario where sampling of the correct population, at the appropriate statistical level, yields data which can be interpreted perfectly with defined confidence limits. This is seldom the case, but the fitness for purpose guidelines do indicate what factors should be taken into account with reference to the validation data. We are in a rather circular argument then, where tests have been validated with less than perfect populations studied, to qualify tests for examination of situations (purpose) that are themselves not easy to understand. It should be remembered that a system is sometimes required comprising a number of techniques and sampling frames rather than a single survey and use of a single test. This is more relevant to certain applications of fitness for purpose that others. Let us re-examine the elements shown in Table VI and estimate where NSP tests can be used in more detail.

## 5.2. Re examination of fitness for purpose criteria of NSP testing

### 5.2.1. Free with vaccination

This is one of the main uses of NSP testing and determined by OIE as mandatory to show that a population is free from infection. There is a good amount of validation data on the use of NSP tests in this situation.

It must be emphasised that: **A population can be free from disease but not free of infection.**

This is very important since vaccination can limit the effect of infection so that clinical signs are reduced or non apparent. This is relevant to contact animals during infection such as sheep and goats that may become infected without clinical signs. The pathogenicity factors involved are not understood but obviously involve particular selected mutants from an FMDV population 'infecting' other species and innate immunity. The NSP offers a test to determine post replication events through detection of specific antibodies. So in any exercise to show lack of disease, all susceptible animals must be examined. Sero monitoring then is at the basis of the proof of a population's FMD innocence. If animals are observed to be NSP antibody positive but have never shown clinical signs, then the control measures taken are highly debatable. The main threat perceived form such animals (excluding pigs) is that they are a source of virus to re-infect others and that are or can become carrier animals.The use of vaccine complicates testing since we have a background of post vaccination antibodies against structural proteins. These are only present in some of the population and vary in titre and quality and depend on the vaccine control programmes being used. Seldom is there good data and marking (e.g. ear tags) of vaccinated animals so that histories are scant. Vaccine quality is paramount and local made vaccines may produce a high prevalence of antibodies against NSP. Where more modern techniques of vaccine purification for vaccines are used, the prevalence of anti-NSP has been shown to be zero. Establishing the effect of the vaccine used is important so that an expected prevalence of false positives is needed. Systems involving confirmatory tests e.g. western blotting of samples to assess antibody profile (dealt with later) are important and add confidence to data.

Diagnostic sensitivity becomes the most important feature along with the sampling design and increasing this in tests will reduce the specificity. This balance has to be realised and determined for each country using NSP tests. The implications of this are that increasing

sensitivity means more re-testing of samples to eliminate false positive results. Survey design is critical. Surveys have to be relatively large scale and involve more than a single exercise.

### 5.2.2. Historical freedom

In such scenarios there has never been vaccination, or it has not been practiced for many years, so that antibodies to FMDV are not expected unless there is infection. In these situations the sampling regimes are far less stringent as compared to 5.2.1. Here, a false positive rate of the test is needed. The diagnostic specificity has been established on many of the NSP tests using negative populations only. The cut off criteria then are based on the negative populations studied only so that the validity only extends to the animals examined and adjustments to cut off levels may be necessary to best reflect local conditions. NSP tests have a very good niche for this purpose.

### 5.2.3. Re-establishment of "freedom" after outbreaks

This infers that a country is infected with FMD. After control measures which could include vaccination (limited or extensive) there is no clinical disease observed. Serological surveys form the basis of establishing freedom from infection. This differs from historical freedom in that we have had recent infection and that vaccination may have been practiced. The NSP test provides the means of assessing whether a population is showing antibodies evident of post replicative events for FMD. The sampling strategy will depend on the exact control measures used. If there was vaccination the test allows discrimination of vaccinated and infected animals (note that vaccines used may give a false positive rate). Sampling in this situation may well have to be extensive, involve all species and be repeated in the light of the expected, or the measured prevalence of the disease.

### 5.2.4. Demonstrate freedom from infection in individual animals or products for trade

NSP testing strongly favours looking at populations rather than single animals. This section refers more to looking at the antigen or agent itself in products. However, where quarantine is concerned there is a real need to test all individuals at times before, during and after movement. This greatly value adds to the disease status of animals. In this case, the mobile use of pen side test is of value, since these can be used on site and more immediate actions taken if evidence of previous disease is observed in the absence of clinical signs.

### 5.2.5. Confirmatory diagnosis of clinical cases

This points to agent and antigen detection methods, but during an outbreak in the face of vaccination the NSP allows discrimination of true positives via the antibodies (produced e.g. 7–10 d post infection). NSP tests in tandem with other agent detection systems are powerful.

### 5.2.6. Eradication of infection from defined populations

The definition of the population is important where there is, for example, zoning in a country. Again this emphasizes infection and not disease, so silent, sub-clinical cases must be identified and appropriate control measures taken. An example in pigs will be shown later. The NSP tests allow identification of animals where replication of FMD has occurred and this includes contact animals and those historically known to be susceptible but which do not show signs, a typical situation where FMD is endemic. Complications ensue where wild life

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such as buffaloes are historically carriers of FMD Defining the highest, at risk, population is important.

*5.2.7. Estimate prevalence of infection to facilitate risk analysis (surveys)*

The percentage of animals infected or post infection can be estimated in a population using the NSP tests. This can be done in the face of vaccination. The NSP does not measure titre so that the VNT or LPBE or equivalent, which is analogous to the VNT, is also needed to measure the antibodies against structural components. In this way the system will determine which titres are as a result of vaccination and which are as a result of previous infection. The risk of infection to surrounding non infected animals as well as to a non- infected population as a whole, can be assessed by determining the number of animals that might survive challenge with FMD (titres in positive vaccinated animals). Survey design is important so that strata depending on geographical location an age have to be examined closely.

*5.2.8. Identifying infected animals or groups toward implementing disease control measures*

This is linked to other sections but specifically identification means that control measures can be made dependant on results of a survey. Infected means showing events related to replication, so that the NSP is ideal. Once again we identify infection with or without clinical signs, which often shows disturbing results as compared with the veterinary picture reported from field.

*5.2.9. Classify herd health status*

This involves all the factors discussed in the other sections. NSP antibody tests identify infection after 6–7d and then in a number of cases show that antibodies prevail indicating a carrier status. Once again, the determination of animals with no clinical signs as positive for NSP is a sure sign that virus is circulating somewhere.

*5.2.10. Immune status in individual animals or populations (post-vaccination)*

Here the antibodies against structural proteins are ideally needed. The efficacy of a vaccine is being measured so that the NSP is not used.

**5.3. Country scenarios for use of NSP tests**

Another way to look at the use of NSP tests is to evaluate needs for countries that: vaccinate/do not vaccinate; and are either threatened by FMD/ have an outbreak of FMD/have endemic FMD; and also consider to what extent control measures are being implemented and what studies are being made allow better planning of control FMD. These cover most requirements for testing.

*FIG. 8. Use of NSP tests where there is no vaccination.*

A country situation where there is a non-immune population since there is no initial vaccination regime. Here the threat is for introduction of disease from outside by live importation of animals or in products. This could also represent a zone within a country where vaccination is not practiced.

◯ = Use of NSP test        ☐ = NSP tested and negative

▲ = Use of Anti structural tests (VNT or LPBE)

*FIG. 9. Use of NSP tests where there is vaccination.*

A country situation where there is vaccination made giving immunity to all or a part of the livestock. Here the threat is higher for disease to be missed clinically; to animals which are not vaccinated or where titres are non-protective due to intervals between vaccinations, the use of the wrong serotype or because of poor vaccine or vaccine practices.

○ = Use of NSP test          □ = NSP tested and negative

△ = Use of Anti structural tests (VNT or LPBE)

Fig. 8 shows a country free from FMDV, e.g. historically or some time after vaccination ceased. The threat is the re-introduction of disease either by livestock or products. Prevention of entry is vital and NSP tests can be used to show lack of infection or infection history in animals either at source or during their transport and in quarantine within the country. In this situation there is either disease introduction or not. Routine serology where there is an historical precedence for lack of FMD is wise. However, in the absence of clinical signs over a long term and with considerations of distance from other countries with FMD and importation of man and animals and their products, there is little impetus to do this. The recent outbreak in the U.K. with its devastating effect on the economics of livestock trade, and public confidence, is a warning about being too complacent about surveillance of imports.

Veterinarians should detect any infection in the field since FMD usually affects a relatively high number of animals in an outbreak (prevalence rate high). In the case or reported disease the outbreak should be dealt with according to a contingency plan. Animals showing clinical signs can be killed, thus removing the threat of infection; or left (segregated/quarantined) or the decision to vaccinate made, in which case the scenario shown in Fig. 9 comes into play. Assessing in contact animals or where tracing has established a link is important and can also be greatly helped with NSP tests. Where there are no clinical signs (by clinical definition non infected) there is a risk that they may become carriers. Such sero-positive animals have to be assessed in terms of the risk of re-introduction and spread of disease.

Where there is no action, the NSP test can be used to determine animals with a past infection, so that another decision to vaccinate, cull or just leave can be made. Negative animals could be regarded as safe and the risk of them causing infection reduced to zero. There is great reluctance to assume zero risk by chief veterinary officers. It would be a more than useful exercise to take a region where carriers were present and perform a large scale experiment to assess different species and different carriers in terms of clinical signs, serology and contact rates to measure the risk of infection. Such situation mixing of for example, sheep that are carriers (never examined) and cattle following outbreaks in cattle have existed for many years in certain countries without any sign of FMD.

### 5.3.1. Vaccination

It is worth discussing mass vaccination adopted by countries in terms of its measured usefulness. Very often vaccination campaigns are mounted and run for a long time without any measure of there efficiency. A serological survey of vaccinated animals using the VNT or LPBE will determine what prevalence of antibody exists in the population. Baring in mind points such as aging of animals and maternal antibodies; it should be easy to examine the population in terms of likely cover against FMD against the serotypes used in vaccines. It is also possible using the combination of these tests and NSP antibody testing to assess whether virus is circulating undetected by clinical examination. Antibody levels are also a component of protection, so that a titration exercise for structural proteins is necessary. Routine blanket vaccination with multivalent vaccines (30 cents to 1 dollar per valency) is very expensive in vaccine and manpower costs. Such vaccines may appear to have a beneficial effect since no disease is recorded where vaccination is made. However, this may not be anything to do with the vaccine. A randomized survey for antibodies at the time (which by definition that whole population is covered can be quite small) in terms of sample number) of a vaccination round may show that only a relatively small proportion of the animals (usually cattle) have any chance of protection. Unless approximately 70% of the total herd is adequately protected

there is a large risk that FMDV will infect that herd, in which case, the vaccination cover is too low and almost useless. Purposive surveys to uncover regions with low total antibody cover are much more beneficial so that vaccine can be administered there to get the required levels. The main influence of vaccination is to limit testing to the use of NSP tests where the differentiation between infection and vaccine generated antibodies is required. As for the situation in Fig. 8 the decisions on control centre on the survey design and assay results. Both of these pose problems concerning risk involving animals developing into carriers and the risk of transporting recently infected (infectious) animals and products within a country.

### 5.3.2. Carrier State

The existence of FMD carriers among recovered cattle, sheep, goats, water buffalo, Cape buffalo, deer and a number of other species in well documented. During the first three months after infection the proportion of FMD carriers with small amounts of FMDV persisting in the throat, may reach 50% of the recovered cattle. This number decreases with time and the percentage of carriers remaining at two years post infection usually is small. Vaccinated cattle exposed to FMD virus can become carriers without showing clinical signs. Carrier cattle are unlikely to have scars on the tongue or foot epithelium and will escape detection at the farm of origin and at ante-mortem or post-mortem inspection. FMD virus is found only in small quantities in the pharyngeal area of carriers. Carriers may have high levels of circulating FMD antibodies and no FMD virus is found in the blood, bone marrow, lymphatic glands or muscle tissue. Such animals can very well be selected and pass all farm and slaughterhouse inspections. Therefore, the question asked repeatedly in discussions with stakeholders concerns the risk posed by healthy carriers.

Only circumstantial evidence suggests that carrier cattle may initiate an outbreak of FMD among susceptible cattle, although experimental proof of this theory has been difficult to obtain. The process of recovering virus in carrier animals involved probing sampling. This is a highly invasive process requiring a great deal of mechanical scraping of cells of the pharyngeal region. It is highly unlikely that this process is present in any animal in the field. Seldom or not at all have animals been seen impaling themselves on branches to scrape out their throats, so the mechanical process required to uncover virus (very intermittently) is not observed. Thus the chance of virus being excreted is very low and there is no evidence that virus is shed from carrier animals.

As stated, carrier cattle may have high antibody levels and do not have virus in the bloodstream, muscles, lymph glands or other organs. However, superficial mechanical contamination of beef by virus present in the throat is a risk to be considered. At the slaughterhouse, the cattle are stunned, hoisted (head down) and exsanguinated. After the skin is removed, the head (including tongue and the pharyngeal area) is removed from the carcass. No further contact occurs between the head and the rest of the carcass. Tongues and adjacent tissues are removed from the rest of the head and processed separately. Pharyngeal tissue, including tonsillar tissue, is removed from the tongue and collected for rendering. Tongues are then rinsed with a strong jet of water and in the unlikely event that the minute amounts of pharyngeal virus contaminate the tongue surface; such virus will be rinsed off or at least diluted to a virtually insignificant quantity. Thus, the risk of mechanical contamination of the carcass or organs with 'carrier virus' from the pharyngeal area during slaughter and processing is negligible.

### 5.4. Problems of NSP testing/ persistently infected animals and vaccination

Countries that are free of FMD are reluctant to use vaccine in the event of an outbreak because of the difficulties this can cause in re-establishing freedom from FMD status to the satisfaction of trading partners. The problem does not lie in distinguishing between vaccinated and recovered animals as vaccinated animals can be tagged or otherwise marked to show that they have been vaccinated. The difficulty is in identifying vaccinated animals that have had contact with live virus and become carriers. The traditional probang test is not sufficiently sensitive and is labour and laboratory intensive, but alternative serological tests provide increased security by reducing the likelihood of trading carrier animals and can be used to help define the limits of an outbreak.

In FMD endemic countries that vaccinate against the disease and in countries which vaccinate during an outbreak of FMD, the possibility is high that clinical disease will be masked in those animals which have only partial immunity and which are exposed to live virus. These animals may show some clinical signs that would be detected by a trained clinician, but such signs will usually be missed by owners and untrained animal health personnel. These animals are likely to remain a source of infection to in-contact susceptible species and the virus can be maintained unobserved in a vaccinated population. Similarly, animals vaccinated during an outbreak of FMD will pass through a period of partial immunity before the vaccine becomes fully effective, during which clinical signs will be reduced or prevented, but infection and virus transmission can still occur. The speed at which vaccination induces protective immunity and prevents transmission depends on the potency of the vaccine against the outbreak strain and the level of viral exposure, but may be as short as four days.

Ruminant animals that have recovered from infection with FMDV and vaccinated ruminants that have had contact with live virus may retain infection in the pharyngeal region for a variable period of time. The carrier is defined as an animal from which live virus can be recovered after 28 d following infection. Over 50% of ruminants exposed to live FMDV become carriers; pigs do not become carriers. The duration of the carrier state depends on the species and individual. The African buffalo (*Syncerus caffer*) may carry virus for over five years, cattle for over three years, sheep for up to nine months (20), goats and wild ruminants for shorter periods of time and for South American camelids, no carrier state exists.

Eventually the carrier does eliminate the virus. The virus persists in the basal layer cells of the pharyngeal epithelium, particularly of the dorsal soft palate. Existing methods do not permit detection of the virus in the more superficial layers of cells and how the virus is excreted into the pharynx is not clear. How this changes from a lytic agent into one that can establish a persistent infection is not known, but is probably linked to the selection of a mutation.

The establishment of the carrier state and the duration of this state depends on the host species, but probably also on the strain and serotype of FMDV and even on the breed of host species. All three serotypes of the South African Territories (SAT) viruses are found in the wild African buffalo populations of Botswana and Zimbabwe, but rarely are the commercially farmed Brahman cattle of the region found to be carrying either SAT 1 or SAT 3. In the last twenty years a series of outbreaks of SAT 2 was observed in the FMD-free zone of Zimbabwe and one Brahman bull in particular remained a carrier of SAT 2 virus for over three years. During the 1991 outbreak of SAT 2 in Zimbabwe, the affected European cattle carried the virus for a shorter period than Brahman cattle. The SAT viruses occasionally spread out of Africa into the Middle East and into Saudi Arabia during 2000. However, although the O, A

and Asia 1 serotypes persist in this region, in spite of limited attempts at control, the SAT viruses died out. This implies that the cattle, sheep and goats are unable to maintain the SAT serotypes, or conversely, these serotypes require particular host species. The distribution of the Asia 1 serotype would also suggest that it has been constrained from establishing itself outside of Asia. Whether the geographical restriction of serotypes and even strains of FMDV is related to the ability of the virus to establish the carrier state in particular susceptible species or breeds is not known, but should that be the case, it presents a powerful argument for considering the importance of the carrier in the epidemiology of FMD.

Transmission of FMDV from a carrier bovine to a susceptible in-contact bovine has never been shown under experimental conditions, despite a considerable number of attempts. An experiment with carrier African buffalo, kept in contact with susceptible cattle and additional susceptible buffalo did succeed, but the results were difficult to explain. A group of three FMD-free buffalo were infected with SAT 2 virus and kept in an enclosure with four susceptible cattle on an island in Lake Kariba. The buffalo developed clinical FMD and recovered without transmitting the disease to the cattle. The buffalo all became carriers and four months later, two further FMD-free buffalo were introduced. Seven weeks after the introduction of the two additional buffalo, the cattle developed clinical FMD, which then spread to the two new buffalo. What triggered the transmission event was not clear, but the cattle were confirmed to be infected with the same virus as that carried by the originally infected buffalo. All the animals were monitored throughout the experiment and regular samples collected from the pharynx to confirm the continuing persistence of the virus. There have been a number of anecdotal accounts of carriers starting new outbreaks of FMD in the field.

The definitive identification of carrier or subclinically infected animals requires recovery of live FMDV from those animals. The predilection of the virus for the epithelium of the pharynx makes this tissue the most suitable to sample, a procedure which can be carried out using the probang sampling cup. This is a hollow metal cup with a slightly sharpened edge, attached from the centre of the bowl by a long wire, approximately half a metre long, to a handle at the free end, which can be pushed into the mouth of the animal being tested, over the base of the tongue into the pharynx. The cup is then withdrawn, collecting as it is pulled out, mucous and superficial cellular material from the pharynx.

The content of the cup is usually mixed with a neutral buffer solution and if not examined immediately, kept frozen over liquid nitrogen or on dry ice (solid carbon dioxide). Live virus can be cultured on sensitive tissue culture such as primary bovine thyroid cells or lamb kidney cells. Carrier animals, which have either recovered from clinical disease, or have been vaccinated and subsequently acquired infection following contact with live virus, will also have high levels of specific anti-FMDV antibody present in the pharyngeal mucous and treatment of the probing sample with chlorofluorocarbon can help dissociate the virus/antibody complexes and increase the possibility of recovering virus on tissue culture. Subclinically infected animals, other than those with partial vaccinal immunity, will not usually have detectable antibody levels at this stage of infection. The quantity of virus present in the pharynx of carrier animals can vary considerably over time and the successful recovery of virus will depend on this and other factors, such as the subsequent handling of the sample and the skill of the operator. Possibly only 50% of carrier animals will be identified from the examination of a single probang sample, but this percentage can be increased by repeating the sampling procedure at two weekly intervals.

The sensitivity of the test can be improved by using the polymerase chain reaction (PCR), which identifies small quantities of viral genome present in the sample. However, the PCR itself can also give false negative results due to the presence of non-specific inhibitors. A comparative study using both tissue culture and PCR on probang samples demonstrated that some samples could give positive results using one method and negative results using the other and that both tests should ideally be used together. There is also the unresolved question on the significance of a positive PCR result. The PCR identifies only part of the viral genome and would be positive even if the genome was itself fragmented and unassociated with any live virus. While a positive PCR is therefore highly suggestive of previous infection, the animal from which the sample was collected could no longer be carrying live virus and no longer represent any risk of causing a further outbreak.

Testing of animals suspected of having subclinical infection may also include animals that have only recently been infected and have not yet developed clinical disease. Mucous samples from the nose and mouth can be collected to detect the low levels of virus present, but because tissue culture techniques for virus isolation may take up to 96 h to complete, by which time these animals would show clear clinical signs, the more rapid PCR test can be used. Methods have been designed to carry out large numbers of PCR tests on 96 well microtitre plates, which would allow rapid screening of at-risk animals at the start of an outbreak, or to determine the extent of a rapidly spreading outbreak. In addition, blood samples can be collected from suspect animals for identification of viraemia, either by PCR or inoculation of tissue culture. During the 2001 outbreak of FMD in the UK, the spread of disease in subclinically infected sheep was responsible for the widespread dissemination and persistence of the virus. Advantage could have been taken of the use of blood samples to help identify infected animals, for although the isolation of virus from blood is restricted to a three day viraemic period, the samples could have been simultaneously tested for the presence of specific anti-FMDV antibody, as a sheep, like any other susceptible species that is or has been recently infected, will either be virus or antibody positive, or sometimes both. Carrier animals also have specific antibodies to FMDV. This is true whether they have recovered from infection or have been vaccinated. In countries that identify vaccinated animals by a brand or an ear tag, there should not be a problem in distinguishing animals that are antibody positive as a result of vaccination from those that are positive following recovery from infection. However, the difficulty lies in identifying those vaccinated animals that have had contact with live virus and become carriers.

Carrier animals have antibodies to FMDV that can be detected in the serum and also in the saliva. Where specific immunoglobulin A (IgA) is present in recovered or vaccinated cattle and is elevated in the carrier animal. An ELISA has been developed to quantify this elevated level of specific IgA, to indicate the possibility that the animal from which the sample was collected could be a carrier but is not in routine use. Some carrier cattle, fail to produce a level of IgA in their saliva significantly higher than non-carrier cattle and while this test has potential as a herd test, further refinement and increased sensitivity is required.

It is good to remind ourselves here (*See* Fig. 4) that the RNA of FMDV has a positive sense, single-stranded ribonucleic acid genome of 8 400 nucleotides that codes for twelve proteins, four of which are structural and make up the capsid of the virus and eight of which are non-structural, which together allow the virus to replicate in an infected cell. The structural genes are identified as 1A, 1B, 1C and 1D, the non-structural as L, 2A, 2B, 2C, 3A, 3B, 3C and 3D.

The functions of the proteins for which the non-structural genes code have not all been fully identified. However, the 3D gene should be mentioned as coding for the viral polymerase and precipitating antibodies to this protein are detected in the viral infection-associated antigen (VIAA) test. The vaccine used to help control outbreaks of FMD is an inactivated preparation of whole virus particles in an oil or aluminium hydroxide/saponin adjuvant. There is no replication of the virus following vaccination and the vaccinated animal develops antibodies to the structural proteins of the virus present in the viral capsid. Some of these antibodies are neutralising and will protect the animal from subsequent infection. No viral replication means that there is no expression of the non-structural proteins (NSPs) and the animal will not develop antibodies to these proteins, although some vaccines do contain low levels of these NSPs depending on the manufacturing process, in particular 3D, and a low antibody response to the NSPs has been observed. This response is more obvious in animals that have been vaccinated several times.

Most animals that have recently recovered from infection will have variable levels antibodies to the NSPs, because as the virus replicates in the tissues of the animals, these proteins will be expressed and stimulate the production of specific antibodies by the host. The detection of these antibodies can therefore be used to identify those animals that have been infected with FMD and which may still be carrying live virus.

Even in cattle, considerable individual variation has been shown in the amount of antibody produced to each of the NSPs and consequently in the period of time after infection that antibody may be detected. (*See* Figs. 7 and 8*)*. The 2C antibodies may be detectable for twelve months, whilst the 3ABC antibodies persist for longer periods. The severity of the infection is likely to be the major influence on the levels and the subsequent duration of detection of the NSP antibodies.

A 3ABC ELISA was used to define the limits of the 1996 outbreak of FMD in the Balkans and antibody to the 3ABC polyprotein is considered the single most reliable indicator of infection. However, a problem persists with the NSP tests on an individual animal level. Some cattle that have been vaccinated particularly with a high potency vaccine as might be used in an outbreak in a previously FMD-free country, will fail to develop antibodies to the NSPs should they have contact with live virus. This is because their level of immunity prevents any significant viral replication and therefore expression of the NSPs. These animals could, however, become carriers of live virus.

On a herd basis, even potent FMD vaccine will not protect 100% of the cattle and should the herd become exposed to live virus, some will support replicating virus, even though they do not show clinical disease and sero-convert to some of the NSPs, in particular to 3ABC. Thus, testing an entire herd makes it possible to diagnose a previous encounter with live virus and determine the potential for the presence of carriers, assuming, of course, that the entire herd was exposed to the same challenge. The test may fail if only a few animals were in contact with live virus, perhaps as an aerosol from a neighbouring infected farm and were all sufficiently immune to prevent the expression of the NSPs.

It can be concluded that the tests for antibodies to NSPs are a significant advance in the detection of carrier animals. However, the test has limitations and cannot be used reliably on individual animals to exclude the possibility that the animal may be a carrier of live virus. Even when used on an entire herd, the test does not constitute a guarantee. The possibility of carrier animals creating fresh outbreaks is probably extremely small and this can be further reduced by probang and serological testing. Nevertheless, however small the risk, if importing

countries have a choice, they are likely to choose to import their live animals and animal products from areas where there is no FMD vaccination or possibility of the presence of carrier animals. Until the identification of carrier animals is 100% certain, FMD will remain the most significant constraint to trade of susceptible animals and their products.

**5.5.    Perspective on use of testing for antibodies against NSP**

A summary of where to use NSP antibody testing in context with other testing is shown in Fig. 10. This divides historically free non-vaccinating countries (initially) from countries that vaccinate the total population or have zones of freedom from infection that are not vaccinated.

The risk assessments of low, medium and high are ascribed. This assessment depends on the proximity of diseased areas; the quality of services and data in such areas, the importing criteria; the veterinary services in the country (clinical surveillance) and economic considerations. Again the design of sero-surveys to take account of likely FMD prevalence rates are important. The distinction between measuring disease freedom (clinical assessment at field level and confirmation of disease in the laboratory) and showing absence of infection (virus multiplication) is paramount.
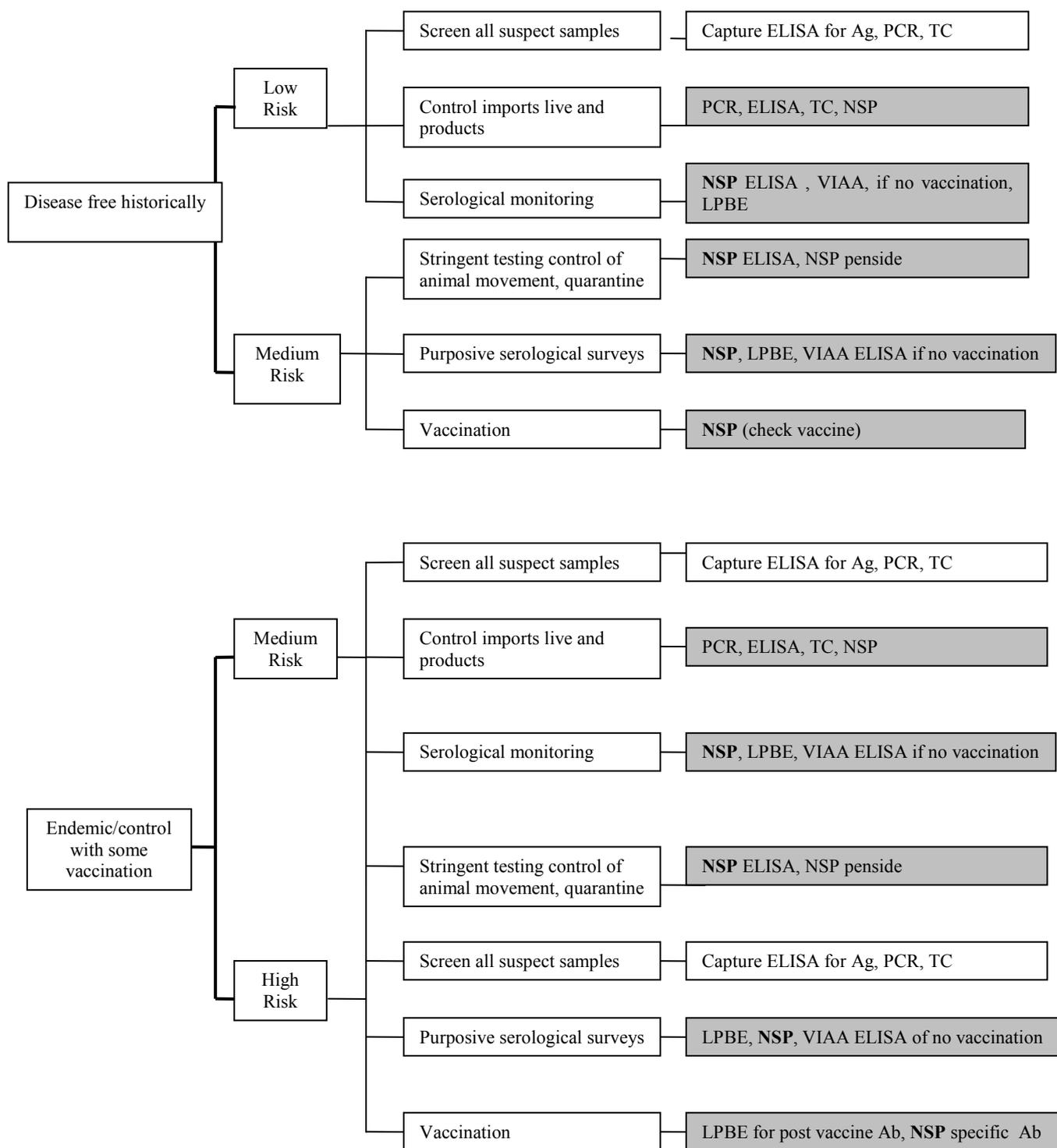
*FIG. 10. Outline of use of NSP based tests in disease monitoring and control.*
The grey boxes indicate applications where NSPs are used in testing.

6.      DATA FROM VALIDATION STUDIES

Data has been collected from many sources during the development of tests involving NSP including:

- Institutional sources at a more research level (particularly in the early stages of interest in NSP
- National and international reference laboratories for FMD
- Companies developing kits in association with institutions and international reference laboratories
- International organizations such as the IAEA who ran a coordinated research project on validation of methods involving NSP and also harmonization exercises.
- EU commission including harmonisation exercises
- Companies providing kits for control campaigns; (data from campaigns)
- Company dossiers for national ratification of tests
- OIE publications
- Published papers

The developments in devising methods for differentiating infected and vaccinated livestock (at the core of the need) has been very fragmented. Attempts to coordinated efforts have been made but there have been underlying problem of:

- Miscellaneous associations of various institutional and commercial concerns
- Independent use of tests that were partially-validated in the field for use in campaigns
- Claims of test performance that cannot be substantiated
- Lack of experimental data to substantiate field findings
- Poorly planned validation exercises
- Lack of reference material for livestock populations
- Lack of quality controls in test kits
- Poor knowledge about validation requirements
- Lack of proper registration processes to stimulate quality
- Guidelines dealing with principles rather than knowledge

The ultimate aim is to provide validated kits. Perfect kits for are defined in Table X where comments are included to relate to the current situation.

TABLE X. DEFINITION OF PROPERTIES OF PERFECT KITS AND PRESENT RELATIONSHIP TO PRESENT SUPPLY

| Ideal property | B | S | C | U | PA* |
|---|---|---|---|---|---|
| (1) Available in bulk (assessment of likely need worldwide are crucial to supply). | + | + | + | + | To S. America only |
| (2) Available and distributed on demand (available immediately) | + | + | + | + | S. A. |

| Ideal property | B | S | C | U | PA* |
|---|---|---|---|---|---|
| (3) Costed - high costs will prohibit use in developing countries. Price needed by buyers. | + | + | + | + | - |
| (4) Quality controlled in terms of day to day running. (IQC and EQA needed). | +/- | -/+ | +/- | +/- | +/- |
| (5) Robust (stable reagents on supply) | | | | | |
| (6) Validated in terms of diagnostic sensitivity. | + cattle ? pigs | +/-cattle | +cattle + pigs | ++ pigs | ++ cattle |
| (7) Validated in terms of diagnostic specificity. | + cattle + pigs | +/-cattle | +cattle + pigs | ++ pigs + cattle | ++ cattle |
| (8) Fit for purpose (linked to estimates of sensitivity/specificity and this defined by producer, or at least criteria of validation, so far, described in full** | - | - | - | - | - |
| (9) Contain control sera ++ = strong positive, + = weak positive; +/- = very weak positive; - = negative | ++; + - | ++; - | ++ ; - | ++; +, - | ++, +, +/-, - |
| (10) Contain everything necessary to fully perform the assay. This includes plates, conjugates, tips, etc. | - tips | - tips | - tips | - tips | ? |
| (11) Data from kits sought in EQA to update validation in planned exercise | - | - | - | - | +/- |
| (12) Have agreed reference standards. | - | - | - | - | - |

B = Bommeli/IDEXX; S = Svanova (cattle only); C = CEDI; U = UBI; PA = PANAFTOSA

* = PANAFTOSA is not commercial kit but produced in large scale for S. American campaigns. It is used as the Index test for screening then in association with IETB confirmation method

** The guidelines and registration process have only just been introduced. It is likely that each commercial kit can be recognised for some fitness for purpose. General screening after infection is possible for all.

## 6.1. Analysis of fitness for purpose criteria for tests to detect antibodies to NSP

Expanding on Table X, the opinions here are of the author only based on his contacts and data. There is a continuous up dating of data by the companies and efforts to improve performance based on feed back. Over the past few years there have been improvements to the end user.

*Point 1*

The manufacturing base for each is good and there are supplies readily. However, in the event of a large-scale operation there may be a short fall in test kits from all suppliers. In this event there may be the temptation to mix kits and there is still no statement as to the relative analytical and diagnostic sensitivities and specificities. Exercises and publications do give general statements for some comparisons of the assays. This situation can be improved

through development of standards that is mentioned in point 12. The latest comparative data is reviewed later in the paper.

### *Point 2*

This is linked to point 1 and kits are supplied in good time.

### *Point 3*

Initially it was difficult to get the cost of kits in an open way. Kits are priced now and there is room for negotiation of prices subject to the scale of needs. All the commercial firms have supplied kits free and at reduced costs where there have been comparative exercises e.g. to the IAEA CRP mentioned by UBI (a good supporter), Bommeli, Svanova and CEDI. This is still going on and Point 12 will illustrate the latest developments. Representatives from all companies and PANAFTOSA have always been available to attend many meetings concerning NSP testing.

### *Point 4*

This area can be considerably strengthened in all cases. The IAEA developed simplified methods for recording untreated and processed data to allow day-by-day control and examination of variation in test performance. Charting methods and manuals for Indirect and competitive ELISA, for their use have not been taken, so far, by any manufacturer. These allow a graphic continuous display of all aspects of the performance (variation) of the tests. Without this principle there can be no justification in giving confidence to any data from any test from any operator(s). It is hoped that soon such methods can be incorporated in all the test kits. This is one of the elements seen in the validation criteria for the OIE so this might well accelerate the process.

### *Point 5*

This is difficult to assess. Kits have been provided which seem to work (consult point 4 to see why this is important). Where there have been difficulties was in efficient transportation under the optimum condition. Certain reagents and formulations in the test kit can be predicted to be susceptible to 'stress' from transportation or storage. Again the OIE validation criteria ask this question.

### *Points 6, 7 and 8*

These are at the heart of proving what the test can do, the performance based on analysis of data from populations reflecting all the variations of FMD infection and vaccination. Data is available but often not directly comparable. This area has been covered in the rest of the paper. Submission for registration will allow companies to define fitness for purpose and stimulate the data needed to prove that the tests are valid.

Point 9.

There are problems here with some kits in the way data is processed and the relationships of data that are being compared. It is not useful to open wounds here but, allied to IQC methods for the continuous analysis of test performance (point 4), some general points about assays can be made.

(a) In indirect ELISA the results have to be based on the relationship of test samples to a control positive that is not too strong. Comparison of reactions to a weaker serum titrating to about half the OD maximum of the system (max no more that 1.4 OD Unit, so around 0.7) is vital. In this control the maximum variation in the test will be observed, so that it can be assessed as the test error. The relationships in I-ELISA are based on a ratio or percentage of

positivity value. If the maximum serum C++ is used then the relationships of weaker OD to this are nonsense and bare no relationship to the effective antibody concentrations based on a single dilution (every kit uses a single dilution to test).

(b) In competitive assays the high OD value for a negative also affects the performance and improvements in the sensitivity/specificity relationships could be made with more careful adjustments of competitive conditions.

***Point 10.***

Tips are not included with tests possibly because of the c various tip requirements for users. This can be a major problem in developing countries where tips are hard to purchase and are often reused adding to the variables in performance of a kit.

***Point 11***

Data are constantly being obtained from users. The manufacturers might gain great information if they collected this data and allowed this to provide evidence that changes might be necessary. This would allow validation criteria to be obtained. This is noted in OIE guidelines where highest category of validation is needed.

***Point 12***

Reference standards are not available so far. This is a major problem to kit developers and in harmonization exercises. The IAEA has made several sera (bovine anti infection SAT 1, 2 and three from S. Africa) to help in this area and will make more. There is a need for a universal set of activity standards to 'calibrate' analytical sensitivities to allow a relative analytical sensitivity to be assessed. Qualitative issues can be addressed where standards reflect typical situation in the field. Once again we are seeking a relative performance comparison. Commercial concerns are all providing kits to help in the IAEA exercise. The EU is also making standards and there are several institutions that provide panels from various epidemiological niches.

7.    OUTLINE OF CRP DEVELOPMENTS

**7.1. Coordinated Research Project (CRP), D3 20.20 of Joint FAO/IAEA Division of the International Atomic Energy Agency (IAEA), Vienna**

A CRP was funded by the Joint FAO/IAEA Division from 1999 to 2004. The title was "Use of non-structural proteins of foot and mouth disease (FMD) virus to differentiate vaccinated and infected animals".

The CRP funds individuals in laboratories to make research on a given theme. Each contract holder was funded for $5000. Agreement holders also were involved and supplied expert advice. The research contract and agreement holders have changed during the 5 years. The idea was to try and examine test performance of the reagent sets available.

Note that in 2000 there were no commercial kits available, although several initiatives had begun which have resulted in such kits. During the time of the CRP the various kits have been altered and this has led to a fragmented set of date and no full validation exercises. However, the basic construction of kits with regard to antigen and systems in general does mean that we can compare data in time and arrive at some valid statements.

During the time of the CRP there have been three Research Coordination meetings (RCM) where research contract holders presented findings and planned future work.

The data here reflect that given in the RCMs.

The overview of the CRP in the Tables XI-XIII helps us to see the developments more clearly and highlights the countries in which the work was done, importantly it emphasized that validation studies require a greater width of use of tests and analysis of more populations.

The tables also show the contacts with commercial concerns and international laboratories and puts this against a time frame for world developments.

TABLE XI. OUTLINE OF THE RCMS FOR CRP DEALING WITH USE OF NSP

| Year | Event | Countries attending |
|------|-------|---------------------|
| 1999 | Setting up CRP | |
| 2000 | RCM 1. Rio de Janeiro, Brazil. | **RC Holders** Argentina (SENASA); Argentina (INTA); Brazil (CPVDF); Colombia;Hong Kong; Lao; Malaysia; Myanmar Paraguay; Philippines; South Africa; Thailand (Pakchong); Uruguay |
| | | **Agreement Holders** Italy, Brescia;  UK, WRL; Denmark, (Lindhold); Brazil (PANAFTOSA); USA (Plum Island), Australia (Geelong) |
| | | **Commercial firms** United Biomedical Inc. (UBI), USA |
| 2000 | RCM 2. CSIRO, Geelong, Australia | **RC Holders** Argentina (INTA); Argentina (SENASA);  Brazil (CPVDF); China (Castle Peak, Hong Kong); Colombia (ICAS); Lao P.D.R; Malaysia; Myanmar;  Peru;  Philippines;  Thailand |
| | | **Agreement Holders** Australia (CSIRO-Geelong); Austria (Vet Univ. Vienna) TC Brazil (PANAFTOSA); Denmark (Lindolm) TC; Italy (Brescia); United Kingdom (WRL) |
| | | **Commercial firms** Intervet-Bommeli (Netherlands) United Biomedical Inc. (UBI), USA |
| | | **Invited experts** Netherlands |
| 2004 | RCM 3. Cebu, Philippines | **RC Holders** Argentina (INTA) (paper sent); Argentina (SENASA); Brazil (CPVDF); China (Castle Peak, Hong Kong); Colombia (ICAS); Lao P.D.R. (paper sent); Myanmar; Peru; Philippines; Thailand |
| | | **Agreement Holders** Australia (CSIRO-Geelong) TC, Austria (Vet Univ. Vienna) TC United Kingdom (WRL), Italy (Brescia) |
| | | **Commercial firms** Intervet-Bommeli (Netherlands), United Biomedical Inc. (UBI), USA CEDI Diagnostics (Lelystad) Netherlands, Svanova (Upsalla) Sweden |

### 7.2. Overview of reagents and samples used in time

The laboratories involved had different interests and needs. The 5 year time span meant supply of reagents as kits and some instability in protocols etc. The results therefore are based on changing reagents, although conclusions may be reached from earlier work since the basic antigens used were similar to the current preparations.

TABLE XII. REAGENTS USED BY CONTRACT HOLDERS TO ANALYSE SERA 1999-2000

| Supplier | Basis |
|---|---|
| (1) Brescia | Technical contract to supply MAb captured *E. coli* expressed 3ABC, Indirect ELISA Bovine test |
| (2) UK, WRL test | Indirect ELISA *E. coli* 3ABC (as for Brescia) directly on plate |
| (3) UBI | Gift supply. 3B Indirect ELISA for pigs |
| (4) Lindholm | C-ELISA using hyperimmune guinea pig detector competed for by test sera, 3ABC a baculo expressed 3ABC |

### 7.3. Conclusions from RCM 2000

- Available tests need more validation and need to build in better IQC control.
- There are differences in relative analytical sensitivity and diagnostic sensitivity/ specificity.
- The Indirect ELISAs (Pirbright, Brescia, S. America, UBI) suffer from problem of the anti-species conjugate and individual serum samples backgrounds.
- There is too much test to test variation in controls for the assays.
- There is little data on sheep/goat sera.
- Supply of large numbers of kits is a limiting factor. Quality control and distribution as well as robustness of reagents has not been addressed.
- Competitive assays should be developed rather than Indirect assays.
- Vaccinated animals pose major problem to tests since they may allow carrier state. Results suggest that Ab against replicating virus is present at "good" levels in cattle and sheep, in carrier state, and can be detected by NSP tests. Further work is needed on pigs.
- The sampling frames for animals in various epidemiological situations has to be considered from the point of view of testing (kit needs, cost, manpower) with assays. This will determine the needs for tests (capacity needed) and is linked to rules of trade.

TABLE XIII. REAGENTS USED BY CONTRACT HOLDERS TO ANALYSE SERA 2000-2002

| Supplier | Basis |
|---|---|
| (1) WRL-Bommeli Diagnostics | Indirect ELISA *E. coli*. 3ABC directly on plate. |
| (2) UBI | Gift and purchased supply. 3B Indirect ELISA for pigs |
| (3) Lindholm | C-ELISA using hyperimmune guinea pig detector competed for by test sera, 3ABC a baculo expressed 3ABC, modified conditions. |

## 7.4. General observations developments in Geelong meeting 2002

At the beginning of the CRP there existed a variety of reagents and systems. Some conclusions based on data from the CRP can be put forward.

- Reagents had been produced put together to form the basis of tests to differentiate vaccinated and infected animals.
- Various systems have been examined then changed.
- Some systems could be regarded as approaching kits, some not.
- Data suggested that differentiation of vaccinated and infected livestock was possible when herds were examined, not individual animals.
- The internal quality control (IQC) aspects have not been addressed too well.
- Commercial considerations are important and complicating with regard to supply and cost for developing countries.
- The purpose of the tests needs to be clearly defined and tests "fit for purpose" are needed with appropriate activities defined to arrive at the required test performance. This requires agreement on diagnostic sensitivity and specificity criteria.
- Reference sera are badly needed.

Note: As from March 2000 there are only three viable "kits" available from the point of view of costings, sustainability and distribution, namely:

- The kits from S. America, PANAFTOSA (bovine, caprine, ovine, porcine?).
- The kits from UBI (bovine, caprine, ovine as well as porcine). Also reported was a confirmatory kit.
- The kits from BOMMELI DIAGNOSTICS (bovine, caprine, ovine as well as porcine).

## 7.5. Discussion points

### 7.5.1. Kits

Discussions were made as to the content of kits. As a beginning a true kit was defined by the TO as below.

- Available in bulk. (Assessment of likely need worldwide is crucial to supply).
- Available and distributed on demand. (available immediately)
- Costed-high costs will prohibit use in developing countries. Price needed by buyers.
- Quality controlled in terms of day to day running. (IQC and EQA needed).
- Robust (stable reagents with defined performances).
- Validated in terms of diagnostic sensitivity.
- Validated in terms of diagnostic specificity.
- Fit for purpose (linked to estimates of sensitivity/specificity and this defined by producer, or at least criteria of validation, so far, described in full.
- Contain control sera and have agreed reference standards.
- Contain everything necessary to fully perform the assay. This includes plates, conjugates, tips, etc.

This was discussed by the commercial company representatives in the light of their current policies. Not all the criteria above could be agreed. In particular the completeness of the kits with regard to plastics was contentious (supply of tips, etc.). It was thought more

necessary by the contract holders to supply everything to developing countries where supply was a problem. There was little doubt that the reagents produced were of high quality and that the quality control at source was excellent. The question of costing kits was also problematic and there was a reluctance to cite costs. Individuals should feel confident to contact companies to state their needs and negotiate prices based on guidelines offered on company brochures. It should be noted that the production of kits is expensive and that there are many overheads. This is not always fully understood by users who may obtain kits free of charge through donor agencies while receiving aid through projects. It should be always understood that there is a real price for kits and that this should be strongly taken into account where testing beyond the projects support is to be maintained. UBI indicated that they will start manufacturing in China and Taiwan to help reduce costs at no loss in quality.

Discussions on the commercial kits use of a single positive of high value were made. It was thought that a better estimate of variability in tests could be gained by using a weaker control positive. The use of two control positives (strong and weaker) is recommended by the IAEA. Some discussion as to the applicability of reagents to robotics was made. It was agreed that sample problems are inherent in the exercise whereby whole blood or plasma is preferred.

The area of large scale testing needs to be considered in the light of possible large scale sampling needs if countries adopt strategies using vaccination without slaughter, where animals have to be certified free from replicative events at various times following an outbreak with or without vaccination.

### 7.5.2. CRP advantages

The advantages of having contract holders from many laboratories involved in many countries was discussed. This highlighted that the CRP covered different specific problems of FMD with respect to various species in countries the and with certain needs for assays dependent on the distinct epidemiology and control measures used.

This also was considered in the light of the different status of countries with regard to disease control measures, vaccination history and different threats. It was concluded that the diversity in the CRP was an advantage where we are trying to assess performance of any assay and increasing validation data. This data is part of the need to justify the use of such assays in disease control.

### 7.5.3. Epidemiological considerations

It is clear that there is a need to define different situations with regard to FMD since this has a direct influence on the desired diagnostic sensitivity and specificity of tests. It was concluded that an attempt must be made to define the different situations in each country in a simple way and relate this to the existing test performances.

### 7.5.4. Vaccines/ vaccine quality

The effect on the performance of the NSP assays in the face of vaccination was discussed. The main point is that commercial highly purified vaccines show few problems in all species, in that there is no development of antibodies against NS proteins, even following multiple vaccinations. This is confirmed through commercially sponsored work and some of the data presented. However, the use of locally produced vaccines may cause problems producing antibodies against contaminating NSP. Therefore the extent of the problem has to

be measured if locally produced vaccines are used. The situation therefore whereby both locally used and purified vaccines are used for disease control, is complicated. Base line data on the immune status of vaccinated animals should be obtained. The experience in S. America indicated that the measurement of contaminating NS in vaccines is vital to allow the NSP test to be maximized in use.

### 7.5.5. Species

Though it was accepted that the Indirect ELISAs developed so far worked to detect antibodies against NS proteins, the format did produce some problems with regard to all species. The development of competitive assays was favoured, both from the point of view of not being dependent on a detecting anti-species conjugate and also since the statistics for analyzing data from competitive methods are more straight-forward. The development of a competitive assay using baculo expressed 3ABC and antibodies prepared in chickens as described which is through a technical contract from the CRP to Geelong and Vienna University.

### 7.5.6. Use of NS tests

Generally the tests all work. There are differences in sensitivity due to the exact test systems used. Thus, the precise antigen used, the conjugate and the concentrations of the reagents all affect the assays. Results from the previous studies using the peptide antigen or 3ABC for determining antibodies from cattle, sheep and pigs, generally agree, but there are differences in certain sera. This reflects that certain samples, taken at different times following infection, contain antibodies at different concentrations. The 3ABC tests are generally more analytically sensitive as compared to the peptide assays for cattle sera. This is seen in the comparative analytical studies as well as in field samples examined. The opposite is generally true for pig samples. Conversely the higher sensitivities offer less specificity. No conclusions should be made in determining the best test at this stage. All work and all are designed to examine sera samples on a herd basis. The precise use of the assays depends on exactly what situation is being examined.

### 7.5.7. Charting methods for IQC

This was demonstrated and it was agreed that data should be processed in this way. An Excel data sheet was provided for results.

TABLE XIV. REAGENTS USED BY CONTRACT HOLDERS TO ANALYSE SERA 2002–2004

| Supplier | Basis |
|---|---|
| (1) Bommeli Diagnostics I_ELISA | Purchased |
| (2) UBI 3B I ELISA | Purchased |
| (3) CEDI C-ELISA | Purchased |
| Australia | Technical contact to develop C-ELISA using chicken anti-baculo 3ABC |
| Austria | Technical contact to develop C-ELISA using chicken anti-baculo 3ABC |

8.    DISCUSSION

There are number of commercial kits available to measure antibodies against NSP of FMDV. The kits rely on either Indirect ELISA or competitive ELISA for screening. The antigens used for detection are either *E. coli* or baculo virus expressed 3ABC or synthesised NSP individual peptides. The reliability of the kits is the same in terms of consistency. Indirect ELISAs have separate kits for ruminants and pigs whereas the competitive format has the advantage that all species can be detected.

At the heart of the use of kits to detect antibodies to NSP is the meaning and usefulness of data produced. There are many different epidemiological scenarios for FMD and many complications in diagnostic applications. The variability of the humoral response in terms of the actual antibodies produced against specific antigens; the differences in avidity of these specific populations; the quantities of antibodies produced and at what time after infection, all complicate any conclusions certainly where individual animals are concerned. The use of vaccines also confuses results since they may raise antibodies against NSP in certain cases. A further complication is the common state of persistently infected ruminants following contact with FMDV. The so called 'carrier animals' may be a risk to non-immune animals and whether they are or not, seems to be academic, since a zero risk mentality by senior veterinary authorities precludes such animals from any trade links. An obstacle to identifying such animals is that, although most produce long lasting antibodies against NSP, not all do. This means that no test, so far, can assure that a single animal with no clinical signs can be excluded as a carrier.

On a herd basis some of the criteria listed in the guidelines for test validation by the OIE can be met for all the NSP tests. The basis of validation, which is a continuous process, is to establish test performance in terms of diagnostic sensitivity and diagnostic specificity. The standard way to do this is though studying populations of animals where the conditions required justifying validation are inherent. Here lays the major problem and paradox with FMD, since it is nigh on impossible to examine field sera where there is any surety of data in terms of known infectious state or history to form a valid population. Experimentally derived sera offer data which approximate to needs for validation studies, but numbers are low and these lack the many parameters which complicate and influence serum taken from animals in the field. All the tests detect antibodies against NSP; the relative analytical sensitivities are not too different. Tests should be used in countries as reagent sets and the cut offs set by producers only used as guidelines for use. The controls given in tests to define performance should be used mainly for internal quality control and validation per se for a country use has to be defined better by the country using the test. This requires a far higher standard of users to understand the principles of testing and data analysis and far greater attention to animal identification coupled with far better designed surveys. The use of NSP tests as a continuous surveillance probe on higher numbers of animals and the selective application to quarantine and movement of animals might offer a very useful approach to spotting disease earlier. In this light the use of mobile tests such as strips which can be used directly in the field or a border controls is recommended. The normally high prevalence rate of FMD offers the chance to make judgments without the need for massive sample numbers. Conversely, in more purposive sampling there is the possibility to test and retest whole herds or congregations of animals in quarantine or markets in a rapid way to remove any sampling statistic.

Use of NSP antibody testing is part of the OIE guidelines for declarations of freedom from FMD, the disease as well as virus circulation. The system quoted is that of the

PANAFTOSA involving an Indirect ELISA screening test and a confirmatory immunoblotting test. This combination allows identification of some of the doubtful positives/negatives, with the caveat that not all carriers do produce antibodies. This offers a test system that is optimal particularly for the purpose of showing freedom from disease and restoration of status following outbreaks. The use of confirmatory tests is to be encouraged and a simpler alternative to the blotting technique might be sought, e.g. as with UBI kits. It is vital that reference standards to measure relative analytical sensitivity of kits on a variety of sera are made and agreed protocols drawn up for their use. Some developments are being made in this direction (e.g. the IAEA) and attention has been paid to the large volumes possibly needed; the safely of products (gamma irradiation); single point (laboratory based) reference testing and storage and distribution of standards (lyophilisation, robustness etc.). There should be more attention paid to Internal Quality Control (IQC) and the setting up of Eternal Quality Assurance (EQA) exercises. The evolution of standards should improve these possibilities. The testing for antibodies against NSP of FMD will continue to be a major diagnostic tool for assuring that populations are free of both disease and virus. The latter is particularly important in vaccinated pig populations. Increase in the use of mobile testing will greatly help local veterinary tools to map FMD in transit situations and new systems for this would be welcome for validation. It will be interesting to see what applications for test registration are submitted through the new OIE procedure involving the established as well as new developments in kits.

Reference to the impact of the research made in the CRP is made in the in the final paper: Conclusions by the Technical Officer.

# REPORTS

# VALIDATION OF COMMERCIALLY AVAILABLE NSP ELISA KITS FOR FOOT AND MOUTH DISEASE SURVEILLANCE TO SUPPORT THE CONTROL AND ERADICATION PROGRAMME IN THE PHILIPPINES

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**Abstract**

This paper summarizes five years of validation exercises using different ELISA kits to detect antibodies against non-structural proteins (NSP) of foot and mouth disease virus (FMDV) in an FAO/IAEA coordinated research project (CRP): "The use of non-structural protein of foot and mouth disease virus (FMDV) to differentiate between vaccinated and infected animals." One thousand five hundred seventy serum samples from ruminants and pigs were examined for the presence of antibodies against the NSP of FMDV using firstly four NSP ELISA kits from United Biomedical Incorporated (UBI); Lindholm Denmark; Brescia and Plum Island, USA. These were made available in the first two years of the CRP. Two hundred and forty three serum samples from ruminants and pigs were tested later in the CRP using kits from Bommeli Diagnostics, Switzerland; CEDI Diagnostics (Lelystad, Netherlands) and Svanovir (Upsalla, Sweden. The origin, species, distribution and number of samples tested were dependent on the type and volume of NSP ELISA kits made available during the validation exercises. Based on the results from the cumulative laboratory data, the different NSP ELISA kits can detect antibody against FMDV NSP beginning seven days post infection (CEDI test) and up to twenty months post infection (Plum Island) in known FMDV post infected animals, both in pigs and in swamp buffalos. However, significant variations were observed between the different assays in terms of diagnostic sensitivity and specificity. These can be attributed to the previous findings that the indirect ELISA can suffer from conjugate problems. At present there are stable commercial NSP ELISA kits available and improvements were observed during the CRP in all kits in terms of diagnostic sensitivity and specificity

## 1. INTRODUCTION

Massive outbreaks of foot and mouth disease (FMD) caused by FMD virus (FMDV) virus type O (porcinophilic type) started in 1994 affecting mainly the island of Luzon and some parts of the Visayas region. During the peak of the outbreak in June–July 1995, local pig producers lost P 2 billion (US $45 million) due to reduction of sales and buyer's fears about eating infected pork [1]. During that period, part of the Government aims in line with the 5–year (1993–1998) Medium Term Livestock Development Plan (MTLDP) was to be self-sufficient in livestock products and to become an exporter of pork and poultry products to some neighbouring countries [1].

The 1994 FMD outbreak resulted in the temporary setback of the MTLDP thus, the National Government through the Bureau of Animal Industry, Department of Agriculture initiated a National FMD control and eradication programme with the assistance from AUSAID and FAO. At present, with the FMD control and eradication programme in place, sporadic infection has now been confined to some areas of central Luzon involving only four regions (regions CAR, 3, 4 and NCR) from the nine regions initially infected. The National FMD programme has four components.

The first component is disease monitoring where the FMD laboratory plays a crucial role. Diagnosis of FMD is made using an Indirect Sandwich ELISA for antigen detection and antibodies measured using the Liquid Phase Blocking ELISA (LPBE) for against FMD serotypes O, A and C. These have been the standard tests used by the FMD laboratory since 1994. The virus neutralization (VN) test is not a routine test and is only used as confirmatory test on samples which give enigmatic results on LPBE. The NSP ELISAs, was a new diagnostic assay used to detect antibody to NSP of FMDV and thus had have not been adopted and used as a routine diagnostic test by my laboratory. The FMD laboratory, through the FAO/IAEA interregional coordination research project participated in a 5 year exercise examining different NSP ELISA kits. The results add data to the present day situation with regard to assessment of the performance of kits.

## 2.    MATERIALS AND METHODS

Kits were provided through gifts from companies or by purchase using CRP funds. The working protocols were followed closely. Sera were collected from various epidemiological niches and examined using the ELISAs.

### 2.1    Kits

Kits used in early work were:

Phase 1

- Monoclonal capture of *E. coli* expressed 3ABC then Indirect ELISA from World Reference Laboratory (WRL) Pirbright, UK.
- UBI kit, Indirect ELISA using 3B synthetic polypeptide.
- Competitive format ELISA from Lindholm, Denmark using 3AB expressed proteins.
- Indirect ELISA using 3ABC from Plum Island.
  Samples were also tested using a research phase ELISAs from WRL, which examined the reactions of sera to a range of 5 expressed antigens as indicated in Table I.

Phase 2

- CEDI competitive ELISA using baculovirus expressed 3ABC and labelled monoclonal antibody against this.
- Bommeli Indirect ELISA using *E. coli* expressed 3ABC.
- Svanovir kit Indirect ELISA using *E. coli* expressed 3ABC.

### 2.2.    Samples

The samples used in Phase 1 were examined by the first four NSP ELISA kits supplied were as follows:

49.7% were post vaccination
13.9% were post infection and vaccinated/infected
36.4% were from naïve from ruminants (46%) and pigs (54%)

Of the 723 ruminant field samples, 6.6% was from post infection and 20% post infected from the 847 pig samples.

The samples tested for Phase 2 using were as follows; 64.1% vaccinated 21.1% naïve and 14.8% post infected from both ruminants and pigs.

## 3. RESULTS

FMD infection in the Philippines is mostly confined to the pig population with some sporadic cases on large ruminants especially on swamp buffalo population but only account for one percent of the total number of field samples received by the FMD laboratory since the 1994 first reported outbreak.

Serum samples from the first case in 1997 in swamp buffalos were sent to WRL for FMD for profiling against NSP. Initial results helped explain the succeeding NSP ELISA test results of the National FMD laboratory which showed that some Indirect ELISAs had difficulty picking up sera from this species [2].

TABLE I. PROFILE ELISA ANTIBODIES TO NSPS IN 8 SWAMP BUFFALOS

| Sample ID | Species | LPBE titre | Lb | 2C | 3A | 3D | 3AB |
|---|---|---|---|---|---|---|---|
| | | | | **Non-structural FMD proteins** | | | |
| 8 | Buf | 1448 | | | | | |
| 9 | Buf | 2896 | | 0.21 | 0.06 | 0.38 | 0.08 |
| 10 | Buf | 4096 | | 0.11 | 0.06 | 0.01 | 0.26 |
| 10.97.8 | Buf | 2048 | | 0.83 | 0.29 | 0.00 | 0.25 |
| 10.97.2 | Buf | 724 | | | | | |
| 10.97.3 | Buf | 724 | | | | | |
| 10.97.4 | Buf | 724 | | 0.22 | 0.01 | 0.00 | 0.28 |
| 10.97.1 | Buf | 1024 | 0.02 | 0.14 | 0.09 | 0.04 | 0.10 |

All sera shown in Table I were screened for antibody against 3ABC using monoclonal antibody trapping ELISA from Brescia and 5 showed some reactivity. These 5 sera were examined by profile ELISA for antibody to the NSP proteins L, 2C, 3A, 3D and 3ABC. Only animals 10.97.8 and 10.97.4 were positive for both antibodies to 2C and 3ABC which is consistent with exposure to infection. On a group basis therefore, it is likely that these animals were exposed to infection although no single animal shows a profile which is typical of FMDV infection in a naïve animal, (personal communication).

Table II shows the cumulative data from swamp buffalo samples tested using UBI, 3AB (Denmark), 3ABC (Pirbright) and 3ABC (Plum Island) kits. Based on our laboratory cumulative data, from the four different NSP ELISAs, 3AB had the highest sensitivity but had very low specificity. The 3ABCPB and UBI have the highest specificity but with very low sensitivity on post infected Philippine swamp buffalos.

TABLE II. BUFFALO SERA COMPARED USING DIFFERENT NSP ELISA KITS

|  | UBI + | UBI - |  | UBI | D sens | 3/32 | 9.3 |
|---|---|---|---|---|---|---|---|
| LP + | 3 | 29 |  |  | D spec | 5/5 | 100 |
| LP - | 0 | 5 |  |  |  |  |  |
|  | 3AB + | 3Ab - |  | 3AB | D sens | 28/32 | 88 |
| LP + | 28 | 4 |  |  | D spec | 1/5 | 20 |
| LP - | 4 | 1 |  |  |  |  |  |
|  | 3ABC PB + | 3ABC PB - |  | 3ABC PB | D sens | 2/32 | 6 |
| LP + | 2 | 30 |  |  | D spec | 5/5 | 100 |
| LP- | 0 | 5 |  |  |  |  |  |
|  | 3ABC PI + | 3ABC PI - |  | 3ABC PI | D sens | 20/43 | 46.5 |
| LP + | 20 | 23 |  |  | D spec | 6/12 | 50 |
| LP - | 6 | 6 |  |  |  |  |  |

TABLE III. FMD CASES IN TEN SWAMP BUFFALOS IN PAMPANGA PROVINCE

| FMD Infection in Buffalo (Pampanga, 2003) | | | | | | |
|---|---|---|---|---|---|---|
| Collection Date after infection | No. of Samples | LPBE + | VNT + | NSP ELISA Positive | | |
|  |  |  |  | CEDI | Bommeli | Svanovir |
| 21d | 10 | 10 | 10 | 4 (2) | 3 (2) | 0 |
| 60d | 38 | 22 | 17 | 2 | 2 | 0 |

Table III compares results on ten swamp buffalos more recently infected with FMDV. In the absence of vesicular epithelium samples for antigen detection and for virus isolation due to late field reports, serum samples from ten FMD field-infected animals were collected 21 d post infection to determine if animals were truly infected with FMDV. By gross examination of animals, old, healed lesions were observed inside the mouth and some animals with vesicular lesions on the feet. All ten animals were positive for antibody to FMDV $O_1$ Manisa by both LPBE and VNT. Four animals were positive to non-structural proteins by CEDI and three animals were positive by Bommeli.

The two assays agreed only in two cases of the positive animals. Unfortunately no single animals showed reaction using the Svanovir kit. The 60 d post infection samples were from animals that were within the 5 km radius of the infected premises. The idea was to see the extent of infection in carabaos. From thirty eight animals sampled, only two animals were positive to NSP of FMDV. But whether the infection spread or not cannot be elucidated given if one the percentage diagnostic sensitivity and specificity of the NSP ELISAs on swamp buffalos.

Table IV shows the results from known post infected pig serum samples. The 3AB and UBI had an average DSn of 96% but had statistically relevant different diagnostic specificities; however; both assays could detect antibodies to NSP from 7 d to a maximum of 6 m post infection based on our laboratory's cumulative data from known FMD post infected pigs. The 3ABCs (Pirbright and Plum Island) both had lower diagnostic sensitivities and specificities compared to 3AB and UBI but could detect antibodies for up to 20 m post infection.

The 2003–2004 validation exercises using three different tests are summarized in Table V. Samples comprised approximately 64% vaccinated; 21% naïve and 15% infected animals from both pigs and ruminants. Variation in the diagnostic sensitivity of assays was significant. Results from 21 to 30 d known post-infected pigs showed 96.2% diagnostic sensitivity for CEDI and only 57.6% for Bommeli. Three animals from the vaccinated population showed some reactivity in some of the ELISAs but all animals from free zone areas that were negative for antibody against FMDV by LPBE were also negative in all ELISAs.

TABLE IV. PIG CUMULATIVE DATA ON FOUR DIFFERENT NSP ELISA KITS

| Tests | | | | DSn and DSp% | | | |
|---|---|---|---|---|---|---|---|
| | | UBI + | UBI - | UBI | D sens | 92/92+3 | 96.8 |
| LP + | | 92 (TP) | 3 (FN) | | D spec | 25/25+4 | 86.2 |
| LP - | | 4 (FP) | 25 (TN) | | | | |
| | | 3AB + | 3Ab - | 3AB | D sens | 90/93 | 96.7 |
| LP + | | 90 | 3 | | D spec | 24/31 | 77.4 |
| LP - | | 7 | 24 | | | | |
| | | 3ABC PB + | 3ABC PB - | 3ABC WRL | D sens | 69/93 | 74 |
| LP + | | 69 | 24 | | D spec | 30/31 | 96.7 |
| LP- | | 1 | 30 | | | | |
| | | 3ABC PI + | 3ABC PI - | 3ABC Plum Island | D sens | 64/91 | 70.3 |
| LP + | | 64 | 27 | | D spec | 20/28 | 71.4 |
| LP - | | 8 | 20 | | | | |

TABLE V. VALIDATION EXERCISES ON THREE ELISAS IN 2003 AND 2004

| Sample Origin | Species | | Positive result | | | | |
|---|---|---|---|---|---|---|---|
| | | | Bommeli | CEDI | Svanovir | LPBE | VNT |
| Vaccination | Sui | 120 | 1 | 1 | | 103 | Not tested |
| | Bov | 30 | 0 | 0 | 1 | 30 | |
| | Cap | 6 | 1 | 0 | 0 | 6 | |
| Infected | Sui | 26 | 15 | 25 | - | 26 | 26 |
| | Buf | 10 | 3 | 4 | 0 | 10 | 10 |
| Naïve | Sui | 51 | 0 | 0 | - | 0 | Not tested |

## 3.1. Internal quality control

Monitoring the performance of the different NSP ELISA kits to measure antibodies against NSP of FMDV was assessed using charting methods with reference to all test controls incorporated in every NSP ELISA kit. In summary, test controls for every NSP ELISA kit was within the acceptable limits established by each NSP ELISA kit producer.

## 4. CONCLUSIONS

Countries wishing to be declared free from FMD within the terms of the OIE Code must carry out surveillance to demonstrate freedom from infection [3]. In the Philippines a total stamping out policy is unlikely during the last stages of any eradication programme due to economic, social and moral considerations. Thus, a test is required to discriminate antibody due to infection from that of vaccination for its surveillance activities in parallel with a routine test such as the LPBE, which measures total antibodies against SP. The use of such test system particularly the 3ABC based NSP ELISA on a herd basis would detect viral circulation either among vaccinated or previously infected population [3]. This is greatly beneficial to the national FMD control and eradication programme. Previously published research data indicates that the detection of antibody to the 3ABC polypeptide is the single most reliable indicator of infection [4]; [5]; [6]. Based on the five year validation exercises using different NSP ELISAs with Philippines field samples from different origins, the use of a particular NSP ELISA kit will depend on the animal species affected and the epidemiologic situations (FMD status zones) in which it might be applicable. In summary therefore, recommendations to policy makers in the livestock industry on the use of NSP ELISA will greatly depend on the performance of a particular NSP ELISA kit against field samples during the entire 5 year FAO/IAEA collaborative research validation exercises.

Future directions
(1) Collaborative studies with international research institutes to determine the possible role of swamp buffalo in the epidemiology of FMD in the Philippines.
(2) Further studies to determine the level of sampling that would be statistically acceptable in clinically and subclinically infected swamp buffalos.

(3)    Recommendation to the policy makers for the use of NSP ELISA in parallel with the LPBE as routine test system in the eradication phase of the FMD programme in the country.

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# USE OF NON-STRUCTURAL PROTEINS OF FMDV TO DIFFERENTIATE BETWEEN INFECTED AND VACCINATED LIVESTOCK IN EXPERIMENTAL AND FIELD STUDIES

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**Abstract**

Work on various aspects related to measuring antibodies against NSP of FMDV was made in Argentina. These are reviewed in 1.1 to 1.4 of the Introduction. Work under the CRP was made in two phases according to the various kit formulations. The results and examination of data from are shown under Phase 1 and Phase 2 research.

## 1. INTRODUCTION

### 1.1. Detection of antibodies to RNA polymerase by ELISA using 3D bioengineered protein

In order to improve the sensitivity of the agar gel immunodiffusion test (AGIDT) and to use a biologically safe antigen, a liquid-phase blocking sandwich ELISA was developed to detect antibodies to the 3D protein in sera from foot and mouth disease virus (FMDV) infected animals. The assay uses a non-structural 3D recombinant protein and two polyclonal antisera, one for capture (bovine) and the other for detection (guinea pig). The specificity of the assay was demonstrated by negative results with 101 sera of cattle from the FMD-free zone in Argentina and with bovine and porcine sera raised against various RNA and DNA viruses. Analysis of sera from a group of 51 animals vaccinated and/or re-vaccinated with commercial inactivated vaccines from FMDV strains O1, A79, A87 and C85 showed that they produced antibodies against the virus infection-associated antigen (VIAA). The responses to protein 3D and the VIAA were detected using AGID and a liquid-phase blocking sandwich ELISA for anti-3D antibodies (ELISA-3D). No anti-VIAA or anti-3D antibodies were detected after the initial vaccination however, following re-vaccination; animals giving positive results were detected by both methods. Antibodies could not be detected between 60-120 d post-revaccination (dprv) by the AGID method and between 90-180 dprv using the ELISA-3D. Samples of oesophageal-pharyngeal (OP) fluid obtained from animals that remained positive for anti-VIAA antibodies at 90-120 dprv gave negative results for viral isolation, indicating that the transitional antibody response induced by the vaccine was due to the presence of non-structural antigens in the vaccine and not to viral infection. The method was also applied in epidemiological surveys in an FMD endemic area in cattle aged 6 to 12 m (n = 542) collected before an autumn vaccination campaign in 1993/1995. Results suggested that the ELISA-3D could be applied as a complementary method in epidemiological studies assuming that good recording systems for vaccination are available and for certification for international trade.

### 1.2. Studies in the persistence of foot and mouth disease virus in bovines, ovines and Llamas (lama glama)

In order to obtain information on the incidence of inapparent and persistent infections with FMDV in bovines, ovines and South American camelids (Llamas, Lama Glama),

experimental infections mimicking field outbreaks of FMD, were made. Under the experimental conditions with naïve and vaccinated animals showing different levels of anti-FMDV antibodies, viral replication in the upper respiratory tract occurred in 100% of exposed bovines between 3 and 5 d after exposure (dae), no matter what the initial immune status of animals. Approximately 80% of the animals became carriers. The proportion of carriers was significantly higher among the animals with previously low levels of antibodies (100%) than in those with high titres of antibodies (42%). The frequency of the carrier state in the sheep was low. Vaccination prevented infection and thus the establishment of the carrier state in this species. The information presented shows that an adequate level of vaccinal protection not only avoids the clinical manifestations of disease, but also diminishes the probability of establishing the carrier state. Llama have very low susceptibility to infection with FMDV and have apparently little or no relevance with regard to transmission inter-or intra-species. Sequencing analysis of virus isolated from carrier animals showed only point mutations, with no distinct pattern of variation between animals. This results indicate that the transmission of FMDV from carriers to susceptible animals occur is extremely rare occurrence and difficult to verify in experimental conditions.

## 1.3.   Surveillance system for FMDV-studies of anti-FMDV antibodies in bovines in Argentinean national plan for eradication

A sero epidemiological survey was made in three counties between 1993-1998 to provide additional information on the epidemiology of FMD; to assess the level of immunity in cattle populations and assess any circulation of FMDV. It included more than 800 cattle in each county/year. As part of the final stage of this survey, a comparison was made of the results obtained by the ELISA and agar gel immunodiffusion techniques. Levels of antibody against four types of virus included in the vaccine increased progressively during the period of the survey. During the three-year study, there was a clear tendency for viral activity to diminish.

## 1.4.   Sero epidemiological study in susceptible non-vaccinated species

In order to obtain information about prevalence of FMD in Argentina a sero epidemiological study made on susceptible non-vaccinated species, prioritising border areas or where information was poor. The status of the population was determined using serological methods based on the detection of antibody to structural and non-structural proteins of FMD (3D, 3ABC and 2C). Serum samples from goats, sheep, South American camelids, cattle, deer and pig, from different locations (Buenos Aires, La Pampa, La Rioja, Chaco, Formosa, Corrientes, Misiones, Salta, Jujuy and Rio Negro) were obtained, at two times. The first from November of 1997 to February of 1998 (n = 2488) and the second from October to December of 1998 (N=939).The results indicated that all the animals were negative and that there was no viral activity or animal carriers in the studied populations. This information confirms the minimal internal risk for the next stage in Argentina.

## 1.5.   Relevant publications to Introduction

FONDEVILA, N., O'DONNELL, V., DUFFY, S., LEÓN, E., SMITSAART, E., SCHUDEL, A.A., Sero epidemiological indicators for evaluating foot and mouth disease control campaigns. Rev. Sci. Tech. Off. Int. Epiz., 16 (3): 784–792. (1997).

O'DONNNELL V., SMITSAART E., CETRA B., DUFFY S., FINELLI J., BOYLE D., DRAGHI G., FONDEVILA N., SCHUDELL, A.A., Detection of virus infection associated

antigen and 3D antibodies in cattle vaccinated against foot and mouth disease. Rev. Sci. Tech.Off. Int. Epiz. , 16 (3): 833–840 (1997).

O´DONNELL V., BOYLE D., SPROAT K., FONDEVILA N., FORMAN A., SCHUDEL A.A., SMITSAART E., Detection of antibodies against foot-and- mouth disease virus using a liquid-phase blocking sandwich ELISA (LPBE) with a bioengineered 3D protein. J. Vet. Diag. Invest., 8:143–150 (1996).

FONDEVILA N., SANCHEZ, A., SMITSAART, E., SAMUEL, A., RODRIGUEZ, M., PRATO MURPHY, M., SCHUDEL, A.A., Studies in the persistence of Foot-and-Mouth-Disease Virus in bovines, ovines and Llamas (Lama glama). Session of the Research Group of the European Commission for the Control of Foot and Mouth Disease. Ma'ale Hachmisha, Israel (1996).

SMITSAART, E., O'DONNELL, V., BOYLE, D., FONDEVILA, N., CETRA, V., DUFFY, S., SCHUDEL, A.A., Detection of antibodies against RNA polymerase of foot and mouth disease virus by ELISA using a bioengineered 3D protein. Session of the Research Group of the European Commission for the Control of Foot and Mouth Disease. Ma'ale Hachmisha, Israel (1996).

## 2.    PHASE 1 RESEARCH

### 2.1.    Objective

To compare ELISAs to detect antibodies against FMDV NSP using sera from uninfected, vaccinated, vaccinated and infected and field samples of different status populations.

### 2.2.    Materials and methods

*2.2.1. Immunoassays*

Serum samples were examined using two ELISA kits:

(1) The UBI FMDV (United Biomedical Inc., New York, USA) is based on synthetic 3B peptide.
(2) FMD-3ABC Reagents developed by Instituto zooprofilactico sperimentale della Lombadia e dell'Emilia (I.Z.S.L.E).

In all the tests the means and standard deviation of the OD were calculated and plotted using charting methods developed by the IAEA.

*2.2.2. Cattle samples*

In order to examine the antibody response after vaccination, 33 cattle vaccinated and revaccinated with different formulations used in the Argentine FMD control programme were studied. All the vaccines had passed the official control.

Serum samples were obtained after vaccination:
0 d
30 d post vaccination (dpv),
120 dpv and 7 d post re-vaccination (dpr)
150 dpv and 37 dpr
180 dpv and 67 dpr

## 2.2.3. Vaccinated cattle

Sixty sera from vaccinated cattle were sampled at 30 dpv.

## 2.2.4. Challenged cattle and sheep

The sera from 4 cattle and 3 sheep challenged with FMDV (type O1 Campos) and sampled at different days post infection were titrated.

Eight cattle, sero negative for FMDV challenged with FMDV (type O1 Campos) by intraepithelial inoculation in the tongue with $10^4$ TCID$_{50}$ were sampled weekly during the first month post infection.

## 2.2.5. Antibody responses in cattle

The antibody responses were studied in 16 cattle after experimental infections by contact with 2 calves infected with FMDV type O. The experimental groups were 3 cattle free of FMDV antibodies; 7 with high and 10 with low titers of antibodies respectively. Animals were infected by contact with two calves sero negative for FMDV which had been intradermoligually inoculated with $10^4$SMLD$_{50}$ of FMDV O1 Campos. In all cases, blood and oesophageal-pharyngeal-fluid (OPF) were obtained at different times after exposure. Any virus in samples was detected using virus isolation (three blind passages in primary cultures of foetal bovine thyroid or foetal ovine kidney in roller tubes) and confirmed by typing ELISA. Samples negatives by tissue culture isolation were retested with RT-PCR.

## 2.2.6. Tongue-infected cattle

Cattle were infected with FMDV strains O1 Campos, A79, A87 or C3 Rezende by intraepithelial inoculation in the tongue, sampled between 10 and 180 d post infection.

## 2.2.7. Persistently infected animals

*Cattle*

Detection of antibodies in sera from infected cattle was made at later stages of persistent infection. Ten vaccinated cattle were challenged by intradermolingual route with $10^4$ bovine infectious doses 50%. Samples of sera were obtained between 7 to 562 dpi. *Sheep*

Sixteen sheep with different vaccination records were contact exposed to two piglets free of FMDV antibodies that had been inoculated with $10^5$ TCID$_{50}$ of FMDV O1 Campos. The vaccination records of the ovines were: 7 unvaccinated, 3 with one dose the week before exposure, 6 with more than 3 doses.

## 2.2.8. Field samples

*Cattle*

Cattle sera were obtained in 1995 from the Pampeana Region. The samples belonged to two groups comprising those younger than 2 and older than 2 y. In the county there had been no recorded outbreaks 3 y previous to the sampling. Vaccination had been made twice a year in cattle younger than 2 y old and then once a year. Sample collection was made simultaneously with the autumn vaccination (the group of <2 y having received one dose 4-6 m before and the animals >2 y, 1 year before).

*Small ruminants*

Twenty samples from sheep and goats were examined.

*Pig samples*

Ten samples from free range pigs were obtained. Forty-four pigs which had been vaccinated and 17 which had been revaccinated were studied (sampled 30 d before the vaccination).

Four FMDV infected pigs (type O1) were sampled at different times post infection in the acute stage of the infection.

3    RESULTS PHASE 1

**3.1.    Quality control**

*UBI kit*

The graphs in Figs 1–4 show the Daily Detailed Data Charts (DDD) and the Summary Data Charts (SDC) for OD of the kits of ruminants and pigs. NRC = non reactive control; RC= reactive controls; sd = standard deviation. The Y axis shows OD units. The x axis contains data from plates.
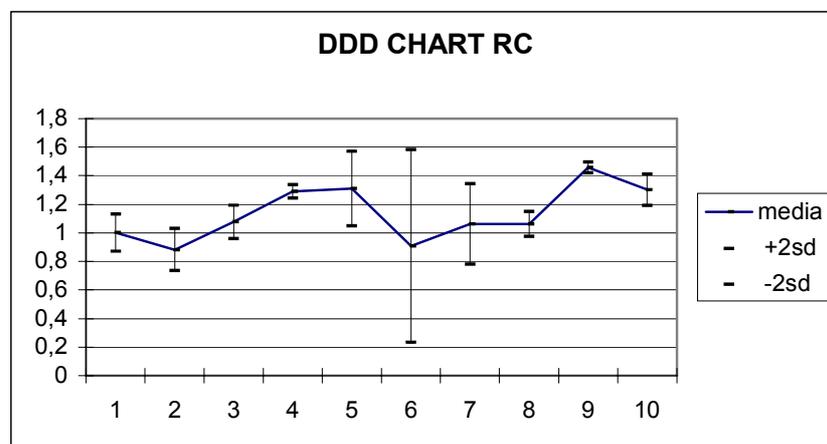


*FIG. 1. Daily Detailed Data Charts (DDD) of the strong control.*

Note: The mean OD and the variation are plotted for each plate control for the serum given as strong positive by the supplier. The graph shows 10 plates.
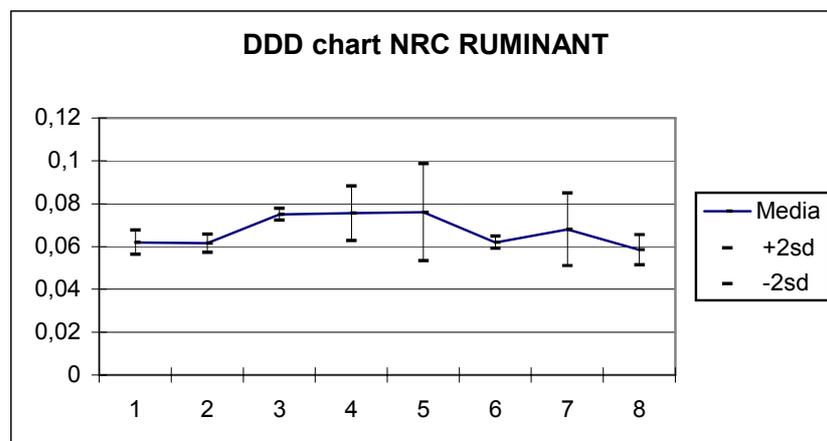


*FIG. 2. Daily Detailed Data Charts (DDD) of the low reactive control.*

Note: The mean OD and the variation are plotted for each plate control for the serum given as weak positive by the supplier. The graph shows 10 plates.
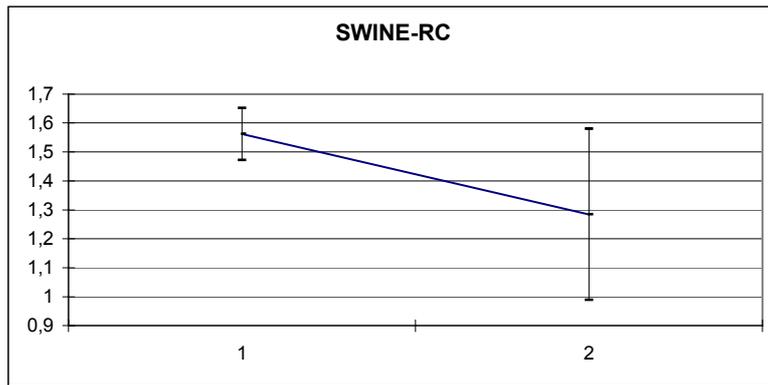
*FIG. 3. Daily Detailed Data Charts (DDD) of the reactive control with swine kit.*

Note: The mean OD and the variation are plotted for each plate control for the serum given as strong positive by the supplier. The graph shows only 2 plates.
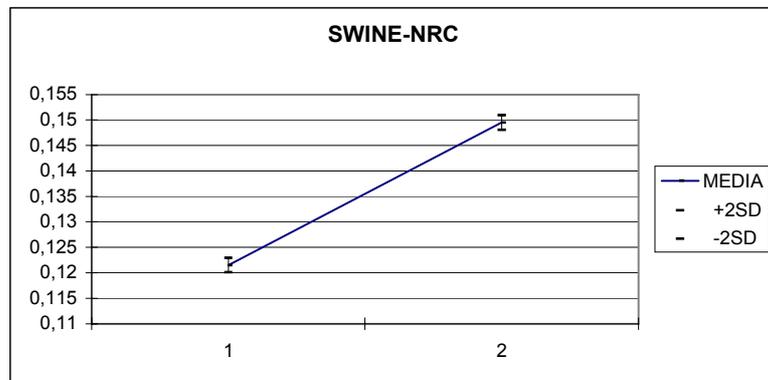


*FIG. 4. Daily Detailed Data Charts (DDD) of the non reactive control with the swine.*

Note: The mean OD and the variation are plotted for each plate control for the serum given as non reactive by the supplier. The graph shows 2 plates.

### 3.2. Brescia 3ABC kit

The graphs in Figs 5–7 show the Daily Detailed Data Charts (DDD) for OD of the kit. WCS= weak control serum; PCS= positive control serum; NCS= negative control serum; SD = standard deviation. The Y axis shows OD units. The x axis contains data from plates.
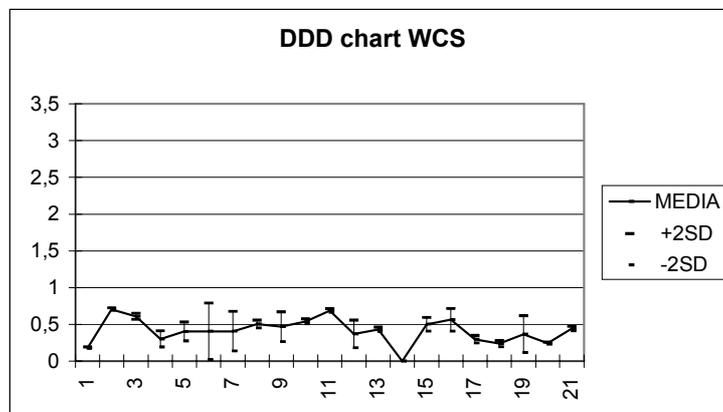


*FIG. 5. Daily Detailed Data Charts (DDD) of the weak control serum.*

Note: Data from 21 plates is shown for the OD mean and variation of the weak control serum.
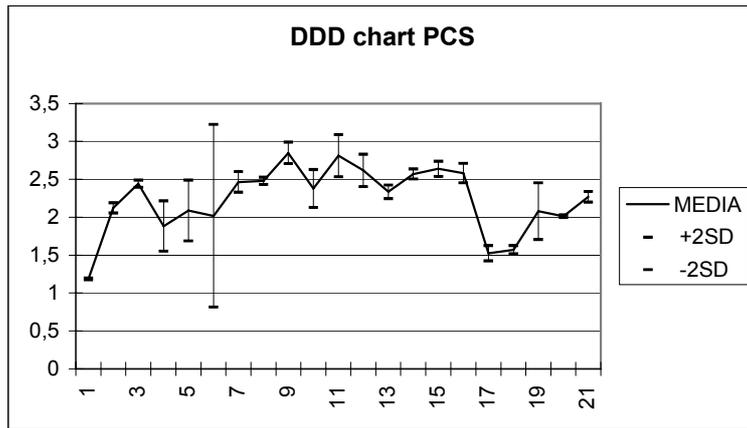
*FIG. 6. Daily Detailed Data Charts (DDD) of the positive control serum.*

Note: Data from 21 plates is shown for the OD mean and variation of the strong control serum.
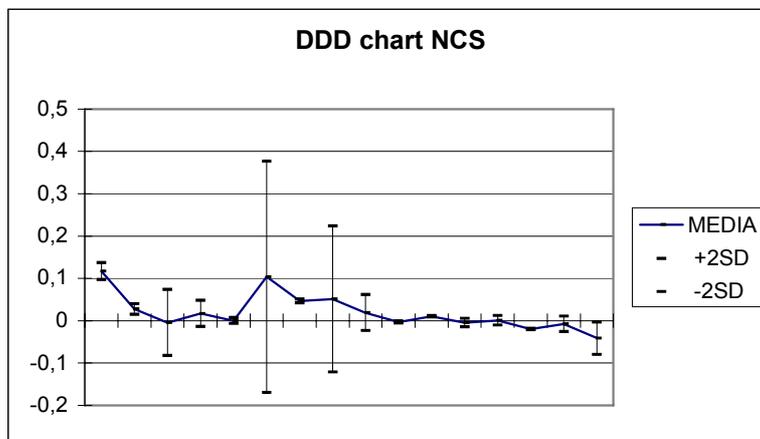


*FIG. 7. Daily Detailed Data Charts (DDD) of the negative control serum.*

Note: Data from 21 plates is shown for the OD mean and variation of the negative control serum.

### 3.3. Bovine samples

*3.3.1. Antibody response in vaccinated and revaccinated cattle*

TABLE I. DATA FROM ANALYSIS OF CATTLE BY VARIOUS ELISAS

|                        | 3ABC | UBI | VIAA | ELISA3D |
|------------------------|------|-----|------|---------|
| 0d                     | 9    | 0   | 1    | 8       |
| 30d pv                 | 7    | 0   | 2    | 4       |
| 120 dpv and 7 d (dpr)  | 6    | 0   | 5    | 13      |
| 150 dpv and 37dpr      | 3    | 0   | 3    | 18      |

*3.3.2. Post vaccinated cattle*

From 60 post vaccinated cattle sera sampled at 30 dpv; 1/60 gave positive result with UBI kit and 0/60 with 3ABC.

*3.3.3. Titration of antibodies in sera from infected cattle*

Four cattle sera and three sheep sera were titrated in a two fold dilution range and tested under standard conditions with the UBI and Brescia ELISA. The dilution end point was calculated for the sera (the last dilution which showed as positive in the ELISAs).

TABLE II. COMPARISON OF ANALYTICAL SENSITIVITIES OF TWO TESTS

| Assay | | Brescia | UBI | Ratio Brescia/UBI |
|---|---|---|---|---|
| Bovine | dpi | | | |
| | 21 | 1/6400 | 1/20 | 320/1 |
| | 21 | 1/20 | 1/160 | 1/8 |
| | 21 | 1/20 | 1/40 | 1/2 |
| | 21 | 1/400 | 1/20 | 40/1 |
| Sheep | | | | |
| | 90 | 1/1600 | 1/320 | 5/1 |
| | 32 | 1/1600 | 1/320 | 5/1 |
| | 32 | 1/3200 | 1/640 | 5/1 |

dpi= days post infection

The analytical sensitivity of the Brescia ELISA was higher in 5 sera than the UBI. The UBI was higher in 2 cases. The extent of the differences was very high in two sera (40 to 320 times in favour of Brescia.

*3.3.4. Analysis of animals after vaccination and infection*

TABLE III. ISOLATION OF VIRUS FROM PROBANGS OR DETECTION BY PCR FROM POST INFECTED CATTLE

| | | Days after infection | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Antibody titre | Bov No | 5 | 7 | 20 | 34 | 48 | 62 | 76 | 90 | 104 | 118 | 132 | 180 | 233 |
| VNT titre | | | | | | | | | | | | | | |
| 2.5–2.8 | 29 | - | - | V | - | - | - | - | - | - | - | - | - | - |
| 2.5–2.8 | 14 | - | V | - | - | - | - | - | - | - | - | - | - | - |
| 2.5–2.8 | 12 | V | V | V | - | V | - | V | V | V | V | V | V | - |
| 2.5–2.8 | 1 | V | V | - | - | - | - | - | - | - | - | - | - | - |
| 2.5–2.8 | 2 | V | - | - | V | V | V | V | V | V | V | V | - | V |
| 2.5–2.8 | 8 | V | V | V | - | | | - | - | - | - | - | - | - |
| 2.5–2.8 | 17 | V | V | - | - | - | - | - | - | - | - | - | V | - |
| 1.9–2.2 | 5 | V | V | V | - | V | - | - | - | - | - | - | - | - |
| | | | | | | | | | | | | | | |
| 1.9–2.2 | 6 | V | V | - | V | - | - | - | - | - | - | V | - | - |
| 1.9–2.2 | 9 | V | V | V | V | - | V | V | V | - | - | V | V | V |
| 1.9–2.2 | 10 | - | V | - | - | - | V | - | V | - | - | - | - | V |
| 1.9–2.2 | 15 | V | V | V | - | PCR | - | - | - | - | - | - | V | - |
| 1.9–2.2 | 25 | V | V | - | - | V | - | V | - | - | - | - | - | - |
| 1.9–2.2 | 3 | V | - | - | - | PCR | - | - | - | - | - | - | V | - |
| 1.9–2.2 | 4 | V | V | - | - | PCR | PCR | - | - | - | - | - | - | - |
| 1.9–2.2 | 7 | V | V | - | - | - | PCR | - | - | - | - | - | V | - |
| 1.9–2.2 | 13 | - | V | V | V | V | - | V | - | - | - | - | - | - |
| | | | | | | | | | | | | | | |

| Antibody titre | Bov No | Days after infection | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 5 | 7 | 20 | 34 | 48 | 62 | 76 | 90 | 104 | 118 | 132 | 180 | 233 |
| 0 | 16 | V | V | - | V | - | V | - | V | - | - | - | - | - |
| 0 | 18 | V | V | V | - | PCR | PCR | - | - | - | - | - | V | - |
| 0 | 19 | V | V | V | V | V | V | V | V | - | - | - | V | V |

V= virus isolated
P = PCR positive
- = no virus or PCR

While the 3 sero negative exposed calves showed clinical signs typical of FMD, only 1 of the 17 vaccinated exposed animals presented a vesicular lesion in the palate. FMDV was detected in OPF of all animals at 5 and 7 d, with rapid sero conversion. Of the 20 animals of the group, fifteen were identified as carriers by virus isolation from OPF. Two additional carriers were detected by PCR. Altogether, 17 out of 20 animals (85%) became carriers in the experimental conditions employed. Among these, 13 were sero negative or had low titres of antibodies at the beginning of the experiment. Only 4 of the 7 animals with high levels of antibodies became carriers.

## TABLE IV. NSP ELISA DATA ON INFECTED CATTLE USING BRESCIA REAGENTS

| BRESCIA | Number | Days post infection | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 5 | 7 | 20 | 34 | 48 | 62 | 90 | 104 | 132 | 180 | 233 |
| Log$_{10}$ Ab titer | 29 | + | + | | + | + | + | + | + | + | + | + |
| 2.5–.8 | 1 | - | - | + | + | + | + | + | | + | + | + |
| 2.5–2.8 | 12 | + | | + | + | + | + | + | + | + | + | + |
| 2.5–2.8 | 8 | - | - | + | + | + | + | + | + | + | + | + |
| | | | | | | | | | | | | |
| 1.9–2.2 | 5 | | + | + | + | + | + | + | + | + | + | + |
| 1.9–2.2 | 6 | - | + | - | + | + | | + | + | + | + | + |
| 1.9–2.2 | 9 | - | - | | | + | + | + | + | + | + | + |
| 1.9–2.2 | 10 | - | - | + | + | + | + | + | + | + | + | + |
| 1.9–2.2 | 15 | + | - | + | + | + | + | + | + | + | + | + |
| 1.9–2.2 | 3 | - | - | + | + | + | + | + | + | + | + | + |
| 1.9–2.2 | 4 | - | - | + | + | + | + | + | + | + | + | + |
| 1.9–2.2 | 7 | - | - | + | + | + | + | + | + | + | + | + |
| 1.9–2.2 | 13 | - | - | + | + | + | | + | + | + | + | + |
| | | | | | | | | | | | | |
| 0 | 16 | - | - | | + | + | + | + | + | + | + | + |
| 0 | 18 | + | | + | + | + | + | + | + | + | | + |
| 0 | 19 | | + | + | + | + | + | + | + | + | + | + |

TABLE V. NSP ELISA DATA ON INFECTED CATTLE USING UBI REAGENTS

| UBI | | Days post infection | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Number | 5 | 7 | 20 | 34 | 48 | 62 | 90 | 104 | 132 | 180 | 233 |
| Log$_{10}$ Ab titer | 29 | | + | + | + | | + | + | + | + | + | + |
| 2.5–2.8 | 1 | - | - | + | + | + | | + | | + | + | + |
| 2.5–2.8 | 12 | - | | + | - | - | - | - | - | - | - | - |
| 2.5–2.8 | 8 | - | - | - | - | | + | + | + | - | - | - |
| | | | | | | | | | | | | |
| 1.9–2.2 | 5 | | - | + | + | + | + | + | + | + | + | + |
| 1.9–2.2 | 6 | - | - | + | + | + | + | + | + | + | + | + |
| 1.9–2.2 | 9 | - | - | + | + | + | + | + | + | + | + | + |
| 1.9–2.2 | 10 | - | - | + | + | + | + | + | + | + | + | + |
| 1.9–2.2 | 15 | - | - | + | + | + | + | + | + | + | + | + |
| 1.9–2.2 | 3 | - | + | + | + | + | + | + | + | + | + | + |
| 1.9–2.2 | 4 | - | - | - | | + | - | - | - | - | - | - |
| 1.9–2.2 | 7 | - | - | + | + | + | + | + | + | + | + | + |
| 1.9–2.2 | 13 | - | - | + | + | + | + | | + | + | + | + |
| 0 | 16 | - | - | - | - | + | + | + | + | + | - | - |
| | | | | | | | | | | | | |
| 0 | 18 | - | | + | + | + | + | + | + | + | + | |
| 0 | 19 | | | + | + | + | + | + | + | + | + | + |

TABLE VI. COMPARISON OF COMBINED NSP ELISA RESULTS CATTLE

| Ab titre | No | 5 | 7 | 20 | 34 | 48 | 62 | 90 | 104 | 132 | 180 | 233 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2.5-2.8 | 29 | B+ | B+ U+ | | B+ U+ | | B+ U+ | B+ U+ | B+ U+ | B+ U+ | B+ U+ | B+ U+ |
| 2.5-2.8 | 12 | **B+** U- | | B+ U+ | **B+** U- | **B+** U- | **B+** U- | **B+** U- | **B+** U- | **B+** U- | **B+** U- | **B+** U- |
| 2.5-2.8 | 1 | B- U- | B- U- | B+ U+ | B+ U+ | B+ U+ | B+ | B+ U+ | B+ | B+ U+ | B+ U+ | B+ U+ |
| 2.5-2.8 | 8 | B- U- | B- U- | **B+** U- | **B+** U- | B+ U+ P | B+ U+ P | B+ U+ | B+ U+ | B+ U- | B+ U- | B+ U- |
| 1.9-2.2 | 5 | | **B+** U- | B+ U+ | B+ U+ | | B+ U+ | B+ U+ | B+ U+ | B+ U+ | B+ U+ | B+ U+ |
| 1.9-2.2 | 6 | B- U- | **B+** U- | B- **U+** | B+ U+ | B+ U+ | | B+ U+ | B+ U+ | B+ U+ | B+ U+ | B+ U+ |
| 1.9-2.2 | 9 | B- U- | B- U- | | | B+ U+ | B+ U+ | B+ U+ | B+ U+ | B+ U+ | B+ U+ | B+ U+ |
| 1.9-2.2 | 10 | B- U- | B- U- | B+ U+ | B+ U+ | B+ U+ | B+ U+ | B+ U+ | B+ U+ | B+ U+ | B+ U+ | B+ U+ |
| 1.9-2.2 | 15 | B+ U- | B- U- | B+ U+ | B+ U+ | B+ U+ P | B+ U+ | B+ U+ | B+ U+ | B+ U+ | B+ U+ | B+ U+ |
| 1.9-2.2 | 3 | B- U- | B- **U+** | B+ U+ | B+ U+ | B+ U+ P | B+ U+ | B+ U+ | B+ U+ | B+ U+ | B+ U+ | B+ U+ |
| 1.9-2.2 | 4 | B- U- | B- U- | **B+** U- | | B+ U+ P | **B+** U- P | **B+** U- | **B+** U- | **B+** U- | **B+** U- | **B+** U- |
| 1.9-2.2 | 7 | B- U- | B- U- | B+ U+ | B+ U+ | B+ U+ | B+ U+ P | B+ U+ | B+ U+ | B+ U+ | B+ U+ | B+ U+ |
| 1.9-2.2 | 13 | B- U- | B- U- | B+ U+ | B+ U+ | B+ U+ | B+ U+ | | B+ U+ | B+ U+ | B+ U+ | B+ U+ |
| 0 | 16 | B- U- | B- U- | | **B+** U- | B+ U+ | B+ U+ | B+ U+ | B+ U+ | B+ U+ | **B+** U- | **B+** U- |
| 0 | 18 | **B+** U- | | B+ U+ | B+ U+ | P B+ U+ | P B+ U+ | B+ U+ | B+ U+ | B+ U+ | | |
| 0 | 19 | | | B+ U+ | B+ U+ | B+ U+ | B+ U+ | B+ U+ | B+ U+ | B+ U+ | B+ U+ | B+ U+ |

B+ = Positive by Brescia test; B- = Negative by Brescia test; U+ = Positive by UBI test; U- = Negative by UBI test; P = PCR positive. Black boxes = agreement with both tests as negative. Grey boxes = Brescia and UBI positive.

## TABLE VII. TESTS AT DIFFERENT DAYS POST INFECTION-COMBINED RESULTS CATTLE

| | Animal | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Days post infection | b1 | b10 | b11 | b12 | b2 | b3 | b4 | b5 | b6 | b8 | b9 |
| 10 | B+ U+ | B + U- | | | B + U+ | B + U+ | B+ U+ | B + U- | B + U- | | B- U- |
| 16 | | | | | | | | | | B + U+ | |
| 19 | | | U- | B + U+ | | | | | | | |
| 30 | | | | | | B + P | B + P | | | | |
| 33 | | B + U+ | | | | | | | | | B + U+ |
| 40 | | | | | | B + U- | | | | | |
| 63 | | B + U+ | | | | | B + U+ | | | | B + U+ |
| 70 | | | | | | B + U- | B + U+ | | | | |
| 90 | B + U+ | | | | B + | | B + U- | | | | B + U+ |
| 100 | | | | | | B + U- | | | | | |
| 120 | | | | | B + U+ | | B + U- | | | | |
| 150 | B + U- | | | | B+ U+ | | | | | | |
| 180 | | | | | B- U+ | | | | | | |

Brescia ELISA = B+ = positive and B– = negative. UBI ELISA U+ =Positive, U- = negative. Black boxes = agreement with both tests as negative. Grey boxes = Brescia and UBI positive.

TABLE VIII. NSP ANTIBODIES AT LATE STAGES PERSISTENCE IN CATTLE (BRESCIA)

| | Brescia ELISA | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Number | 1119 | 635 | 1057 | 337 | 80–970 | 336 | 1060 | 440 | 319 | 082–1111 |
| Days after infection | 7–14 | + | + | - | - | + | + | + | + | | + |
| | 21–30 | + | + | - | - | + | + | + | + | + | +D |
| | 125–150 | | - | | - | | + | | - | | |
| | 470 | - | - | + | | + | | | | + | + |
| | 535 | - | - | + | | | | + | | - | - |
| | 562 | | - | - | | | | + | - | - | |

| | UBI ELISA | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Number | 1119 | 635 | 1057 | 337 | 80–970 | 336 | 1060 | 440 | 319 | 082–1111 |
| | Days post infection | | | | | | | | | | |
| Days after infection | 7–14 | + | + | - | - | + | + | + | + | | + |
| | 21–30 | + | + | - | - | + | + | + | + | + | - |
| | 125–150 | | - | | - | | - | | - | | |
| | 470 | - | - | - | | + | | | | - | + |
| | 535 | - | - | - | | | | - | - | - | + |
| | 562 | | - | - | | | | - | | - | |

TABLE IX. LATE STAGES PERSISTENT INFECTION-COMBINED ELISA DATA CATTLE

| Days post infection | Animal | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1119 | 635 | 1057 | 337 | 80–970 | 336 | 1060 | 440 | 319 | 082–1111 |
| 7–14 | + B + U | + B + U | - B | - B | + B + U | + B + U | + B + U | + B + U | | + B + U |
| 21–30 | + B + U | + B + U | - B - U | - B - U | + B + U | + B + U | + B + U | + B + U | + B + U | + **B** - U |
| 125–150 | | - B - U | | - B - U | | + B -U | | - B - U | | |
| 470 | - B -U | - B -U | + **B** -U | | + B + U | | | | + B - U | + B + U |
| 535 | - B - U | - B - U | + **B** - U | | | | + **B** -U | | - B - U | - B + **U** |
| 562 | | - B - U | - B - U | | | | + **B** - U | - B | - B - U | |

+B = positive by Brescia test; -B = negative by Brescia test;
+ U = positive by UBI test, - U = negative by UBI test;
Single results indicate that only this test was made


TABLE X. ANTIBODIES TO NSP IN INFECTED CATTLE, ACUTE STATE (UBI)

| UBI | DPI | 0d* | 7d | 14d | 21d | 30d |
|---|---|---|---|---|---|---|
| Animal | | | | | | |
| 31 | | - | - | + | + | |
| N | | | - | | + | |
| 1 | | | - | | | |
| 2 | | - | + | | | |
| sc | | | + | + | + | + |
| 091 | | | | | + | + |
| D1 | | | - | | | |
| D2 | | | - | | | |

TABLE XI. ANTIBODIES TO NSP IN INFECTED CATTLE, ACUTE STATE

| Brescia | 0d* | | 7d | | 14d | | 21d | | 30d | |
|---|---|---|---|---|---|---|---|---|---|---|
| Animal | OD | TP ratio | OD | TP ratio | OD | TP ratio | OD | TP ratio | OD | TP ratio |
| 31 | - | - | + | + | + | + | + | + | | |
| N | | | | | | | + | + | | |
| 1 | | | - | - | | | | | | |
| 2 | | | - | + | | | | | | |
| sc | | | + | + | + | + | + | + | + | + |
| 091 | | | | | | | + | + | + | + |
| D1 | | | - | - | | | | | | |
| D2 | | | - | D | | | | | | |

TABLE XII. VIRUS ISOLATION FROM EXPERIMENTAL INFECTIONS IN SHEEP

| Treatment | No | Days after infection | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0 | 2 | 4 | 6 | 8 | 15 | 24 | 32 | 39 | 46 | 56 | 66 |
| UnV | s/c | | | | V | V | | | | | | | |
| UnV | 195 | | | V | V | V | V | | | | | | |
| UnV | 198 | | | | | | V | | | V | | | |
| UnV | 202 | | | | V | | | | V | | | V | |
| UnV | 203 | | | | V | V | V | V | V | V | | | |
| UnV | 212 | | | | | | | | | | | | |
| UnV | 224 | | | | V | V | V | | | | | | |
| 1 dose | 209 | | | | | | | | | | | | |
| 1 dose | 215 | | | | | | | | | | | | |
| 1 dose | 216 | | | V | V | | V | | | | | | |
| > 3 doses | 35 | | | | | | | | | | | | |
| > 3 doses | 75 | | | | | | | | | | | | |
| > 3 doses | 84 | | | | | | | | | | | | |
| > 3 doses | 96 | | | | V | | | | | | | | |
| > 3 doses | 98 | | | | | | | | | | | | |
| > 3 doses | 135 | | | | | | V | | | | | | |

UnV = not vaccinated; V= virus isolated

TABLE XIII. EXPERIMENTAL INFECTION SHEEP COMBINED ELISA DATA

| | Previous high levels of antibodies | | | | | | No antibodies | | | | | Low antibodies | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 35 | 75 | 84 | 96 | 98 | 135 | 195 | 198 | 224 | 202 | 203 | 209 | 215 | 216 |
| Dpi | | | | | | | | | | | | | | |
| 0 | U- B- | U- B- | U- B- | U- B- | U- B- | U- B- | | | U- B- | U- B- | U- B- | U- B- | U- B- | U- B- |
| 2 | | | | | | | U- B- | U- B- | | | | | | |
| 8 | | | | | | | U- B- | U- B- | U- B- | U- B- | U- B- | | U- B- | U- B- |
| 15 | | | | | | | U+ B+ | U- B- | U+ B+ | U- B- | U+ B+ | U- B- | | U+ B+ |
| 32 | U- B- | U- B- | U- B- | U- B- | U- B- | U- B- | U+ B+ | U+ B+ | U+ B+ | U+ B+ | U+ B+ | U- B- | | U+ B+ |
| 56 | | | | | | | U+ B+ | U+ B+ | U+ B+ | U+ B+ | U+ B+ | | | |
| 92 | | | | | | | | U+ B+ | U- **B+** | U+ B+ | U+ B+ | | | |
| 105 | | | | | | | U- **B+** | U- **B+** | U+ B+ | | U+ B+ | | | |
| 137 | U- B- | U- B- | U- B- | U- B- | U- B- | U- B- | | U- **B+** | U- **B+** | U- **B+** | U+ B+ | | U- **B+** | |

U- or U + is negative or positive by UBI test
B- or B+ is negative or positive with Brescia test

TABLE XIV. RESULTS OF ANALYSIS OF FIELD SAMPLES

| UBI | P | N TOTAL | |
|---|---|---|---|
| <2 | 5 | 12 | 42 |
| >2 | 4 | 25 | 16 |

| Brescia | P | N TOTAL | % |
|---|---|---|---|
| <2 | 9 | 12 | 75 |
| >2 | 9 | 25 | 36 |
| TOTAL | 18 | 37 | 48 |

TABLE XV. RESULTS OF ANALYSIS OF FIELD SAMPLES BY AGID

|  | P | N TOTAL | % |
|---|---|---|---|
| <2 | 12 | 12 | 100 |
| >2 | 8 | 25 | 32 |
| TOTAL | 20 | 37 | 54 |

**Small ruminants**

Twenty Samples of ovine and goats were studied.

1/20 sample result positive (UBI), 8/20 samples were positive with ELISA 3D.

**Pig samples**

*Free pigs*

10 sera from free (wild) pigs gave negative results.

*Vaccinated Pigs*

44 swine vaccinated and sampled at 30 dpv, gave negative results.

17 swine revaccinated and sampled 30 dpr gave negative results.

*Infected Pigs*

Four infected pigs were sampled at different times post infection in the acute stage of the infection. A total of 21/26 samples of 4 infected swine were positive. The test failed to detect antibodies collected at 2, 3, 6, and in one animal at 8 dpi.

4.      DISCUSSION AND CONCLUSIONS from PHASE 1 research

Both tests were reliable and reproducible assays to measure antibodies to FMDV NSP. Both tests are adequate epidemiological tools for evaluation of campaigns for eradication and control of FMDV.

*Advantages*

UBI gave a high specificity, even with sera from recently vaccinated animals.
The 3ABC Brescia was highly sensitive, it can present positive results with sera from infected animals since 5–7 dpi up to more than 1 y pi.

*Disadvantages*

The UBI was less sensitive compared to the 3ABC Brescia, especially with serum samples taken at long intervals after exposure to virus. The Brescia 3ABC test was less specific when vaccinated animals were evaluated, especially with samples taken shortly after vaccination.

5.      PHASE 2 RESEARCH

**5.1.    Material and methods**

Three ELISAs for detection of antibodies directed against non-structural proteins (NSP ELISAs) were compared.

(A)   CHEKIT-FMD-3ABC bo-ov enzyme immunoassay based on the detection of antibodies binding with a recombinant FMDV protein 3ABC (the kit used ABTS as chromogen and had to be read at wavelength of 405nm).

(B)   UBI FMDV ELISA based on the detection of antibodies binding a synthetic peptide of 3B.

(C)   CEDI TEST FMDV-NSP based on the detection of antibodies binding with a recombinant FMDV protein 3ABC.

Tests B and C were performed at the same time, Test C was made separately because it had a different protocol. In all tests, controls were made in duplicate; the means and standard deviation of the OD were calculated and plotted.

## 5.2.   Serum samples

The following serum samples were used:

*Sera from disease free animals*

A1-51 are serum samples from cattle from the FMDV-free area of Argentina (south of 42°S, Patagonia Region), that are free of antibodies to type O, A, C of FMDV as measured by liquid phase blocking sandwich ELISA (LPBE). A2-13 are serum samples of naive cattle exposed to FMDV carriers, sampled at 30-150 and 230 d post contact.

*Vaccinated animals*

Twelve sera from cattle from the FMDV free area of Argentina vaccinated and sampled 30 d post vaccination.

*Sera from FMDV infected animals*

Samples from cattle challenged by inoculation or contact were taken from 10; 11–15; 16–20; 21–30; 31–90; 104–150 and 180–233 dpi.

*Field samples*

Fourteen cattle that had clinical FMD 2y before that had received 4 doses of oil-adjuvanted FMD vaccine. Cattle field samples comprising 180 sera obtained from the infected and surveillance zone, 1 y post-outbreak.

# 6. RESULTS PHASE 2 RESEARCH

## 6.1. Performance of the tests

The graphics of the Daily Detailed Data Charts for three kits are shown in Fig. 8.
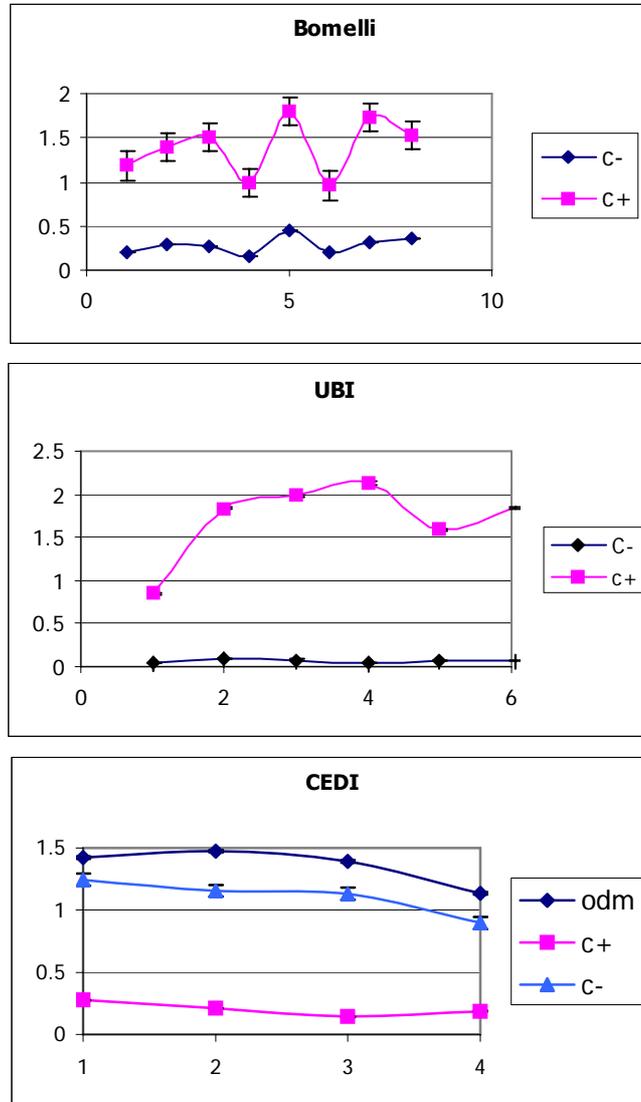


*FIG. 8. Graphs show the Daily Detailed Data Charts (DDD).*
OD are shown of Bommeli (a), UBI (b) and CEDI test (c).C+= positive control serum; C-= negative control serum; odm= Maximum optical density. The Y axis shows OD units. The x axis contains data from plates.

## 6.2. Results obtained with experimental and field samples

### 6.2.1. Bovine free of FMDV

All 51 cattle sera from the FMDV free zone gave negative results in the three kits.

The cattle exposed to FMDV carriers remained negative by the three kits until 230 d post contact.

### 6.2.2. Antibody response in vaccinated animals

In order to examine the antibody response after vaccination, 12 bovines free of FMDV vaccinated with commercial formulations used in the Argentine FMD control programme were studied.

All the sera gave negative results in the three kits.

### 6.2.3. Infected cattle

Results from studying all results of infected animals (135 samples) showed differences among the 3 kits, especially between CEDI and UBI.
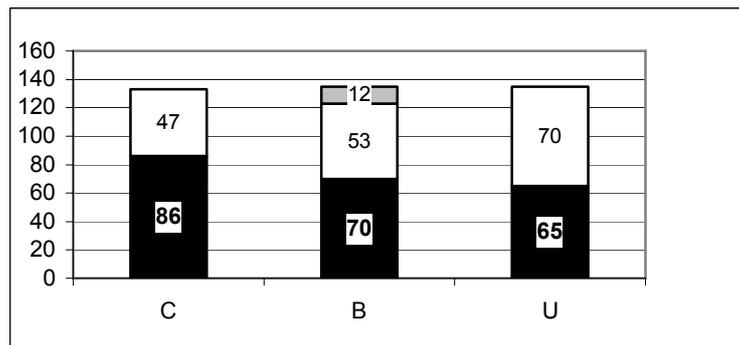


Y axis is number samples
Black box = positive, white box = negative, grey box = ambiguous

|   | P | N | A | % CI |
|---|---|---|---|---|
| C | 86 | 47 |  | 64(56-72) |
| B | 70 | 53 | 12 | 56(47-65) |
| U | 65 | 70 |  | 48(39-56) |

*FIG. 9. Results of infected animals.*

C = CEDI, B= Bommeli, U= UBI, P= positive, N= negative, A= ambiguous.
Prevalences are represented as percentages, 95% confidence intervals are between parentheses.

When we grouped the sera by dpi, we found that the differences are observed in early times of infection or in samples obtained up 150 dpi.
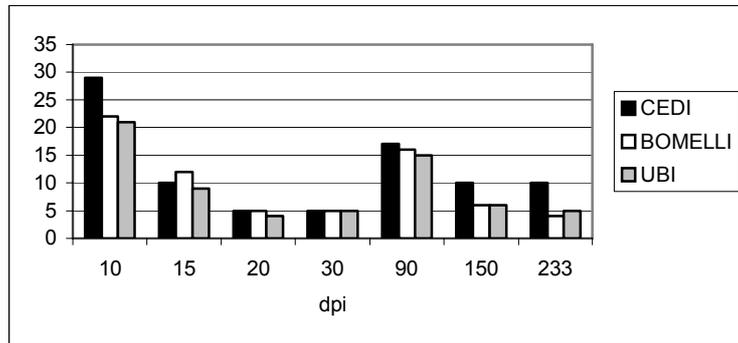


*FIG. 10. Results of infected animals grouped by d post infection.*

The x axis shows dpi, y axis shows the number of positive sera detected.
Black box = CEDI, White box = Bommeli, Grey box = UBI

### 6.2.4. Field samples

Fourteen cattle were sampled after 2 y post clinical signs. The results with test A (CEDI) were all negative, test B (BOMMELI) 1/14 positives and test C (UBI) 14/14 positives.
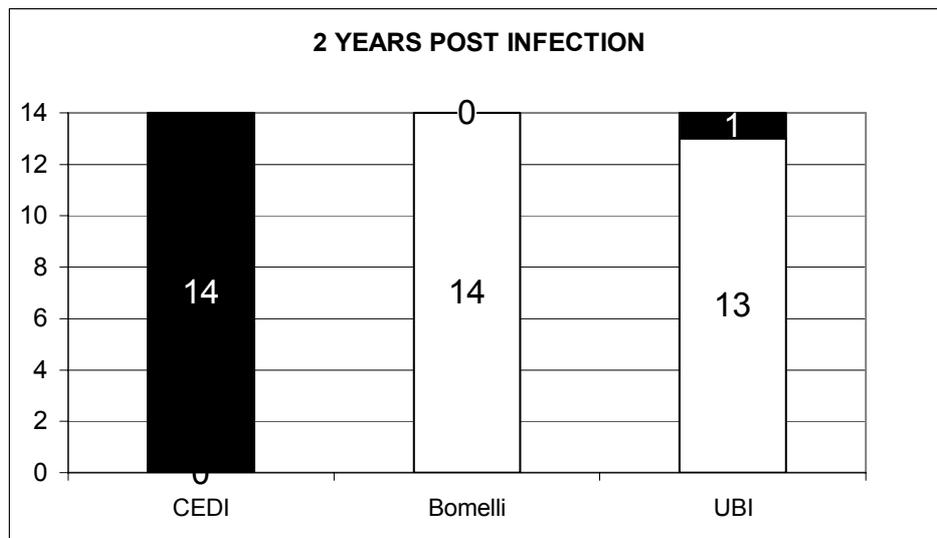


*FIG. 11. Results of bovines samples 2 years after clinical signs.*

Black box = positive, white box = negative

The results from 200 cattle samples from a surveillance zone are shown in Table XVI

TABLE XVI. RESULTS ON FIELD SAMPLES FROM SURVEILLANCE ZONE

| | Negative | Positive | Ambiguous | % |
|---|---|---|---|---|
| CEDI | 99 | 107 | | 52(45-59) |
| Bommeli | 160 | 32 | 8 | 16 (11-22) |
| UBI | 140 | 40 | | 28 (20-36) |

Prevalences are represented as percentages, 95% confidence intervals are between parentheses. Note the increased number of positives detected by CEDI.

Statistically differences were observed between CEDI and BOMMELI/UBI.

## 7. DISCUSSION AND CONCLUSION PHASE 2 RESEARCH

Analysing the results obtained in this work is possible reach the following conclusions.

The specificity was similar between the three kits.

The antibodies against NSP can be detected for a long term, more than a year.

The 3 kits showed differences in their sensitivity particularly in samples in early periods of the infection or obtained up 6 m post infection.

For a better control of the evolution of FMDV status, it is convenient to have annual epidemiological controls. Serological diagnosis of calves of 6 m to one year of age should be adequate to assess the status of viral circulation in the previous year to sampling. It is known that in this group of animals the colostral antibodies have already disappeared and also, this would avoid an overestimation caused by positive samples from previous periods.

# THE USE OF NON-STRUCTURAL PROTEIN OF FOOT AND MOUTH DISEASE VIRUS ELISA KITS IN DIFFERENT SEROLOGICAL EVALUATIONS IN BRAZIL

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**Abstract**

The work was made in two phases. In the first phase, sera from buffalo and cattle from different farms and counties were tested using three non-structural protein (NSP) foot and mouth disease virus (FMDV) ELISA kits. In the second part sera from selected vaccinated cattle were tested using four commercial NSP-FMDV ELISA kits. Sera from two different outbreaks were also tested in both parts.

## INTRODUCTION

Foot and mouth disease (FMD) is a highly contagious disease, caused by a virus of the genus Aphtovirus which is member of the family Picornaviruses [1]. The disease affects susceptible cloven-hoofed animals causing severe economic losses for the agricultural sector of countries. The disease is considered a global animal health problem especially related with trade. The disease cannot be differentiated from other vesicular diseases only by clinical signs [2] thus, it is very important the laboratory diagnosis is made rapidly. Serological tests detecting antibody to the capsid proteins of FMDV are currently used but cannot differentiate vaccinated animals from those that have been infected, once antibodies to this protein are induced by vaccination and infection [3]. During serological surveys it is very important to differentiate vaccinated from infected animals. Differentiation among these two groups of animals can be based on the detection of antibody to non-structural proteins (NSP) using ELISA and enzyme-linked immunoelectrotransfer blot (EITB) [4]. The use of serological diagnostic tests capable to detect viral activity in a vaccinated animal population it is a very important step for recognition of a country sanitary status as FMD free with vaccination.

PHASE 1. COMPARISON OF THREE NSP OF FMDV ELISA KITS USING BUFFALO
          AND BOVINE SERA SAMPLES

1.     MATERIALS AND METHODS

In total, 950 serum samples were taken from buffalo from 19 farms in 9 counties and 800 serum samples from cattle on 34 farms of 17 counties of the Rio Grande do Sul state. All were collected between 1999 and 2001 from animals raised in the field.

The FMD situation in Rio Grande do Sul state changed during this period. After 6 y and 3 m without outbreaks and with recognition by the OIE as a free zone from FMD with vaccination in April of year 2000 and the withdrawal of vaccination in August of that year, an outbreak occurred [5]. Some sera from this outbreak and from another in May 2001 are included in the total samples.

Ten positive samples were kindly provided by Dr. Emiliana Brocchi from Istituto Zooprofillattico Sperimentale della Lombardia e dell' Emilia Romagna (IZSLER). The number of sera tested in each kit varied according with the quantity of plates which was sent allowing work however, the comparison of results of the three kits was made on data only when the same samples were tested, with the exception of data on the positive samples from IZSLER due to the low volume of the samples and because samples from the second outbreak were collected after all plates of UBI kit were used. All samples sera were kept frozen at -20º C until use.

**1.1.    NSP FMDV ELISA kits**

ELISA kits from Brescia/Italy (IZSLER), Denmark (Danish Veterinary Institute for Virus Research Diagnostic and Pathology) and United States of America (United Biomedical Incorporation-UBI) were used according with the protocol of each manufacturer.

2.     PHASE 1 RESULTS

**2.1.    UBI kit**

A total number of 1,656 sera samples were tested and these comprised:

Buffalo: 911 from 19 farms from 9 countries.

Results: 905 negative sera and 6 positive sera (0.65% of tested buffalo sera).

Cattle 745 sera from 34 farms from 17 counties.
Results. 708 negative sera and 37 positive sera (4.96% of tested bovine sera).

Repeatibility
Sera (62) were retested. From 43 positive sera (total number of positive sera of ruminants), 36 were retested and confirmed the result of the first. However, 7 sera were positive in the first test and negative at retest. Nineteen samples negative in the first test were confirmed on retest.

Results of positive samples: four were tested and one retested. All sera were positive and the one retested was positive by both tests. The result are shown in Table I.

## 2.2. Results from Brescia (IZSLER) kit

The total number of tested sera samples was 1,436 samples and the samples were divided as follows:

Buffalo: 854 sera from 19 farms from 9 counties.
Results: 833 negative sera and 21 positive sera (2.45% of buffalo sera).

Cattle: 582 sera from 34 farms from 17 counties.
Results: 479 negative sera and 103 positive sera 17.69% of bovine sera).

Repeatibility: Sera (154) were retested. From these 124 positive sera (total number of positive samples, including twenty five positive as T/P ratio, 59 positive samples were retested.

Fifty-three positive sera were confirmed and six positive samples did not confirm at retest. Ninety-five negative sera were retested and all were confirmed negative.

TABLE I. RESULTS (IN OPTICAL DENSITY) OF POSITIVE SAMPLES FROM IZSLER

| SAMPLE | UBI | BRESCIA |
|--------|-----|---------|
| EMC1 | NT* | 1.996 (0.200)** |
| EMC2 | NT | 2.100 |
| EMC3 | NT | 1.987 |
| EMC4 | NT | 1.697 |
| EMC5 | 0.868 (0.138)** | 1.267 |
| EMC6 | 0.911(0.138)** | 1.854 |
| EMC7 | NT | 1.940 |
| EMC8 | NT | 2.087 |
| EMC9 | 1.315(0.138)** | 2.238 |
| EMC10 | 0.229/0.261(0.141/0.138)** | 1.902 |

* NOT TESTED
** CUTOFF - UBI: OD/per plate and BRESCIA: 0.200
All ten sera samples were positive.

## 2.3. Results from Denmark kit

The number of tested sera (total) using this kit was 966 samples divided as follows:

Buffalo: 636 sera from 19 farms from 9 counties.
Results: 603 negative sera and 33 positive sera (5.18% of tested buffalo sera).

Cattle: 330 sera from 34 farms from 17 counties.
Results: 278 negative sera and 52 positive sera (15.75% of tested bovine sera).

Repeatibility: Sera (41) samples were retested. From these 20 samples were positive at test and retest, 19 were positive at first test and negative at retest and 2 sera were negative at first test and retest.

The results of positive samples from first outbreak (year 2000) and second outbreak (year 2001) are shown in Tables II and III.

TABLE II. POSITIVE SAMPLES AT OUTBREAK /2000 — ANIMALS WITH CLINICAL SIGNS

| SAMPLES | UBI (R)* | BRESCIA(R)* | DENMARK (R)* |
|---------|----------|-------------|--------------|
| TM07 | + (+)* | + | +(+)* |
| TM02 | + (+) | + | +(+) |
| TM06 | + (+) | + | +(+) |
| TM09 | + (+) | + | +(+) |
| TM01 | + (+) | + | +(+) |
| TM05 | + (+) | + | +(+) |
| TM2 | + (+) | + | + |
| RB2 | + (+) | + | + (+) |
| 1182SM | + (+) | + | + |
| 20SM | + (+) | + | + |
| HS14 | + (+) | + | + (+) |
| HS15 | + (+) | + | + (+) |
| HS16 | + (+) | + | + (+) |

*(R) RETEST

TABLE III. POSITIVE SAMPLES AT OUTBREAK/2001 — ANIMALS WITH CLINICAL SIGNS

| SAMPLES | UBI | BRESCIA (R)* | DENMARK (R)* |
|---------|-----|--------------|--------------|
| 2 | NT** | + (+)* | + |
| 93 | NT | + (+) | + (+)* |
| 15 | NT | + | + |
| 032 | NT | + (+) | + |
| B18 | NT | + | + (-) |
| B05 | NT | + | + (-) |
| J08 | NT | + | + (-) |
| J06 | NT | + | + (-) |

* (R) RETEST
** NOT TESTED

3.      PHASE 1 DISCUSSION AND CONCLUSIONS

Data from samples from animals with clinical signals from the outbreak in 2000 showed that all 3 kits had the same performance (Table II). From the second outbreak the Brescia kit (8 samples animals which showed clinical signals) were positive) was more

sensitive than the Denmark kit (only 4 samples were positive) when tested in a single well. Results for the remaining four samples remained negative on a double well retest

## 3.1. Comparison between the 3 kits in relation to other positive samples

The objective of this experiment was to look the performance of each kit at the laboratory over one year and not to establish the specificity or sensitivity of each kit, but

We conclude that during this phase, the UBI kit was the easiest test because it was the less time consuming and reagents were ready to use. All the plates were valid according to the UBI criteria even when it was used close to the expiry date. There was no significant variation in the controls during the period of work.

The Brescia test was also easy to perform but was more time consuming than the UBI and some reagents had to be prepared by the user. All the plates passed the validation criteria and there was not a significant variation of the controls during the time of the experiment.

The Denmark test was less validated when received and laborious in relation to the others. It was necessary to prepare several reagents. Several plates did not pass at the validation criteria and the repeatability was not as good as the two others test systems.

In relation to the repeatability criteria all 3 kits showed the same performance for negative samples (100%) but for re-tested positive samples the best performance was with the Brescia kit (89.83%) followed by UBI (83.72%) and Denmark (51.28%)

**UBI**

From 17 positive samples from several areas: 8 were positive in UBI and Brescia but negative in the Denmark kit. Three sera were positive in the UBI, negative in the Brescia kit and positive on initial tests test and negative at retest for the Denmark kit. Six sera were positive only with the UBI kit.

**BRESCIA**

From 93 positive sera, 8 were positive only with the UBI and Brescia kits. Two were positive in the UBI first test and negative at retest and for the Brescia for these two samples, only one was positive at first test and retest and one was positive at first but negative at retest. Three negative sera including a retest ain the UBI were positive on test and retest in the Brescia. Seventy-seven samples were positive only in the Brescia test (thirty eight of them were retested). Four samples were positive in the Brescia and Denmark tests, but only two were positive at retest in the latter kit.

**DENMARK**

Of the remaining 58 positives, 2 were positive in the Brescia and Denmark kits (non-tested at UBI). One serum was positive in the Denmark kit, negative in the Brescia and positive on first testing with the UBI, but negative on retest. Fifty-five sera were positive only using the Denmark kit (15 were not tested at UBI). Other results: Two were positive in the Brescia kit but only positive on first test and negative at retest in the Denmark kit (not tested in UBI kit). Three were positive in the UBI kit but positive only at first test in the Denmark kit. Nine sera were positive in the Denmark kit on first test and negative on retest and all were negative in the Brescia kit (not tested in the UBI kit).

PHASE 2. THE USE OF NON-STRUCTURAL ANTIGENS OF FMD VIRUS TO ASSESS ANTIBODIES IN VACCINATED AND INFECTED LIVESTOCK

## 4.     MATERIALS AND METHODS

### 4.1.    Vaccinated cattle

Non-vaccinated cattle from a vaccine challenge test came from 9 different farms registered and controlled by the Ministry of Agriculture of Brazil (MAPA). These animals had an age range from 18 to 24 m and were born to vaccinated cows. Selected animals were all negative for antibodies against FMDV in the Liquid Phase Blocking ELISA (LPBE). The animals were transferred to the MAPA farm where vaccinations were made. The cattle were divided into groups of 18 which received trivalent (A, O, C) vaccines from 4 different commercial vaccine companies on the same day. For one commercial vaccine two batches were used. All vaccines were produced in 2002 and were approved in the official control for potency, stability and safety. Two animals were not vaccinated to act as controls and kept together with the vaccinated group. They were sampled at the same intervals as the vaccination group. Samples were collected at day zero and 28 d post vaccination (dpv). At 56 dpv the cattle were revaccinated and subsequent samples collected at 28, 84 and 112 d post revaccination (dpr).

Samples from 105 non-vaccinated cattle were tested with ages ranging from 18 to 24 m coming born from vaccinated cows and taken from different farms registered and controlled by the MAPA. All the selected animals were negative in the LPBE and passed clinical examination by veterinarians of MAPA.

Serum samples from cattle involved in 2 different outbreaks (13 samples and 14 samples respectively) were also tested. All animals of the first outbreak showed clinical signs and they were not recently vaccinated. The samples came from 4 different farms. From the second outbreak the cattle came from 2 different farms and only one animal showed clinical signs. The sera were collected less than a month after the second outbreak started. All the animals from these two outbreaks were sacrificed.

Pools of ten FMDV antibody positive serum samples, kindly provided by Dr. Emiliana Brocchi (IZSLER) were titrated in a dilution range of 1/12.5, 1/25, 1/50 and 1/75) using all kits. All samples had showed a very high positivity optical density (test done in year 2001) when they were tested using an IZSLER ELISA kit. The lowest optical density was 6.33 times higher than the cut-off. All samples were kept frozen at -20$^{\circ}$C until be used.

### 4.2.    ELISA

ELISA plates from BOMMELI, CEDI and UBI were used on the same day. The work was done on one plate of each ELISA kit starting at the same day in a controlled temperature room (average of 23$^{\circ}$C). All kits were used according to the manufacturer's protocols. The PANAFTOSA kits were received one month after start the work with the other kits. The PANAFTOSA kits were used later at the same average temperature as with the other kits. For the final result for the PANAFTOSA system, inconclusive and positive samples on ELISA test were tested using ELISA the EITB. The 3ABC ELISA and the EITB test were performed according to the manufacturer's protocol. The validation criteria and the results were also done according to the mentioned protocol.

## 5.    RESULTS FROM PHASE 2

The first outbreak data showed that the BOMMELI and UBI tests gave positive results for all samples (100%). The PANAFTOSA ELISA also found all samples positive but one sample was inconclusive when on the EITB system, thus the overall sensitivity was given as 92.31%. Two samples were negative using the CEDI test (84.61% positive). Samples which showed some discrepancy were retested at least in duplicate and the final result for ascribing the antibody status was considered that of the re-test. The final results are shown in Fig. 1.

**FIRST OUTBREAK – 2000**

**TOTAL 13 SAMPLES**



| Classific | BOMMELI | CEDI | PANAFTOSA | 3ABC/EITB | UBI |
|-----------|---------|------|-----------|-----------|-----|
| | % | | | | |
| Negative | 0.00 | 15.39 | 0.00 | 0.00 | 0.00 |
| Suspect | 0.00 | 0.00 | 0.00 | 7.69 | 0.00 |
| Positive | 100.00 | 84.61 | 100.00 | 92.31 | 100.00 |
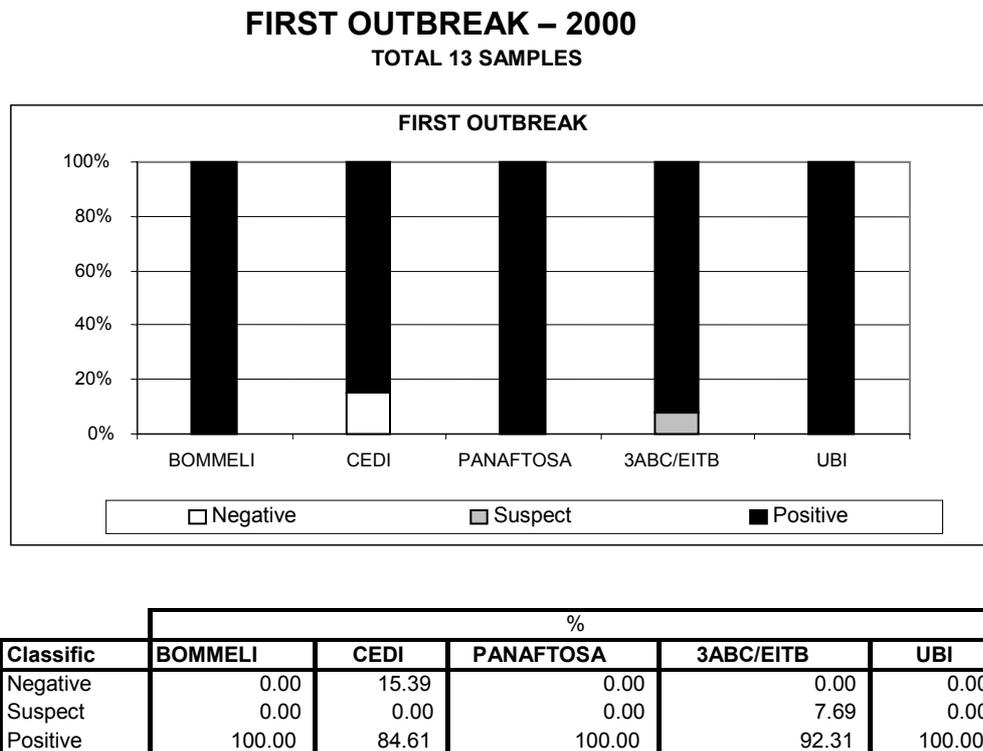
*FIG. 1. Analysis of sera from an intial outbreak of FMD by commercial kits.*

For the second outbreak the BOMMELI kit gave the highest number of positives (92.86%) followed by CEDI and PANAFTOSA, 78.58%. All positives samples in the PANAFTOSA kit were examined by the EITB test and the final result was 71.42%. The lowest number of positives was observed for the UBI kit with 57.15%. Several samples of this group were retested and the final result was considered when more than one well per sample were used. The final results are presented in Fig. 2.

## SECOND OUTBREAK – 2001
### TOTAL 14 SAMPLES
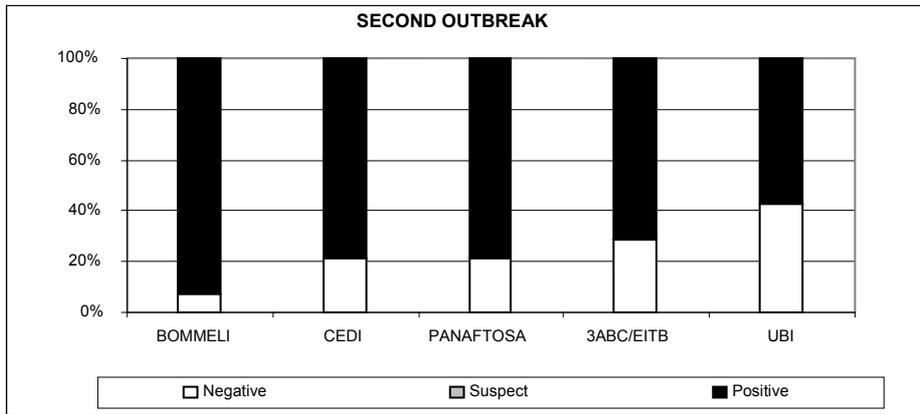


| Classific | BOMMELI | CEDI | PANAFTOSA | 3ABC/EITB | UBI |
|---|---|---|---|---|---|
| | | | % | | |
| Negative | 7,14 | 21,42 | 21,42 | 28,57 | 42,85 |
| Suspect | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| Positive | 92,86 | 78,58 | 78,58 | 71,42 | 57,15 |

*FIG. 2. Analysis of sera from a second intial outbreak of FMD by commercial kits.*

CEDI and PANAFTOSA gave the same results for the negative group, where all were found negative. The UBI showed 99.05% of samples as negative and BOMMELI this percentage 88.58%. The results are presented in Fig. 3.

## NEGATIVE
### TOTAL 105 SAMPLES
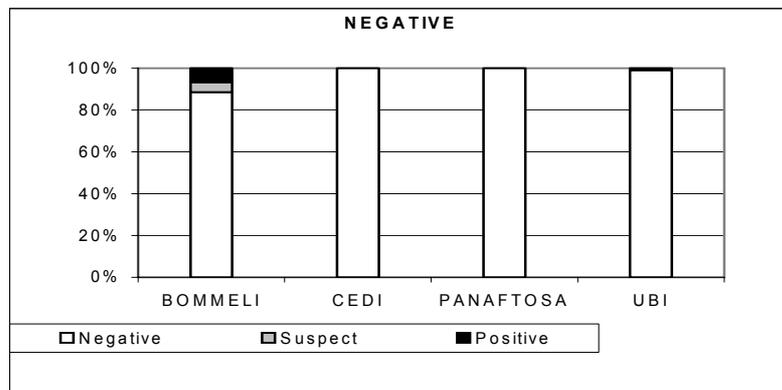


| Classific | BOMMELI | CEDI | PANAFTOSA | UBI |
|---|---|---|---|---|
| | | % | | |
| Negative | 88.58 | 100.00 | 100.00 | 99.05 |
| Suspect | 4.76 | 0.00 | 0.00 | 0.00 |
| Positive | 6.66 | 0.00 | 0.00 | 0.95 |

*FIG. 3. Analysis of 'negative' sera by commercial kits.*

When dilutions of a pool of positive samples were tested the CEDI kit detected the sample as positive in all four dilutions, the BOMMELI kit detected the first two dilutions and PANAFTOSA kit detected only at the first dilution. The UBI kit was not able to detect even the most concentrated dilution. All these dilutions were tested in more than three wells and the final result was the average of the results. The results are presented in Fig. 4.

## DILUTION OF POSITIVE SAMPLE (POOL)
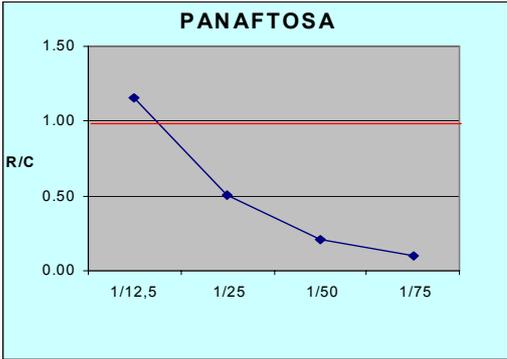


| DILUTION | 1/12,5 | 1/25 | 1/50 | 1/75 |
|---|---|---|---|---|
| R/C | 1.16 | 0.51 | 0.21 | 0.10 |

| DILUTION | 1/12,5 | 1/25 | 1/50 | 1/75 |
|---|---|---|---|---|
| % INH | 78.25 | 71.38 | 62.08 | 54.07 |

| DILUTION | 1/12,5 | 1/25 | 1/50 | 1/75 |
|---|---|---|---|---|
| % INH | 99.29 | 31.22 | 16.76 | 9.74 |

| DILUTION | 1/12,5 | 1/25 | 1/50 | 1/75 |
|---|---|---|---|---|
| O.D. | 0.058 | 0.025 | 0.016 | 0.014 |
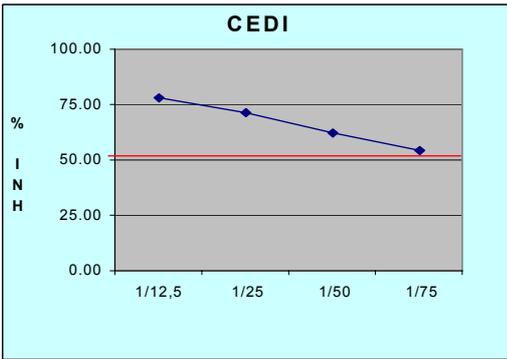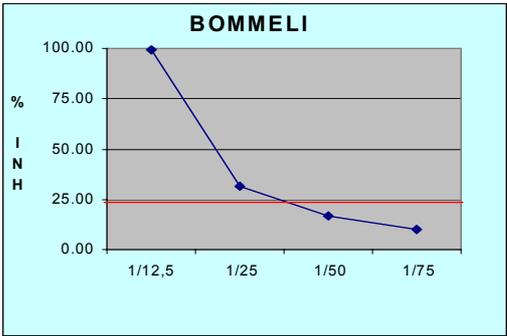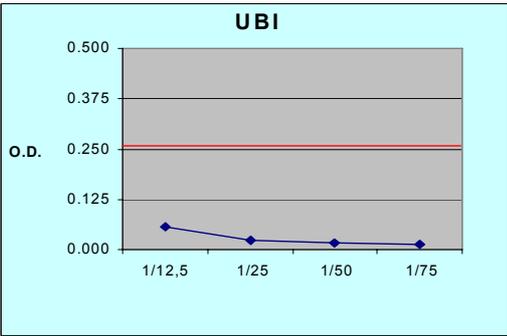
*FIG. 4. Comparison of relative analytical sensitivities of commerical kits.*

For commercial vaccine A at day 0 the results were the same in all 4 kits (100% negative).

At 28 DPV some reactivity was detected, the results of BOMMELI, PANAFTOSA and CEDI tests were the same at 5.56% positive. The UBI kit did not detect any sample as positive.

At 28 d post re-vaccination (28DPR), the CEDI kit detected 66.67% positive; the BOMMELI and PANAFTOSA using the 3ABC-EITB system, 38.89% positive (77.78% of positive without use of the EITB system). The lowest detection was with UBI kit at 22.22% positive.

At 84 DPR the BOMMELI kit gave 44.44% positive; the CEDI kit 22.22% positive; and the PANAFTOSA using the 3ABC-EITB system, 11.11% (44.44% without system). The UBI gave 5.56%.

For the 112 DPR the vaccine reactivity started to disappear and only 11.11% positives were found using the BOMMELI kit, 5.56% using the CEDI and UBI kits. The PANAFTOSA did not detect any positive samples when the ELISA 3ABC-EITB system was used but had an 11.11% positive result without reference to the system. The results of the 4 ELISA kits and the samples related to commercial vaccine A are presented in Fig. 5.

## COMMERCIAL VACCINE  A  Batch 009/02
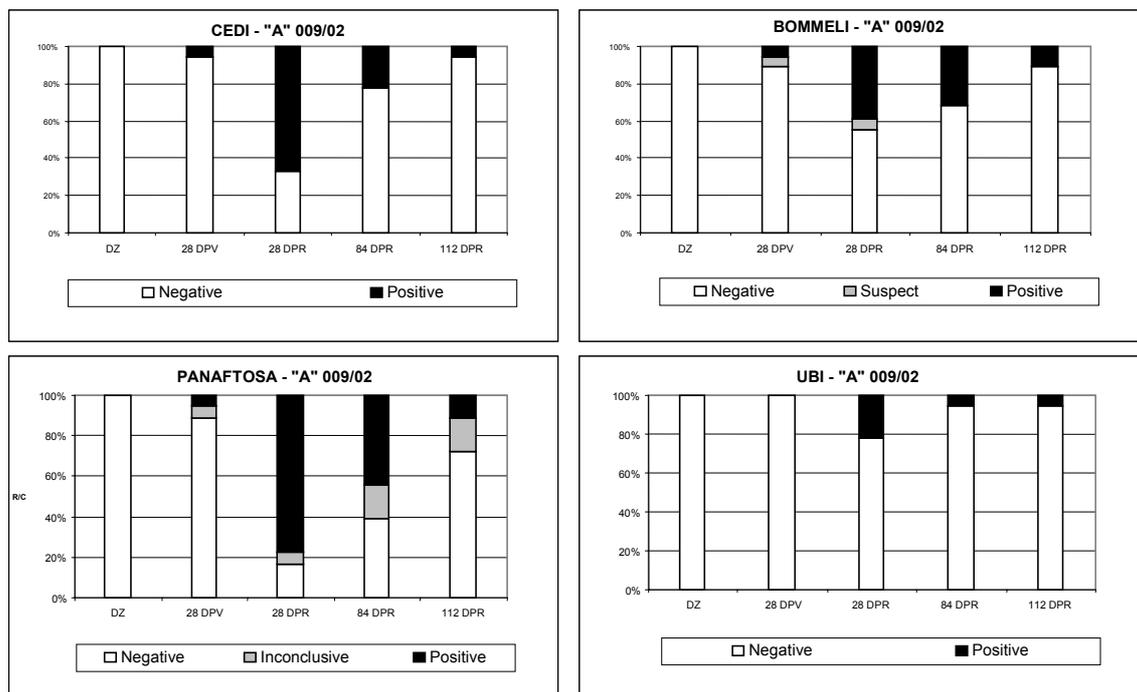


FIG. 5. Examination of antibody responses against commercial vaccine batch A.

For the commercial vaccine B at day 0 the results were the same (100% negative) using all 4 kits.

At 28 DPV only the BOMMELI kits detected some antibodies in one sample (5.56%) as suspicious.

At 28 DPR the highest % of positive samples was observed with the BOMMELI and CEDI kits (33.33%) kits The PANAFTOSA gave 22.22% (27.78% without 3ABC-EITB system). The lowest % was observed with UBI (5.56%).

At 84 DPR the highest level of positive samples was observed with the CEDI test (31.25%). The UBI test gave 12.50% and the lowest positive percentage was observed with BOMMELI and PANAFTOSA systems of 6.25% (same result using the 3ABC-EITB system).

At 112 DPR the highest positive sample number was still observed with the CEDI kits (11.11% of positive), followed by BOMMELI and PANAFTOSA with 5.56% (same result using the 3ABC-EITB system) and the UBI kits did not detect any positive sample. The results of the 4 ELISA kits and the samples related to commercial vaccine B are presented in Fig. 6.

**COMMERCIAL VACCINE  B  Batch 005/02**
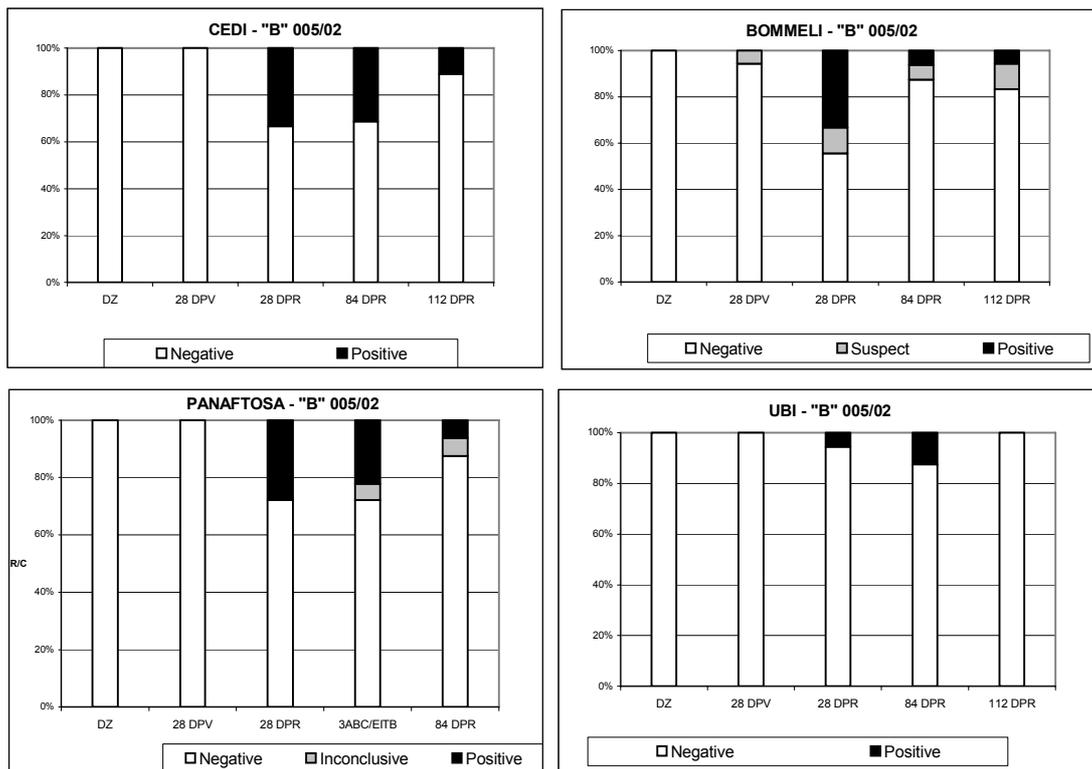


FIG. 6. Comparison of kits assessing antibodies against NSP after vaccine batch B.

For the commercial vaccine C (batch 010/02) at day 0 the results were the same (100% negative) in all 4 kits

At 28 DPV only the CEDI test detected some antibodies (11.11% positive). One sample (5.56%) was suspect and this was detected as positive using the BOMMELI test.

At 28 DPR the highest percentage of positive samples was observed with CEDI and PANAFTOSA kits at 22.22% using 3ABC-EITB system (38.89% without system). The BOMMELI detected 11.11% positive samples. All samples were negative using the UBI kit.

At 84 DPR only the PANAFTOSA system detected one positive sample (5.56% using 3ABC EITB system). BOMMELI did detect 16.67% as suspect only.

At 112 DPR only one sample was positive (5.56%) using the BOMMELI kits. In all others 3 kits all the samples were negative. The ELISA results related to commercial vaccine C batch 010/02 are presented in Fig. 7.

**COMMERCIAL VACCINE  C  Batch 010/02**
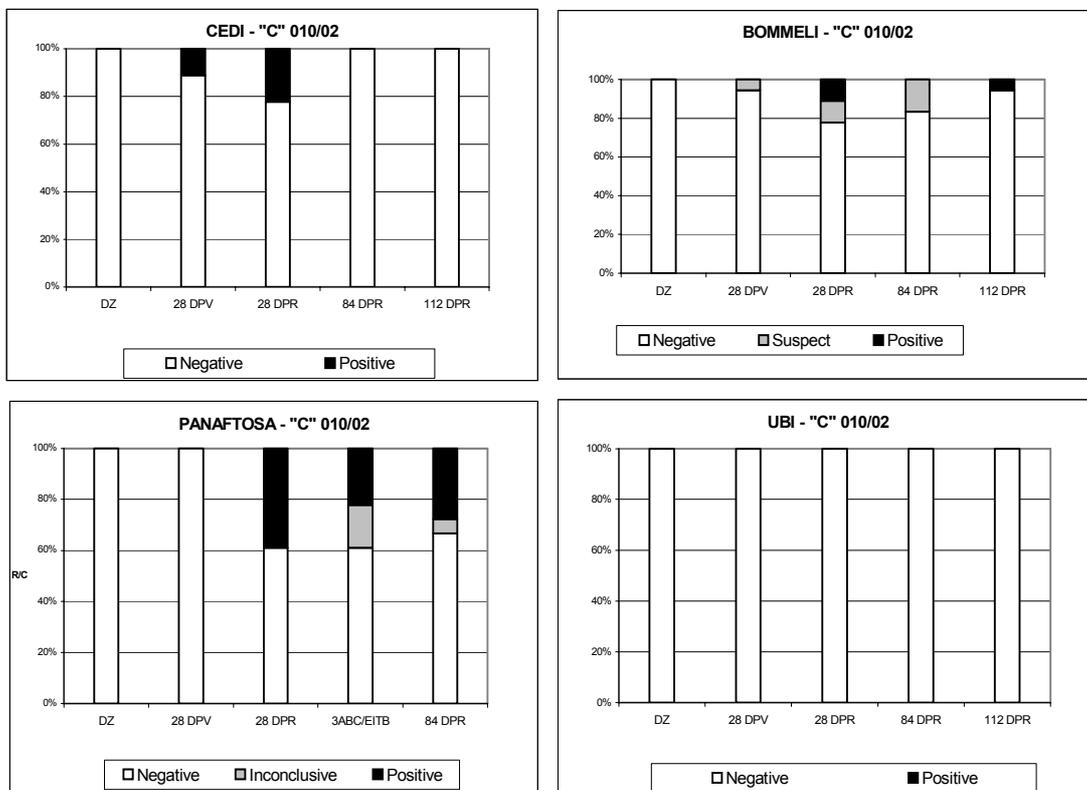


FIG. 7. Comparison of kits assessing antibodies against NSP vaccine batch C.

For the commercial vaccine C (batch 034/01) at day 0 all samples were negative in all 4 kits.

At 28 DPR CEDI kits did detect 5.56% as positive; all the other kits showed the same result with 100% as negative samples.

At 84 DPR only the BOMMELI kits detected some positive sample (5.56%). PANAFTOSA detected 5.56% as an inconclusive and 100% negative when 3ABC-EITB system.

At 112 DPR BOMMELI kits still had the same positive percentage (5.56%) as the previous sampling and in the others 3 kits, all samples were negative. The ELISA results related to commercial vaccine C batch 034/01 are presented in Fig. 8.

**COMMERCIAL VACCINE  C Batch 034/01**
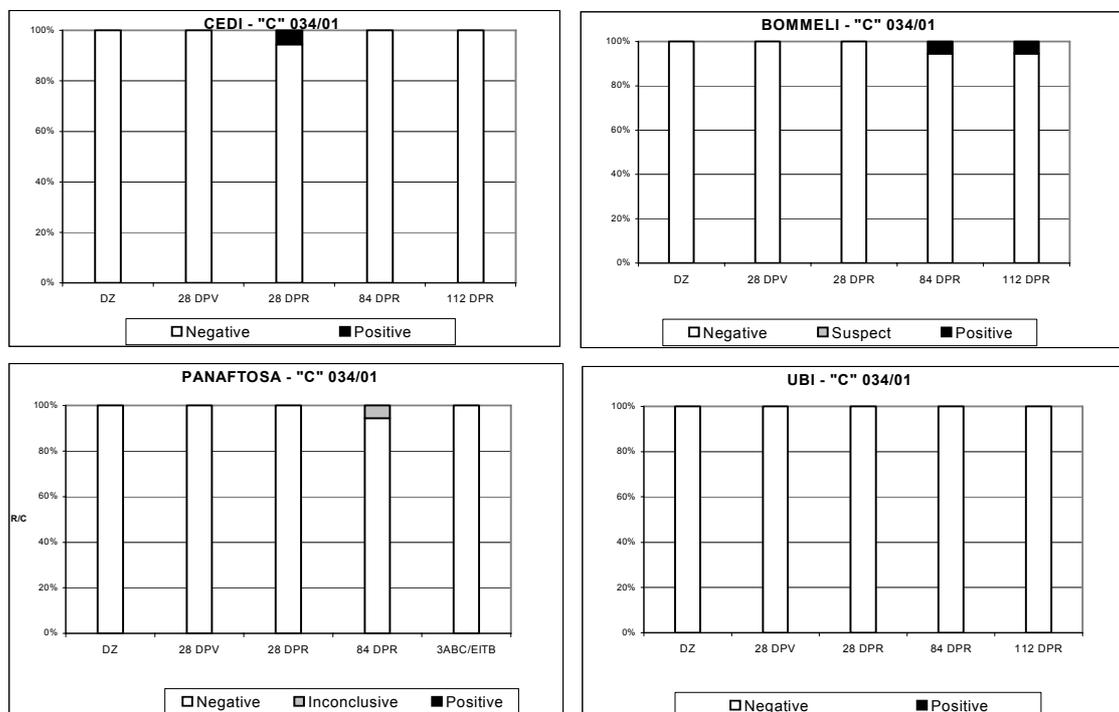


FIG. 8. Comparison of kits assessing antibodies against NSP with vaccine batch C.

For the commercial vaccine D at day 0, all samples were 100% negative.

At 28 DPV in the CEDI 16.67% of the samples were positive. The BOMMELI kits showed 11.11% positive which was the same result observed in PANAFTOSA but when the ELISA 3ABC-EITB system was used, the final percentage of positive was 5.6%. All samples were negative in UBI kits.

At 28 DPR the highest positive percentage was observed in CEDI kits (83.3%) and PANAFTOSA kits, but when the ELISA 3ABC-EITB system where used the final percentage of positive was 61.1%. The BOMMELI kits showed 72.2% positive samples and the lowest level of positive percentage was observed with UBI kits with 22.2%.

At 84 DPR the PANAFTOSA ELISA gave 61.11% positive. However, when the ELISA 3ABC-EITB system was used the final percentage of positive was 11.11%. CEDI gave 55.6% of positive and BOMMELI 16.7%. The positive percentage observed in UBI was 11.1%.

At 112 DPR the highest number of positive samples (44.44%) was observed in PANAFTOSA but when the ELISA 3ABC-EITB system was used the final percentage of positive was zero and 27.8% were classified as an inconclusive samples. CEDI kits gave 27.78% as positive samples, BOMMELI 16.7% also positives and the lowest positive percentage was observed in UBI with 5.6%. The ELISA results related to commercial vaccine D are presented in Fig. 9.

**COMMERCIAL VACCINE  D Batch 006/02**
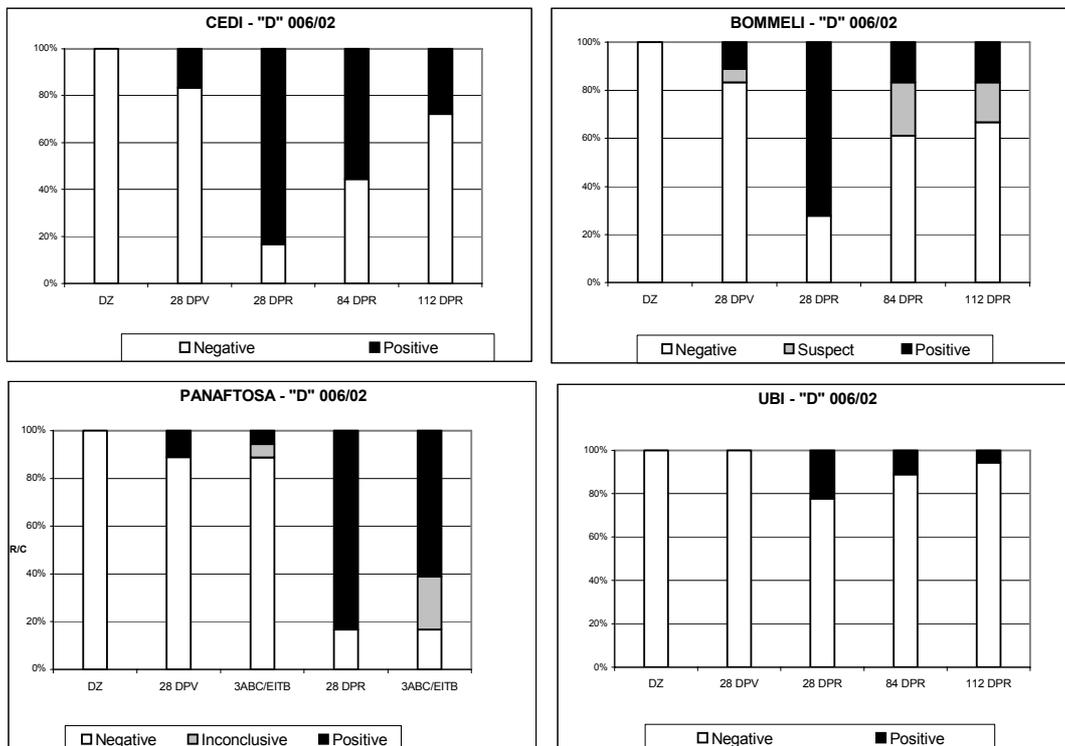


*FIG. 9. Comparison of kits assessing antibodies against NSP with vaccine batch D.*

In relation to the two bovines without vaccination used as controls in the CEDI, BOMMELI and PANAFTOSA kits, the results were negative in all sampling periods. For the UBI, both samples were negative on day zero, 28 DPV, 28 DPR and 112 DPR. At 84 DPR one control was positive. This sample was retested several times and the result was always the same. (Fig.10).

**VACCINE CONTROL**
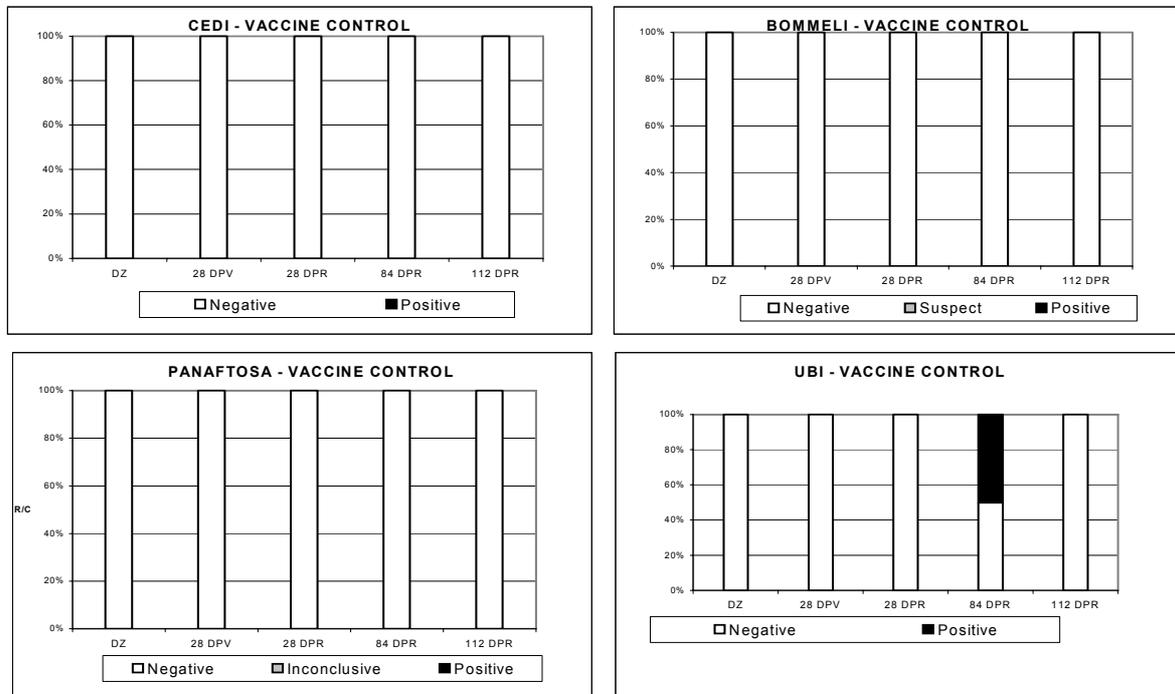


*FIG. 10. Comparison of commercial kits assessing negative population.*

## 7. DISCUSSION AND CONCLUSIONS

Results from analysis of the first outbreak in animals which had shown clinical signs and were not vaccinated; the performance of 3 kits was similar in diagnostic sensitivity (positive percentage was 100%). For the final result of PANAFTOSA using the ELISA 3ABC-EITB system the percentage decrease to 92.31% since one positive sample was shown to be inconclusive in the EITB. The CEDI tests had the lowest diagnostic sensitivity of 84.61%.

For the second outbreak where only one animal showed clinical signs the sensitivity performance ranged from BOMMELI 92.86% to UBI 57.15% positive. CEDI and PANAFTOSA had the intermediary sensitivity both of 78.58%. For the final result of PANAFTOSA using the ELISA 3ABC-EITB system the percentage decreased to 71.42% since one positive sample was negative in EITB. Thus where one is considering the detection of the most positive animals in an infected herd the BOMMELI test was most sensitive test, followed by PANAFTOSA, CEDI and UBI.

The results of the negative samples showed that 3 kits CEDI, PANAFTOSA and UBI were similar with a high specificity (100% of negative samples). The specificity of BOMMELI was 88.58%.

The CEDI kits showed the highest relative analytical sensitivity as shown from results on titration of a pool of positive samples. The BOMMELI and PANAFTOSA tests were lower. The UBI did not any antibodies in the sample. This result indicates possible reasons why the cut off levels set by the producers affect the diagnostic sensitivity obtained with the outbreak situations.

For commercial vaccines all 4 ELISA kits detected some antibodies following vaccination at 28 DPV where the highest levels were observed. The level of reactivity was different among the commercial vaccines and even among batches of the same commercial vaccine. All the animals at d 0 were negative. UBI kits showed the lowest detection rate of antibodies following vaccination, indicating that the specific antigen target in the test (3B) is not recognised as well as other NSP components. Presumably there is a differential production of specific antibodies with the contaminating NSP in vaccines during manufacture. It could also and that the antigenicity of the 3B is low as compared to other NSP The BOMMELI ELISA detected the highest number of animals as positive following vaccination. CEDI and PANAFTOSA showed an intermediary position in relation to this interference. This may reflect the lower specificity of the BOMMMELI test.

The use of the NSP in FMDV ELISA tests was shown to be very efficient in detecting positive cattle in an infected herd. Although some post vaccine antibodies could interfere in the results, the NSP ELISA for FMDV is a very good tool for serological surveys. Once the peak of reactivity caused by vaccination is known it is possible to establish the best period after vaccination to sample animals in order to minimize any vaccine interference. Besides, all positive samples in a vaccinated or non-vaccinated herd must pass a complementary tests and for a fully epidemiological investigation before giving a final result. The use of more highly purified vaccines is very important to help the use of the NSP ELISA kits to achieve good results. Since these results on vaccines in the year 2002, there has been a marked improvement in vaccine quality as far as contamination with NSP. Tests on vaccinated animals more recently indicate far less NSP antibody positive animals. The MAPA and the vaccine producers are working to provide vaccines with better NSP purification and at the same time with good potency to give better immune protection with the lowest induction of antibodies to FMD NSP.

## ACKNOWLEDGEMENTS

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**STUDIES USING TESTS TO DETERMINE ANTIBODIES AGAINST NON-STRUCTURAL PROTEINS OF FOOT AND MOUTH DISEASE IN COLOMBIA**

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**Abstract**

Colombia is a country with free areas without vaccination; free areas with vaccination and endemic areas. Buffer regions also are well surveyed. The various geographical areas where the present vaccination and control policies are being made are being revised in the light of animal movement control and surveillance. The role of NSP antibody testing (fitness for purpose) is very pertinent. The laboratory involved in FMD control is capable of antigen detection through tissue culture antigen capture ELISA; CFT; PCR; probang testing; (carriers) and antibody testing through VNT, VIAA AGID; LPBE and various NSP tests including the PANAFTOSA system using a 3ABC ELISAs and IETB. So we have a complete system for any studies in cattle and pigs. The routine work includes surveillance of cattle both from zones with vaccination and without disease and also testing animals that are moved from buffer zones into vaccination but disease free areas. In 2003, 13,016 and 10,000 samples respectively for the two situations were examined. Results of testing from different populations and comparison of tests are made. Various numbers of sera were tested with a variety of tests including: United Biomedical Incorporated (UBI) NSP ELISA test Chekit-FMD-3ABC (then BOMMELI diagnostics) CEDI test FMDV-NSP (CEDI) I-ELISA-3ABC from PANAFTOSA (I-ELISA) Electroimmunetransfer blot (EITB) VIAA Immunodiffusion test (VIAA AGID).

## 1. INTRODUCTION

Foot-and-Mouth Disease (FMD) is one of the most feared diseases of domestic animals because of its high contagion, wide host range and damaging effects on livestock production and the general economy. It infects valuable farm animals namely cattle, pigs, sheep and goats; as well as all domestic and wild cloven-footed animals. Colombia is affected by O and A types of virus. Since 1997, when the FMD eradication programme was implemented in Colombia, the change in the epidemiological situation in the region emphasized the need for the development and application of rapid and accurate sero diagnostic approaches to assess residual infection-specific antibodies in a livestock population. In order to know the background of the population and detection of antibodies against non-structural proteins (3A, 3B, 2C, 3D and 3ABC) which, are induced only during infection and not upon vaccination, the laboratory used three ELISA tests.

## 2. MATERIALS AND METHODS

### 2.1. I-ELISA-3ABC (CPFA)

This technique used a 3ABC protein as antigen coated in ELISA plates. The steps followed were:

(1)   A 1/20 dilution of test or reference sera in blocking buffer is added to the plate.
(2)   Wash six times with phosphate buffer containing 0.05% Tween 20 (PBST).
(3)   Incubated for 30 min at $37^0$C.
(4)   Wash six times with PBST.
(5)   Add optimal dilution rabbit antibovine IgG peroxidase conjugate in blocking buffer.
(6)   Incubate 30 min at $37^0$C.
(7)   Washed six times with PBST
(8)   Detect using TMB + $H_2O_2$ in phosphate-citrate buffer
(9)   Colour development stopped after 15 min by addition of $H_2SO_4$
(10)  Read absorbance of each well using a dual filter and the wavelength should be within the range of 450 to 620 nm.

*2.1.1. Performance criteria*

For the test system to be valid the following performance criteria were applied. The absorbance of negative controls should be <0.10 after correction for absorption blank wells. The cut-off serum should give absorbance values of 0.15–0.40. Results were expressed as an index derived by dividing the absorbance value of the serum tested (T) by that of the cut-off control (C). The ratio of the weak positive/cut-off should be 2.5 with a coefficient of variation <20%.

Using this ratio the test sera were classified:

- As non-reactive (T/C <0.8)
- Suspect or Indeterminate (0.8<T/C1.0)
- Reactive (T/C> 1.0).

The assay was used as a single dilution screening test. Sera tested as indeterminate and reactive will be run by EITB assay.

## 2.2.   Enzyme-Linked Immunoelectrotransfer Blot (EITB)

The EITB assay detects the binding of specific antibodies to five expressed non-structural proteins of FMD as serological probes. Using only a small amount of serum, the assay detects antibodies against polypeptides 3A, 3B, 2C, 3D and 3ABC, produced by the virus while it replicates in the host

For this study the I-ELISA-3ABC was used only in cattle, and the EITB assay was used in bovines, swine and sheep.

## 2.3.   UBI FMDV NS ELISA

This uses synthetic peptides as the solid-phase immunosorbent for detection of antibodies to infectious FMDV. The peptide mixture contains site-specific antigenic determinants taken from immunoreactive domains of NSP FMDV proteins. They impart the assay with excellent sensitivity; however, the site-specific synthetic peptides of the UBI FMDV NSP EIA (RUMINANT and SWINE) do not posses the serological cross reactivities to vaccine serum samples that have been observed for other NSP polypeptide antigens derived from virus or recombinant organisms. This means that the UBI FMDV NSP EIA is less reactive with vaccines specimens. The use of these synthetic peptides also minimizes the incidence of non- specific reactions originating from antibody reactivities in the specimen towards host cell antigens or expression vector antigens which are co-purified with virus derived or recombinant derived antigens.

## 2.3.1. Criteria for valid assay run

Ensure that the mean absorbance value of the NON-REACTIVE controls (NRL) is <0.2. If in the mean of the NRC is not within this range, the assay is invalid and must be repeated.

Ensure that the FMDV NSP REACTIVE control values (RC) are >0.400, and within the linear response range of the microplate reader. If individual RC values are not within this range, that assay is invalid.

## 2.4.    Samples

Sera represented populations of:

- Non-vaccinated, non infected cattle
- Vaccinated non infected cattle
- Sera from cattle in the field
- Carrier cattle.

TABLE I. RESULTS OF TESTING COLOMBIAN CATTLE SERA

| Stratum | Number | Tests | | | | |
|---|---|---|---|---|---|---|
| | | PANAFTOSA/ EITB | UBI | BOM | CEDI | AGID VIAA |
| Non vaccinated | 45 | 0 100% | 0 100% | 1 97.7% | 1 97.7% | 0 100% |
| Field Sera | 137 | 71 51.8% | 29 21% | 11 8.02% | 74 54.0% | 18 13.1% |
| Carrier | 1 | 1 | 1 | 0 | 1 | 0 |
| Multiple Vaccinated | 42 | 1 97.6% | 1 97.6% | 1 97.6% | 2/ 95.2% | 0 100% |

## 2.5.    Earlier work

This demonstrated the use of the PANAFTOSA system (ELISA and IETB). The Brescia and UBI tests available at the time were also used to analyse a variety of cattle and pig (including wild boar) sera.

## 2.5.1. Analysis of cut off values for different populations

The given cut-off levels for positivity were determined through experimental sera in the producers laboratories. The populations defined in Colombia were used to assess and revise the cut off levels for Colombian conditions

## 2.5.2. Post vaccination cattle, bled between 0 and 180 d following vaccination

Table II show the results of testing post vaccination sera by the UBI kit. Animals were taken from a disease free zone and then vaccinated.

TABLE II. UBI ELISA ON CATTLE, BLED AT 0 AND UP TO 180 D POST VACCINATION

| Number | 210 |
|---|---|
| Mean OD | 0.090 |
| Standard Error | 0.005 |
| Standard Deviation | 0.031 |
| Sample Variance | 0.0009 |
| Skewness | 1.83 |
| Range | 0.13 |
| Minimum | 0.06 |
| Maximum | 0.19 |

A mean value of 0.090 +/- 0.062 (2 x SD) is indicated as cut off for negativity.

### 2.5.3. Examination of non two x vaccinated area cattle

Table III shows the results of testing sera from cattle that had not been vaccinated.

TABLE III. UBI ELISA ON CATTLE NEVER VACCINATED

| Number | 200 |
|---|---|
| Mean OD | 0.086 |
| Standard Error | 0.002 |
| Standard Deviation | 0.020 |
| Sample Variance | 0.0004 |
| Skewness | 1.875 |
| Range | 0.01 |
| Minimum | 0.06 |
| Maximum | 0.16 |

A mean value of 0.086 +/- 0.040 (2 x SD) is indicated as cut off for negativity.

Although there is a slight increase in the mean and variation for the vaccinated population as compared to the non vaccinated, this is not statistically significant. A mean of 0.09 and 2 x *SD* of 0.06 can be taken as representative of a negative population distribution. The upper limit for a negative then would be 0.21.

### 2.5.4. Post challenge results using UBI kit

Table IV shows the data after analysing 2 cows experimentally infected with FMD using the UBI kit.

TABLE IV. DATA FROM POST INFECTED COWS USING UBI NSP TEST

| | **Optical Density (OD) in test** | |
|---|---|---|
| Days PI | Cow 1 | Cow 2 |
| 0 | 0.098 | 0.093 |
| 3 | 0.109 | 0.085 |
| 4 | 0.086 | 0.081 |
| 5 | 0.085 | 0.077 |
| 6 | 0.141 | 0.076 |
| 7 | 0.127 | 0.104 |
| 8 | 0.126 | 0.135 |
| 9 | 0.185 | 0.441 |
| 10 | 0.33 | 0.77 |

| | Optical Density (OD) in test | |
|---|---|---|
| 11 | 0.553 | 1.71 |
| 12 | 0.562 | 0.583 |
| 13 | 1.25 | 1.303 |
| 14 | 0.872 | 1.38 |
| 15 | 0.822 | 1.252 |
| 16 | 0.751 | 1.276 |
| 17 | 0.718 | 1.378 |
| 18 | 0.734 | 1.395 |
| 19 | 0.427 | 1.191 |
| 20 | 0.548 | 1.349 |
| 21 | 0.478 | 1.366 |
| 22 | 0.559 | 1.152 |
| 23 | 0.701 | 1.071 |
| 24 | 0.647 | 1.189 |
| 25 | 0.557 | 1.03 |
| 26 | 0.596 | 0.681 |
| 27 | 0.62 | 1.225 |
| 28 | 0.511 | 1.037 |
| 29 | 0.619 | 1.004 |
| 30 | 0.482 | 0.984 |

The grey boxes indicate where the OD values are above the upper negativity limits established from the negative populations.

### 2.5.5. Assessment of controls in UBI kit

The given controls in the UBI test on 10 plates were assessed statistically and results are shown in Table V.

TABLE V. ASSESSMENT OF PLATE DATA FROM 10 PLATES USING UBI TEST

| Plate | C- | C+ |
|---|---|---|
| 1 | 0.072 | 0.83 |
| 1 | 0.066 | 0.84 |
| 2 | 0.088 | 1.039 |
| 2 | 0.084 | 1.028 |
| 3 | 0.073 | 0.638 |
| 3 | 0.008 | 0.609 |
| 4 | 0.073 | 0.927 |
| 4 | 0.071 | 0.881 |
| 5 | 0.070 | 1.016 |
| 5 | 0.068 | 0.948 |
| 6 | 0.062 | 0.632 |
| 6 | 0.064 | 0.588 |
| 7 | 0.066 | 0.834 |
| 7 | 0.058 | 0.753 |
| 8 | 0.064 | 1.068 |
| 8 | 0.065 | 0.953 |
| 9 | 0.073 | 0.722 |
| 9 | 0.060 | 0.563 |

| Plate | C- | C+ |
|---|---|---|
| 10 | 0.061 | 1.194 |
| 10 | 0.068 | 0.478 |
| Mean | 0.069 | 0.821 |
| Standard Deviation | 0.0078 | 0.197 |
| Median | 0.068 | 0.837 |
| Standard Error | 0.00175 | 0.044 |

*2.5.6. Analysis of pig populations by UBI test*

Two pig populations suspected as free from FMD were examined by the UBI test. The statistics are shown in Table VI.

TABLE VI. ANALYSIS OF DISEASE FREE PIGS BY UBI TEST

| | |
|---|---|
| Mean OD | 0.102 |
| Standard Deviation | 0.029 |
| Standard Error | 0.006 |
| Median | 0.095 |
| Mode | 0.087 |
| Population 2 | |
| Mean | 0.110 |
| Standard Deviation | 0.040 |
| Standard Error | 0.0040 |
| Median | 0.112 |
| Mode | 0.05 |

## 2.6. Use of ELISA and immunoblotting in testing sera

Illustrative results of the use of NSP ELISAs and IETB are given in the Figures 1–28.

The figures have common abbreviations as follows:

| | |
|---|---|
| PID | Post-inoculation days |
| EITB | Electroimmunotransfer blot |
| UBI | United Biomedical. Inc. ELISA Test |
| P/B | Pirbright/Brescia ELISA Test |
| IDGA | Immunodiffusion gel agar test (VIAA) |
| N | Negative |
| P | Positive |
| NR | Non reactive |
| R | Reactive |
| CPFA | Pam American Foot-and-Mouth disease Center I-ELISA-3ABC |
| I | Indeterminate or suspect |
| np | No process |
| Serol 1/4 | Convalescent serum |
| Mix A | Infected from one year OP +, VIAA + |
| Mix D | Epidemiology Control2 years OP - VIAA |
| Mix F | Cut off Import/Export |
| DPI | Days post infection |

| PID | 3A 3B | 2C | 3D 3ABC | UBI | P/B | IDGA |
|-----|-------|----|---------|-----|-----|------|
| 0 | | | | NR | N | N |
| 1 | | | | NR | N | N |
| 2 | | | | NR | N | N |
| 3 | | | | NR | N | N |
| 4 | | | | NR | N | N |
| 5 | | | | NR | N | N |
| 6 | | | | NR | N | N |
| 7 | | | | R | N | P |
| 12 | | | | R | P | P |
| 13 | | | | R | P | P |
| 14 | | | | R | P | P |
| 15 | | | | R | P | P |
| 16 | | | | R | P | P |
| 17 | | | | R | P | P |
| 18 | | | | R | P | P |
| 19 | | | | R | P | P |
| 20 | | | | R | P | P |
| 21 | | | | R | P | P |
| 22 | | | | R | P | P |
| 23 | | | | R | P | P |
| 24 | | | | R | P | P |
| 25 | | | | R | P | P |
| 26 | | | | R | P | P |
| 27 | | | | R | P | P |
| 28 | | | | R | P | P |
| 29 | | | | R | P | P |
| 30 | | | | R | P | P |
| 60 | | | | R | P | P |

**PIG 1 inoculated with FMDV Type O**

← Blot positive / UBI and Agar gel +
← Definite detection of NSP
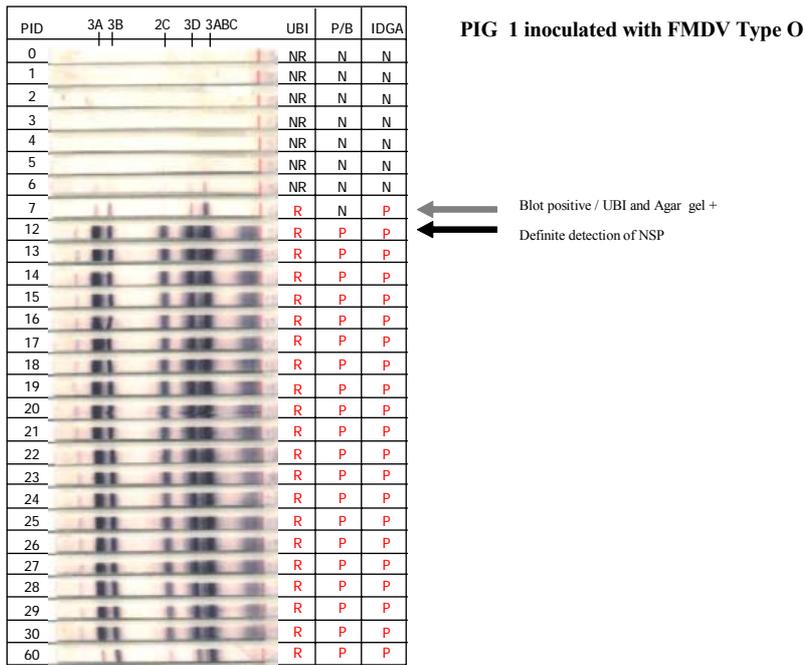
This serum were analyzed by EITB, ELISA-UBI, ELISA-Pirbright/Brescia and Immunodiffusion Agar Test.

The pig was inoculated by via the intradermal footpad with FMDV type O, and sampled daily from 0 d up to 30 d and then up to 60 d. The UBI is most sensitive detecting positivity at 7d, equivalent to the blotting technique. Note that the antibodies are detected by all techniques until 60d.

*FIG. 1. Analysis of response in single pig following infection.*

Gray arrow shows the earliest detection of NSP proteins by blotting. Note ELISA results.

**PIG inoculated with FMDV Type O**

| PID | UBI | R/B | IDGA |
|---|---|---|---|
| 1 | NR | N | N |
| 2 | NR | N | N |
| 3 | NR | N | N |
| 4 | NR | N | N |
| 5 | NR | N | N |
| 6 | NR | N | N |
| 7 | R | N | N |
| 8 | R | N | P |
| 9 | R | np | P |
| 10 | R | np | P |
| 11 | R | np | P |
| 12 | R | np | P |
| 13 | R | P | P |
| 14 | R | P | P |
| 15 | R | P | P |
| 16 | R | P | P |
| 17 | R | P | P |
| 18 | R | P | P |
| 19 | R | P | P |
| 20 | R | P | P |
| 21 | R | P | P |
| 22 | R | P | P |
| 23 | R | P | P |
| 24 | R | P | P |
| 25 | R | P | P |
| 26 | R | P | P |
| 27 | R | P | P |
| 28 | R | P | P |
| 29 | R | P | P |
| 30 | R | P | P |
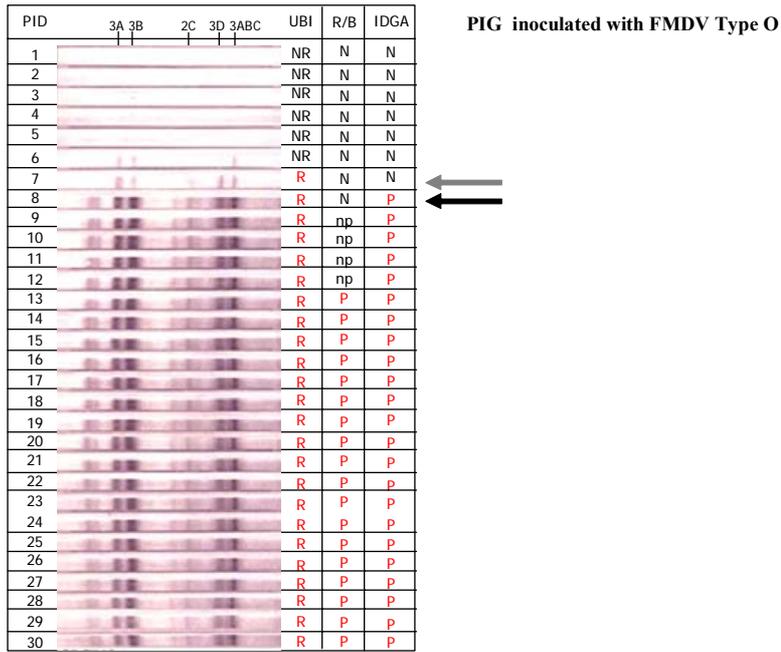
This pig was inoculated by via intradermal pad with FMDV type O, and bled daily from 0 d up to 30 d.

*FIG. 2. Analysis of response in single pig following infection.*

The quantity of specific anti NSP antibodies at day 8 is enough to be detected by the ELISA. The blotting test detects positive animal a day earlier.

| PID | 3A 3B | 2C | 3D 3ABC | UBI | P/B | IDGA |
|-----|-------|-----|---------|-----|-----|------|
| 0 | | | | NR | N | N |
| 1 | | | | NR | N | N |
| 2 | | | | NR | N | N |
| 3 | | | | NR | N | N |
| 4 | | | | NR | N | N |
| 5 | | | | NR | N | N |
| 6 | | | | R | N | N |
| 7 | | | | R | P | P |
| 8 | | | | R | N | P |
| 9 | | | | R | P | P |
| 10 | | | | R | P | P |
| 11 | | | | R | P | P |
| 12 | | | | R | P | P |
| 13 | | | | R | P | P |
| 14 | | | | R | P | P |
| 15 | | | | R | P | P |
| 16 | | | | R | P | P |
| 17 | | | | R | P | P |
| 18 | | | | R | P | P |
| 19 | | | | R | P | P |
| 20 | | | | R | P | P |
| 21 | | | | R | P | P |
| 22 | | | | R | P | P |
| 23 | | | | R | P | P |
| 24 | | | | R | P | P |
| 25 | | | | R | P | P |
| 26 | | | | R | P | P |
| 27 | | | | R | P | P |
| 28 | | | | R | P | P |
| 29 | | | | R | P | P |
| 30 | | | | R | P | P |
| 60 | | | | R | P | P |
| 90 | | | | R | P | P |
| 120 | | | | R | P | P |

**Pig inoculated with FMDV Type O**
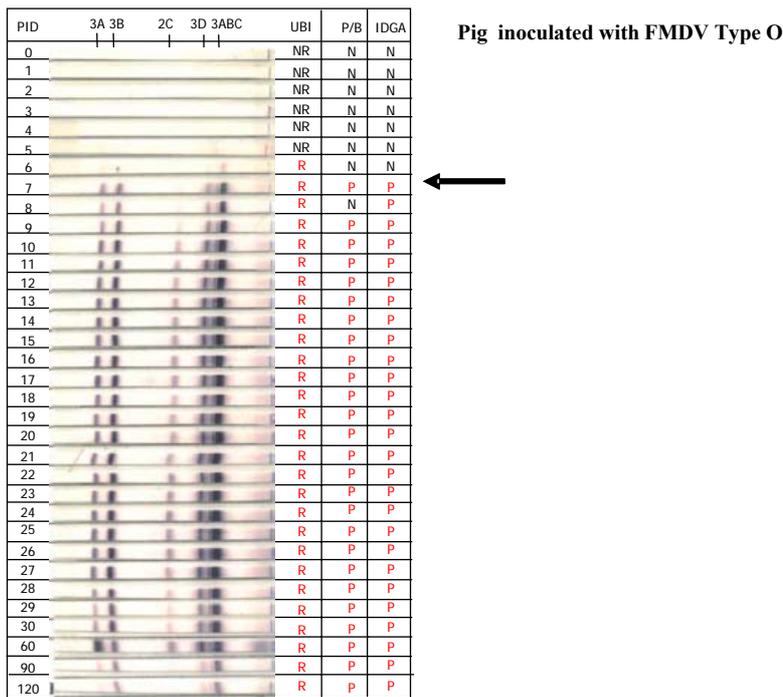
This pig was inoculated by via intradermal pad with FMDV type O then bled daily from day 0 to 30 and through days 60, 90 and 120d post-inoculation.

*FIG. 3. Analysis of response in single pig following infection.*

The UBI was most sensitive (by 1d) compared to P/b test. Is it important to point out that the EITB technique the 2C protein show a signal from 10d to 60d which then disappears 90d and 120d post-contact. Note that the ELISAs are positive at 90 and 120d where there is a weakening of the lines in the blotting technique.
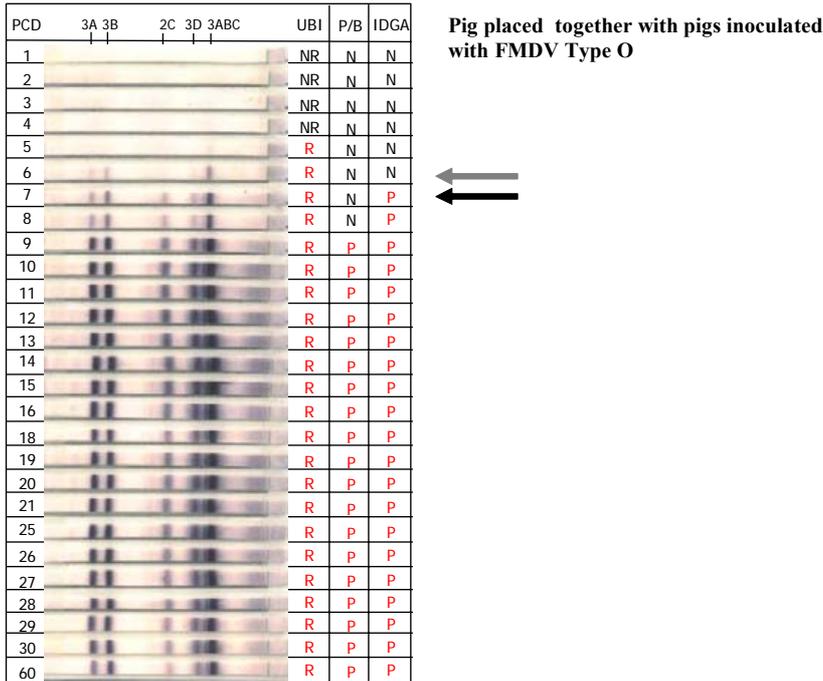
| PCD | 3A 3B | 2C 3D 3ABC | UBI | P/B | IDGA | **Pig placed together with pigs inoculated with FMDV Type O** |
|-----|-------|------------|-----|-----|------|---|
| 1 | | | NR | N | N | |
| 2 | | | NR | N | N | |
| 3 | | | NR | N | N | |
| 4 | | | NR | N | N | |
| 5 | | | R | N | N | |
| 6 | | | R | N | N | ← |
| 7 | | | R | N | P | ← |
| 8 | | | R | N | P | |
| 9 | | | R | P | P | |
| 10 | | | R | P | P | |
| 11 | | | R | P | P | |
| 12 | | | R | P | P | |
| 13 | | | R | P | P | |
| 14 | | | R | P | P | |
| 15 | | | R | P | P | |
| 16 | | | R | P | P | |
| 18 | | | R | P | P | |
| 19 | | | R | P | P | |
| 20 | | | R | P | P | |
| 21 | | | R | P | P | |
| 25 | | | R | P | P | |
| 26 | | | R | P | P | |
| 27 | | | R | P | P | |
| 28 | | | R | P | P | |
| 29 | | | R | P | P | |
| 30 | | | R | P | P | |
| 60 | | | R | P | P | |

This pig was placed in contact with others pigs inoculated by via intradermal pad with FMDV type O, and was bleed daily from 0 d to 30 d and then at 60 d. The UBI test is very sensitive in detecting positive response (5d) even where the blotting technique is negative. The P/B ELISA is negative until 9 d even though the blotting shows high antibodies against all NSP proteins from 7 d.

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*FIG. 4. Analysis of response in single pig following contact with infected pigs.*
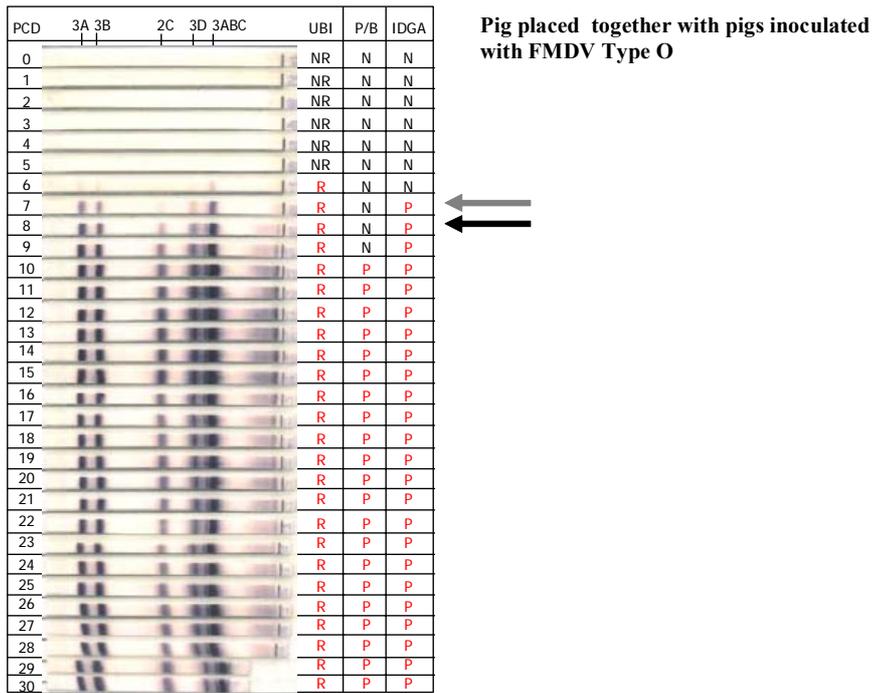
**Pig placed together with pigs inoculated with FMDV Type O**

| PCD | 3A 3B 2C 3D 3ABC | UBI | P/B | IDGA |
|-----|------------------|-----|-----|------|
| 0 | | NR | N | N |
| 1 | | NR | N | N |
| 2 | | NR | N | N |
| 3 | | NR | N | N |
| 4 | | NR | N | N |
| 5 | | NR | N | N |
| 6 | | R | N | N |
| 7 | | R | N | P |
| 8 | | R | N | P |
| 9 | | R | N | P |
| 10 | | R | P | P |
| 11 | | R | P | P |
| 12 | | R | P | P |
| 13 | | R | P | P |
| 14 | | R | P | P |
| 15 | | R | P | P |
| 16 | | R | P | P |
| 17 | | R | P | P |
| 18 | | R | P | P |
| 19 | | R | P | P |
| 20 | | R | P | P |
| 21 | | R | P | P |
| 22 | | R | P | P |
| 23 | | R | P | P |
| 24 | | R | P | P |
| 25 | | R | P | P |
| 26 | | R | P | P |
| 27 | | R | P | P |
| 28 | | R | P | P |
| 29 | | R | P | P |
| 30 | | R | P | P |

This pig was placed in contact with others pigs inoculated by via intradermal pad with FMDV type O and bled daily from 0 d to 30 d. The P/B test is late in detecting the antibodies demonstrated by the blotting technique (at 6d very weakly then days 7 onwards. The UBI detects positives at 6 d then onwards.

*FIG. 5. Analysis of response in single pig following contact with infected pigs.*

| ID | CPFA | 3A | 3B | 2C | 3D | 3ABC | UBI | P/B | IDGA |
|----|------|----|----|----|----|------|-----|-----|------|
| Serol 1/4 | | | | | | | | | |
| Mix A | | | | | | | | | |
| Mix D | | | | | | | | | |
| Mix F | | | | | | | | | |
| T1 | R | | | | | | R | P | P |
| T2 | R | | | | | | R | P | P |
| T3 | R | | | | | | R | P | P |
| T4 | R | | | | | | R | P | P |
| T5 | R | | | | | | NR | N | N |
| T6 | R | | | | | | R | P | P |
| T7 | R | | | | | | NR | N | N |
| T8 | R | | | | | | R | P | P |
| T9 | R | | | | | | R | P | P |
| T10 | R | | | | | | R | P | P |
| T11 | R | | | | | | R | P | P |
| T12 | R | | | | | | R | P | P |
| T13 | R | | | | | | R | P | P |
| T14 | R | | | | | | R | P | P |
| T15 | R | | | | | | R | P | P |
| T16 | R | | | | | | R | N | P |
| T17 | R | | | | | | R | P | P |
| T18 | R | | | | | | R | P | P |
| T21 | R | | | | | | R | P | P |
| T22 | R | | | | | | R | P | P |
| T23 | R | | | | | | R | P | P |

OUTBREAK OF FMDV TYPE O IN CALVES

Farm: CALIFORNIA
Town: GIRARDOT
State : CUNDINAMARCA
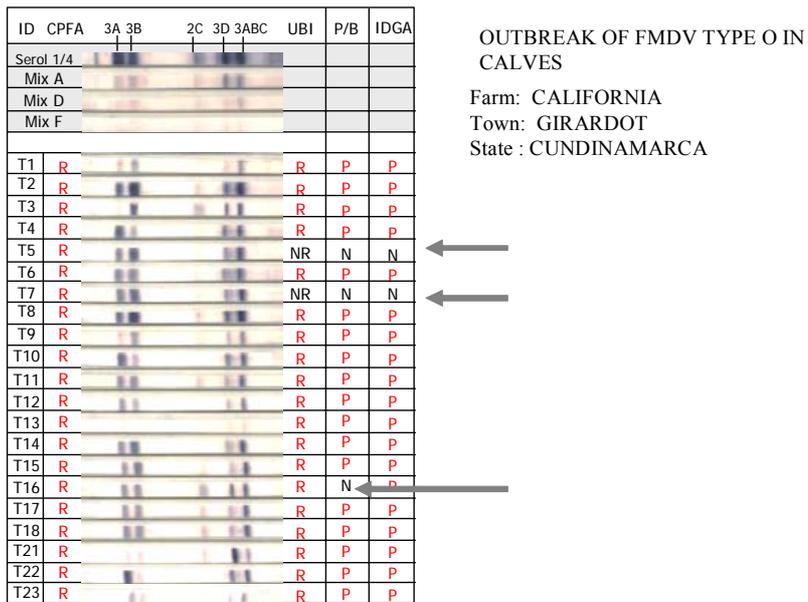
This farm had 170 non vaccinated' and 90 FMDV type O infected cattle (calves). The animals were bled 90 d post-outbreak. Gray arrows denote lack of ELISA positivity in face of blot positive result. In three cases the positive animals are not diagnosed positive (both ELISAs in 2 cases and the P/B in 1 case).

*FIG. 6. Analysis of responses from calves after outbreak of FMD.*

Note some failure in ELISAs to detect positive animals.

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| ID | CPFA | 3A 3B | 2C | 3D 3ABC | UBI | P/B | IDGA |
|---|---|---|---|---|---|---|---|
| Serol 1/4 | | | | | | | |
| Mix A | | | | | | | |
| Mix D | | | | | | | |
| Mix F | | | | | | | |
| N1 | R | | | | np | np | P |
| N2 | R | | | | R | np | P |
| N3 | R | | | | R | np | N |
| N4 | R | | | | R | np | N |
| N5 | R | | | | R | np | N |
| N6 | R | | | | R | np | P |
| N7 | R | | | | R | np | N |
| N8 | R | | | | R | np | N |
| N9 | R | | | | R | np | N |
| N11 | R | | | | R | np | N |
| N12 | R | | | | R | np | N |
| N13 | R | | | | R | np | P |
| N14 | R | | | | R | np | N |
| N15 | R | | | | R | np | P |
| N16 | R | | | | NR | np | N |
| N17 | R | | | | R | np | N |
| N18 | R | | | | R | np | P |
| N19 | R | | | | R | np | P |
| N20 | R | | | | R | np | P |
| N21 | R | | | | R | np | P |
| N22 | R | | | | R | np | P |
| N23 | R | | | | R | np | N |
| N24 | R | | | | R | np | P |
| N25 | R | | | | R | np | N |
| N26 | R | | | | R | np | N |

OUTBREAK OF FMDV TYPE O IN
HEIFERS


FARM: CALIFORNIA
TOWN: GIRARDOT
STATE: CUNDINAMARCA

These sera belong to heifers which were bled 90 days post-outbreak.

*FIG. 7. Analysis of response heifers following a FMD outbreak.*

# OUTBREAK OF FMDV TYPE O IN HEIFERS

**FARM: CALIFORNIA**
**TOWN: GIRARDOT**
**STATE: CUNDINAMARCA**

| ID | CPFA | 3A 3B | 2C | 3D 3ABC | UBI | P/B | IDGA |
|----|------|-------|----|---------|-----|-----|------|
| Serol 1/4 | | | | | | | |
| Mix A | | | | | | | |
| Mix D | | | | | | | |
| Mix F | | | | | | | |
| | | | | | | | |
| N27 | R | | | | R | np | N |
| N28 | NR | | | | NR | np | N |
| N29 | R | | | | R | np | N |
| N30 | R | | | | R | np | P |
| N31 | R | | | | R | np | P |
| N32 | R | | | | R | np | P |
| N33 | R | | | | R | np | P |
| N34 | R | | | | R | np | P |
| N35 | R | | | | R | np | P |
| N36 | R | | | | R | np | P |
| N37 | R | | | | np. | np | P |
| N38 | R | | | | np | np | P |
| N39 | R | | | | np | np | P |
| N40 | R | | | | np | np | N |
| N41 | R | | | | np | np | P |
| N42 | R | | | | np | np | N |
| N43 | R | | | | np | np | N |
| N44 | R | | | | np | np | P |
| N45 | R | | | | np | np | N |
| N46 | R | | | | np | np | N |
| N47 | R | | | | np | np | N |
| N48 | R | | | | np | np | P |
| N49 | R | | | | np | np | P |
| N50 | R | | | | np | np | N |
| N51 | R | | | | np | np | N |

These sera belong to heifers which were bled 90 d post-outbreak. As previously seen, the agar gel test is very insensitive (missing 10 sera clearly positive for NSP); the UBI data is incomplete, but agrees with the blotting data for 10 sera (9 positive and one negative).

*FIG. 8. Analysis of response heifers following a FMD outbreak.*

Note high failure of AGID to detect blot-positive animals.

| ID | CPFA | 3A | 3B | 2C | 3D | 3ABC | UBI | P/B | IDGA |
|----|------|----|----|----|----|------|-----|-----|------|
| Serol 1/4 | | | | | | | | | |
| Mix A | | | | | | | | | |
| Mix D | | | | | | | | | |
| Mix F | | | | | | | | | |
| | | | | | | | | | |
| N52 | R | | | | | | np | np | P |
| N53 | R | | | | | | np | np | N |
| N54 | R | | | | | | np | np | N |
| N55 | R | | | | | | np | np | P |
| N56 | R | | | | | | np | np | N |
| N57 | R | | | | | | np | np | N |
| N58 | R | | | | | | np | np | N |
| N59 | R | | | | | | np | np | P |
| N60 | R | | | | | | np | np | N |
| V1 | R | | | | | | R | np | P |
| V2 | R | | | | | | R | np | P |
| V3 | R | | | | | | R | np | P |
| V4 | R | | | | | | R | np | P |
| V5 | R | | | | | | R | np | P |
| V6 | R | | | | | | R | np | N |
| V7 | R | | | | | | R | np | P |

**OUTBREAK OF FMDV TYPE O IN HEIFERS AND COWS**

**FARM: CALIFORNIA**
**TOWN: GIRARDOT**
**STATE: CUNDINAMARCA**

These sera belong to heifers and cows which were bleed 90 d post-outbreak. Note that all are positive by blotting. The UBI data is incomplete but there is total agreement with blotting data for 6 sera (positive). The agar gel once again has a low diagnostic sensitivity (misses 7 out of 16 positive sera).

*FIG. 9. Analysis of response heifers following a FMD outbreak.*

Note high number blot positive animals are missed by AGID.

| ID | CPFA | 3A 3B | 2C | 3D 3ABC | UBI | P/B | IDGA |
|----|------|-------|----|---------|-----|-----|------|
| Serol 1/4 | | | | | | | |
| Mix A | | | | | | | |
| Mix D | | | | | | | |
| Mix F | | | | | | | |
| 1 | R | | | | R | np | P |
| 2 | R | | | | R | np | P |
| 3 | R | | | | R | np | P |
| 4 | R | | | | R | np | P |
| 5 | R | | | | R | np | P |
| 6 | R | | | | R | np | P |
| 7 | R | | | | R | np | P |
| 8 | R | | | | R | np | P |
| 9 | R | | | | R | np | P |
| 10 | R | | | | R | np | P |
| 11 | R | | | | R | np | P |
| 12 | R | | | | R | np | P |
| 13 | R | | | | R | np | P |
| 14 | R | | | | R | np | P |
| 15 | R | | | | R | np | P |
| B1 | NR | | | | R | np | P |
| B2 | R | | | | R | np | P |
| B3 | R | | | | R | np | P |
| B4 | R | | | | R | np | P |
| B5 | NR | | | | np | np | P |

**OUTBREAK OF FMDV TYPE O**

**FARM: LAS TECAS / BOTIJUELA.**
**TOWN: NECOCLÍ.**
**STATE: ANTIOQUIA**

This farm was affected by FMDV type O, animals recently infected. In the recent infection, there is a perfect correlation of blot and UBI ELISA results. Note also that the agar gel also detects all positive animals in contrast to the earlier results analysing sera from animals at later times post infection.

*FIG. 10. Analysis of animals from an affected farm.*

Here the UBI, AGID and blot results correlate exactly.

NATURALLY INFECTED BOVINES WITH FMDV

FARM: EL CEBO.
TOWN: ARENAL.
STATE: BOLIVAR
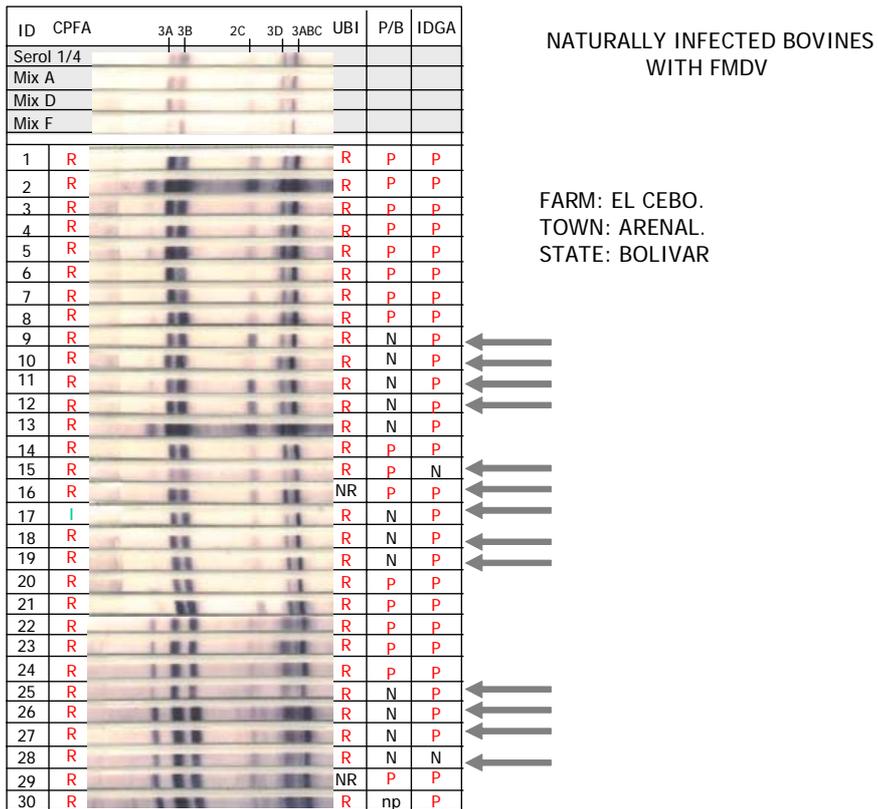
In this farm there were a high number of cattle showing old lesions (scars). Here there is a eider variation in results. The gray arrow denotes differences in test data. The P/B test has a low diagnostic sensitivity for these samples (missing 12 out of 30 sera found positive by other tests). The UBI detects 28 /30 positives found by blotting. The agar gel only misses 2 sera from the 30 found positive by blotting.

*FIG. 11. Analysis of animals showing old scar lesions.*

Note the differences in tests.

**EXPERIMENTAL INFECTED COW WITH FMDV TYPE O**

| DPI | CPFA | 3A 3B | 2C | 3D 3ABC | UBI | P/B | IDGA |
|---|---|---|---|---|---|---|---|
| Serol 1/4 | | | | | | | |
| Mix A | | | | | | | |
| Mix D | | | | | | | |
| Mix F | | | | | | | |
| 0 | NR | | | | NR | np | N |
| 3 | NR | | | | NR | np | N |
| 4 | NR | | | | NR | np | N |
| 5 | NR | | | | NR | np | N |
| 6 | NR | | | | NR | np | P |
| 7 | NR | | | | NR | np | P |
| 8 | I | | | | NR | np | P |
| 9 | P | | | | NR | np | P |
| 10 | P | | | | R | np | P |
| 11 | P | | | | R | np | P |
| 12 | P | | | | R | np | P |
| 13 | P | | | | R | np | P |
| 14 | P | | | | R | np | P |
| 15 | P | | | | R | np | P |
| 16 | P | | | | R | np | P |
| 17 | P | | | | R | np | P |
| 18 | P | | | | R | np | P |
| 19 | P | | | | R | np | P |
| 20 | P | | | | R | np | P |
| 21 | P | | | | R | np | P |
| 22 | P | | | | R | np | P |
| 23 | P | | | | R | np | P |
| 24 | P | | | | R | np | P |
| 25 | P | | | | R | np | P |
| 26 | P | | | | R | np | P |
| 27 | P | | | | R | np | P |
| 28 | P | | | | R | np | P |
| 29 | P | | | | R | np | P |
| 30 | P | | | | R | np | P |
| 31 | P | | | | R | np | P |

AGID VIAA positive?

Blot and ELISA positive

This 18 month, originally tested negative for antibodies against NSP, was inoculated with FMDV type O in the tongue with 10000 DICC, and was bled from 0 d to 31 d post inoculation. The blotting shows a later than expected development of antibodies (9 d). The agar gel in this case detected positive animal at 6 d

*FIG. 12. Response in a cow following infection with FMD.*

Note the AGID gives very early positive reactions.

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| ID | CPFA | 3A 3B | 2C | 3D 3ABC | UBI | P/B | IDGA |
|----|------|-------|----|---------|-----|-----|------|
| Serol 1/4 | | | | | | | |
| Mix A | | | | | | | |
| Mix D | | | | | | | |
| Mix F | | | | | | | |
| 1 | R | | | | R | N | N | ← |
| 2 | R | | | | R | P | P |
| 3 | R | | | | R | P | P |
| 4 | R | | | | R | P | P |
| 5 | R | | | | R | P | P |
| 6 | R | | | | R | P | P |
| 7 | R | | | | R | P | P |
| 8 | R | | | | R | P | P |
| 9 | R | | | | R | P | P |
| 10 | R | | | | R | P | P |
| 11 | R | | | | R | P | P |
| 12 | R | | | | R | N | P | ← |
| 13 | R | | | | R | P | P |
| 14 | R | | | | R | P | P |
| 15 | R | | | | R | P | P |
| 16 | R | | | | R | P | P |
| 17 | R | | | | R | P | P |
| 18 | R | | | | R | N | P | ← |
| 19 | R | | | | R | P | N | ← |
| 20 | R | | | | R | P | P |
| 21 | R | | | | R | P | P |

OUTBREAK OF FMDV TYPE O

**FARM: LAS VEGAS**
**TOWN: NECOCLÍ**
**STATE: ANTIOQUIA.**
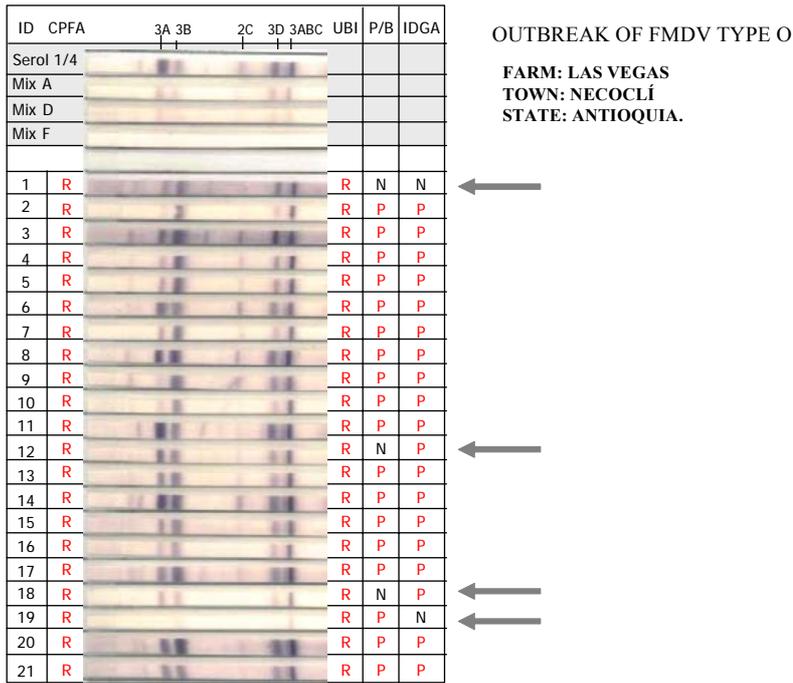
This farm was affected with FMDV type O. Note that the UBI test is in total agreement with the blot results, that the P/B test misses 3 positives and the agar gel test 2 positives.

*FIG. 13. Examination of cattle following FMD outbreak.*

Note discrepancies in results.

OUTBREAK OF FMDV TYPE O

FARM: LAS VEGAS
TOWN: NECOCLÍ
STATE: ANTIOQUIA.

Neighbouring farm

Sera from cattle in Las Vegas farm affected by FMD type O were
examined together with a neighboring farm no showing clinical signs.
This is interesting since the blotting techniques gives very weak positive
bands whereas the UBI ELISA show unambiguous positives in
neighbouring farm. Both tests agree for 1 serum as negative (PV2).

*FIG. 14. Cattle after FMD outbreak and neighbouring farm, animals showing no clinical signs.*

| PVD | CPFA | 3A 3B | 2C 3D 3ABC | UBI | P/B | IDGA |
|-----|------|-------|------------|-----|-----|------|
| Serol 1/4 | | | | | | |
| Mix A | | | | | | |
| Mix D | | | | | | |
| Mix F | | | | | | |
| **Suero: 1** | | | | | | |
| 0 | NR | | | NR | np | N |
| 30 | NR | | | NR | np | N |
| 60 | NR | | | NR | np | N |
| 90 | NR | | | NR | np | N |
| 120 | NR | | | NR | np | N |
| 150 | I | | | NR | np | N |
| 180 | NR | | | NR | np | N |
| 30* | NR | | | NR | np | N |
| **Suero: 2** | | | | | | |
| 0 | NR | | | NR | np | N |
| 30 | NR | | | NR | np | N |
| 60 | NR | | | NR | np | N |
| 90 | NR | | | NR | np | N |
| 120 | NR | | | NR | np | N |
| 150 | NR | | | NR | np | N |
| 180 | NR | | | NR | np | N |
| 30* | NR | | | NR | np | N |
| **Suero: 3** | | | | | | |
| 0 | NR | | | NR | np | N |
| 30 | NR | | | NR | np | N |
| 60 | NR | | | NR | np | N |
| 90 | NR | | | NR | np | N |
| 120 | NR | | | NR | np | N |
| 150 | NR | | | NR | np | N |
| 180 | NR | | | NR | np | N |
| 30* | NR | | | NR | np | N |
| **Suero: 4** | | | | | | |
| 0 | NR | | | NR | np | N |
| 30 | NR | | | NR | np | N |
| 60 | NR | | | NR | np | N |
| 90 | NR | | | NR | np | N |
| 120 | NR | | | NR | np | N |
| 150 | NR | | | NR | np | N |
| 180 | I | | | NR | np | N |
| 30* | NR | | | NR | np | N |
| **Suero: 5** | | | | | | |
| 0 | I | | | NR | np | N |
| 30 | NR | | | NR | np | N |
| 60 | NR | | | NR | np | N |
| 90 | NR | | | NR | np | N |
| 120 | NR | | | NR | np | N |
| 150 | NR | | | NR | np | N |
| 180 | NR | | | NR | np | N |
| 30* | NR | | | NR | np | N |

GROUP OF CATLE LESS THAN 2 Y VACCINATED AGAINST FMDV AND BLEEDED MONTHLY

FARM: LA PAZ
TOWN: ARBOLETES
STATE: ANTIOQUIA.

PVD = Post-vaccination days
* = After booster days

The objective of this study was to know if the vaccination against FMD caused cross reaction with the response to antibodies to non-structural proteins. In Total, 25 cattle less than 2 y old from an FMD free area-with vaccination were analysed. This group was vaccinated against FMD and bled monthly until 180 d post-vaccination and then they were revaccinated and bled 30 d later.

*FIG. 15. Examination of vaccinated and repeat vaccinated cattle.*

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| PVD | CPFA | 3A 3B | 2C | 3D 3ABC | UBI | P/B | IDGA |
|---|---|---|---|---|---|---|---|
| Serol 1/4 | | | | | | | |
| Mix A | | | | | | | |
| Mix D | | | | | | | |
| Mix F | | | | | | | |
| | Suero: 6 | | | | | | |
| 0 | NR | | | | NR | np | N |
| 30 | NR | | | | NR | np | N |
| 60 | NR | | | | NR | np | N |
| 90 | NR | | | | NR | np | N |
| 120 | NR | | | | NR | np | N |
| 150 | NR | | | | NR | np | N |
| 180 | NR | | | | NR | np | N |
| 30* | NR | | | | NR | np | N |
| | Suero: 7 | | | | | | |
| 0 | NR | | | | NR | np | N |
| 30 | NR | | | | NR | np | N |
| 60 | NR | | | | NR | np | N |
| 90 | NR | | | | NR | np | N |
| 120 | I | | | | NR | np | N |
| 150 | NR | | | | NR | np | N |
| 180 | NR | | | | NR | np | N |
| 30* | NR | | | | NR | np | N |
| | Suero: 8 | | | | | | |
| 0 | NR | | | | NR | np | N |
| 30 | NR | | | | NR | np | N |
| 60 | NR | | | | NR | np | N |
| 90 | NR | | | | NR | np | N |
| 120 | NR | | | | NR | np | N |
| 150 | NR | | | | NR | np | N |
| 180 | NR | | | | NR | np | N |
| 30* | I | | | | NR | np | N |
| | Suero: 9 | | | | | | |
| 0 | NR | | | | NR | np | N |
| 30 | NR | | | | NR | np | N |
| 60 | NR | | | | NR | np | N |
| 90 | NR | | | | NR | np | N |
| 120 | I | | | | NR | np | N |
| 150 | I | | | | NR | np | N |
| 180 | I | | | | NR | np | N |
| 30* | NR | | | | NR | np | N |

**GROUP OF BOVINES LESS THAN 2 YEARS VACCINATED AGAINST FMDV AND BLEEDED MONTHLY**

FARM: LA PAZ
TOWN: ARBOLETES
STATE: ANTIOQUIA.

PVD = post-vaccination days
* = After booster days

This group of cattle was bled from 0 d and then 30, 60, 90, 120, 150, 180 d and then animals were revaccinated then and then bled at 30 d. All are negative by all tests.

FIG. 16. Examination of vaccinated and repeat vaccinated cattle.

| PVD | CPFA | 3A | 3B | 2C | 3D | 3ABC | UBI | P/B | IDGA |
|---|---|---|---|---|---|---|---|---|---|
| Serol 1/4 | | | | | | | | | |
| Mix A | | | | | | | | | |
| Mix D | | | | | | | | | |
| Mix F | | | | | | | | | |
| Sera 10 | | | | | | | | | |
| 0 | NR | | | | | | NR | np | N |
| 30 | NR | | | | | | NR | np | N |
| 60 | I | | | | | | NR | np | N |
| 90 | NR | | | | | | NR | np | N |
| 120 | NR | | | | | | NR | np | N |
| 150 | NR | | | | | | NR | np | N |
| 180 | NR | | | | | | NR | np | N |
| 30* | NR | | | | | | NR | np | N |
| Sera 11 | | | | | | | | | |
| 0 | NR | | | | | | NR | np | N |
| 30 | NR | | | | | | NR | np | N |
| 60 | NR | | | | | | NR | np | N |
| 90 | NR | | | | | | NR | np | N |
| 120 | I | | | | | | R | np | N |
| 150 | NR | | | | | | NR | np | N |
| 180 | NR | | | | | | NR | np | N |
| 30* | NR | | | | | | NR | np | N |
| Sera 12 | | | | | | | | | |
| 0 | NR | | | | | | NR | np | N |
| 30 | NR | | | | | | NR | np | N |
| 60 | NR | | | | | | NR | np | N |
| 90 | NR | | | | | | R | np | N |
| 120 | NR | | | | | | NR | np | N |
| 150 | NR | | | | | | NR | np | N |
| 180 | NR | | | | | | NR | np | N |
| 30* | NR | | | | | | NR | np | N |
| Sera 13 | | | | | | | | | |
| 0 | NR | | | | | | NR | np | N |
| 30 | NR | | | | | | NR | np | N |
| 60 | NR | | | | | | NR | np | N |
| 90 | NR | | | | | | NR | np | N |
| 120 | NR | | | | | | NR | np | N |
| 150 | NR | | | | | | NR | np | N |
| 180 | NR | | | | | | NR | np | N |
| 30* | NR | | | | | | NR | np | N |
| Sera 14 | | | | | | | | | |
| 0 | NR | | | | | | NR | np | N |
| 30 | NR | | | | | | NR | np | N |
| 60 | NR | | | | | | NR | np | N |
| 90 | NR | | | | | | NR | np | N |
| 120 | NR | | | | | | NR | np | N |
| 150 | NR | | | | | | NR | np | N |
| 180 | NR | | | | | | NR | np | N |
| 30* | NR | | | | | | NR | np | N |

**GROUP OF BOVINES LESS THAN 2 Y VACCINATED AGAINST FMDV AND BLED MONTHLY**

**FARM: LA PAZ**
**TOWN: ARBOLETES**
**STATE: ANTIOQUIA.**

This group was bled from 0D and then AT 30, 60, 90, 120, 150, 180 days. After revaccination they were bled at 30 d.

*FIG. 17. Examination of vaccinated and repeat vaccinated cattle.*

| PVD | CPFA | 3A 3B | 2C 3D 3ABC | UBI | P/B | IDGA |
|---|---|---|---|---|---|---|
| Serol 1/4 | | | | | | |
| Mix A | | | | | | |
| Mix D | | | | | | |
| Mix F | | | | | | |
| **Suero: 15** | | | | | | |
| 0 | NR | | | NR | np | N |
| 30 | NR | | | NR | np | N |
| 60 | NR | | | NR | np | N |
| 90 | NR | | | NR | np | N |
| 120 | NR | | | NR | np | N |
| 150 | NR | | | NR | np | N |
| 180 | NR | | | NR | np | N |
| 30* | NR | | | NR | np | N |
| **Suero: 16** | | | | | | |
| 0 | NR | | | NR | np | N |
| 30 | NR | | | NR | np | N |
| 60 | NR | | | NR | np | N |
| 90 | NR | | | NR | np | N |
| 120 | NR | | | NR | np | N |
| 150 | NR | | | NR | np | N |
| 180 | NR | | | NR | np | N |
| 30* | I | | | NR | np | N |
| **Suero: 17** | | | | | | |
| 0 | NR | | | NR | np | N |
| 30 | NR | | | NR | np | N |
| 60 | NR | | | NR | np | N |
| 90 | NR | | | NR | np | N |
| 120 | I | | | NR | np | N |
| 150 | I | | | NR | np | N |
| 180 | NR | | | NR | np | N |
| 30* | I | | | NR | np | N |
| **Suero: 18** | | | | | | |
| 0 | NR | | | NR | np | N |
| 30 | NR | | | NR | np | N |
| 60 | NR | | | NR | np | N |
| 90 | NR | | | NR | np | N |
| 120 | NR | | | NR | np | N |
| 150 | NR | | | NR | np | N |
| 180 | NR | | | NR | np | N |
| 30* | NR | | | NR | np | N |

GROUP OF BOVINES LESS THAN
2 YEARS VACCINATED AGAINST
FMDV AND BLEEDED MONTHLY

FARM: LA PAZ
TOWN: ARBOLETES
STATE: ANTIOQUIA.

PVD = post-vaccination days
* = After booster days

This group of cattle was bled from 0 d
and then at 30, 60, 90, 120, 150, 180 d.
After a booster vaccination it was bled
at 30 d.

FIG. 18. Examination of vaccinated and repeat vaccinated cattle.

| PVD | CPFA | | 3A | 3B | | 2C | 3D | 3ABC | UBI | P/B | IDGA |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Serol 1/4 | | | | | | | | | | | |
| Mix A | | | | | | | | | | | |
| Mix D | | | | | | | | | | | |
| Mix F | | | | | | | | | | | |
| Suero: 19 | | | | | | | | | | | |
| 0 | NR | | | | | | | | NR | np | N |
| 30 | NR | | | | | | | | NR | np | N |
| 60 | NR | | | | | | | | NR | np | N |
| 90 | NR | | | | | | | | NR | np | N |
| 120 | NR | | | | | | | | NR | np | N |
| 150 | NR | | | | | | | | NR | np | N |
| 180 | NR | | | | | | | | NR | np | N |
| 30* | NR | | | | | | | | NR | np | N |
| Suero: 20 | | | | | | | | | | | |
| 0 | NR | | | | | | | | NR | np | N |
| 30 | NR | | | | | | | | NR | np | N |
| 60 | NR | | | | | | | | NR | np | N |
| 90 | NR | | | | | | | | NR | np | N |
| 120 | NR | | | | | | | | NR | np | N |
| 150 | NR | | | | | | | | NR | np | N |
| 180 | NR | | | | | | | | NR | np | N |
| 30* | NR | | | | | | | | NR | np | N |
| Suero: 21 | | | | | | | | | | | |
| 0 | I | | | | | | | | R | np | N |
| 30 | I | | | | | | | | R | np | N |
| 60 | NR | | | | | | | | R | np | N |
| 90 | NR | | | | | | | | NR | np | N |
| 120 | NR | | | | | | | | NR | np | N |
| 150 | NR | | | | | | | | NR | np | N |
| 180 | NR | | | | | | | | NR | np | N |
| 30* | NR | | | | | | | | NR | np | N |
| Suero: 22 | | | | | | | | | | | |
| 0 | NR | | | | | | | | NR | np | N |
| 30 | NR | | | | | | | | NR | np | N |
| 60 | NR | | | | | | | | NR | np | N |
| 90 | NR | | | | | | | | NR | np | N |
| 120 | NR | | | | | | | | NR | np | N |
| 150 | NR | | | | | | | | NR | np | N |
| 180 | NR | | | | | | | | NR | np | N |
| 30* | NR | | | | | | | | NR | np | N |
| Suero: 23 | | | | | | | | | | | |
| 0 | NR | | | | | | | | NR | np | N |
| 30 | NR | | | | | | | | NR | np | N |
| 60 | NR | | | | | | | | NR | np | N |
| 90 | NR | | | | | | | | NR | np | N |
| 120 | NR | | | | | | | | NR | np | N |
| 150 | NR | | | | | | | | NR | np | N |
| 180 | NR | | | | | | | | NR | np | N |
| 30* | NR | | | | | | | | NR | np | N |

**GROUP OF CATTEL LESS THAN 2Y VACCINATED AGAINST FMD BLED MONTHLY**

**FARM: LA PAZ
TOWN: ARBOLETES
STATE: ANTIOQUIA.**

PVD = post-vaccination days
* = After booster days

This group of bovines was bled at 0 and then at 30, 60, 90, 120, 150, 180 d. After revaccination, they were bled at 30 d.

*FIG. 19. Examination of vaccinated and repeat vaccinated cattle.*

| PVD | CPFA | 3A 3B | 2C | 3D 3ABC | UBI | P/B | IDGA |
|---|---|---|---|---|---|---|---|
| Serol 1/4 | | | | | | | |
| Mix A | | | | | | | |
| Mix D | | | | | | | |
| Mix F | | | | | | | |
| Suero: 24 | | | | | | | |
| 0 | NR | | | | NR | np | N |
| 30 | NR | | | | NR | np | N |
| 60 | NR | | | | NR | np | N |
| 90 | NR | | | | NR | np | N |
| 120 | NR | | | | NR | np | N |
| 150 | NR | | | | NR | np | N |
| 180 | NR | | | | NR | np | N |
| 30* | I | | | | NR | np | N |
| Suero: 25 | | | | | | | |
| 0 | NR | | | | NR | np | N |
| 30 | NR | | | | NR | np | N |
| 60 | NR | | | | NR | np | N |
| 90 | NR | | | | NR | np | N |
| 120 | I | | | | NR | np | N |
| 150 | NR | | | | NR | np | N |
| 180 | I | | | | NR | np | N |
| 30* | NR | | | | NR | np | N |
| Suero: 26 | | | | | | | |
| 0 | NR | | | | NR | np | N |
| 30 | NR | | | | NR | np | N |
| 60 | I | | | | NR | np | N |
| 90 | NR | | | | NR | np | N |
| 120 | I | | | | NR | np | N |
| 150 | NR | | | | NR | np | N |
| 180 | R | | | | NR | np | N |
| 30* | R | | | | NR | np | N |
| Suero: 27 | | | | | | | |
| 0 | NR | | | | NR | np | N |
| 30 | NR | | | | NR | np | N |
| 60 | I | | | | NR | np | N |
| 90 | NR | | | | NR | np | N |
| 120 | NR | | | | NR | np | N |
| 150 | NR | | | | NR | np | N |
| 180 | R | | | | NR | np | N |
| 30* | NR | | | | NR | np | N |
| Suero: 28 | | | | | | | |
| 0 | R | | | | NR | np | N |
| 30 | I | | | | R | np | N |
| 60 | R | | | | NR | np | N |
| 90 | R | | | | NR | np | N |
| 120 | NR | | | | NR | np | N |
| 150 | R | | | | NR | np | N |
| 180 | R | | | | R | np | N |
| 30* | R | | | | R | np | N |

**GROUP OF CATTLE LESS THAN 2 Y VACCINATED AGAINST FMDV BLED MONTHLY**

**Predio: LA PAZ**
**Municipio: ARBOLETES**
**Departamento: ANTIOQUIA.**

PVD = post-vaccination days
* = After booster days

This group of cattle vaccinated then bled from 0 and at 30, 60, 90, 120, 150, 180 d. After revaccination they were bled at 30 d..,

*FIG. 20. Examination of vaccinated and repeat vaccinated cattle*

| PVD | CPFA | 3A 3B | 2C | 3D 3ABC | UBI | P/B | IDGA |
|---|---|---|---|---|---|---|---|
| Serol 1/4 | | | | | | | |
| Mix A | | | | | | | |
| Mix D | | | | | | | |
| Mix F | | | | | | | |

**Suero: 29**

| PVD | CPFA | UBI | P/B | IDGA |
|---|---|---|---|---|
| 0 | NR | NR | np | N |
| 30 | I | NR | np | N |
| 60 | I | NR | np | N |
| 90 | NR | NR | np | N |
| 120 | I | NR | np | N |
| 150 | NR | NR | np | N |
| 180 | NR | NR | np | N |
| 30* | NR | NR | np | N |

**Suero: 30**

| PVD | CPFA | UBI | P/B | IDGA |
|---|---|---|---|---|
| 0 | NR | NR | np | N |
| 30 | I | NR | np | N |
| 60 | NR | NR | np | N |
| 90 | NR | NR | np | N |
| 120 | NR | NR | np | N |
| 150 | NR | NR | np | N |
| 180 | NR | NR | np | N |
| 30* | NR | NR | np | N |

**Suero: 31**

| PVD | CPFA | UBI | P/B | IDGA |
|---|---|---|---|---|
| 0 | I | NR | np | N |
| 30 | R | NR | np | N |
| 60 | R | NR | np | N |
| 90 | I | NR | np | N |
| 120 | I | NR | np | N |
| 150 | I | NR | np | N |
| 180 | NR | NR | np | N |
| 30* | I | NR | np | N |

**Suero: 32**

| PVD | CPFA | UBI | P/B | IDGA |
|---|---|---|---|---|
| 0 | NR | NR | np | N |
| 30 | NR | NR | np | N |
| 60 | I | NR | np | N |
| 90 | NR | NR | np | N |
| 120 | NR | NR | np | N |
| 150 | NR | NR | np | N |
| 180 | NR | NR | np | N |
| 30* | NR | NR | np | N |

**Suero: 33**

| PVD | CPFA | UBI | P/B | IDGA |
|---|---|---|---|---|
| 0 | I | NR | np | N |
| 30 | I | NR | np | N |
| 60 | I | NR | np | N |
| 90 | I | NR | np | N |
| 120 | I | NR | np | N |
| 150 | I | NR | np | N |
| 180 | I | NR | np | N |
| 30* | NR | NR | np | N |

**GROUP OF CATTLE GREATER THAN 2 Y VACCINATED AGAINST FMDV BLED MONTHLY**

**FARM: LA PAZ**
**TOWN: ARBOLETES**
**STATE: ANTIOQUIA.**

PVD = post-vaccination days
* = After booster days

This group of bovines was bled at 0 and then 30, 60, 90, 120, 150, 180 d. After revaccination they were bled at 30 d.

FIG. 21. Examination of vaccinated and repeat vaccinated cattle

| PVD | CPFA | 3A 3B | 2C | 3D 3ABC | UBI | P/B | IDGA |
|---|---|---|---|---|---|---|---|
| Serol 1/4 | | | | | | | |
| Mix A | | | | | | | |
| Mix D | | | | | | | |
| Mix F | | | | | | | |
| Suero: 34 | | | | | | | |
| 0 | NR | | | | NR | np | N |
| 30 | NR | | | | NR | np | N |
| 60 | NR | | | | NR | np | N |
| 90 | NR | | | | NR | np | N |
| 120 | NR | | | | NR | np | N |
| 150 | NR | | | | NR | np | N |
| 180 | I | | | | NR | np | N |
| 30* | I | | | | NR | np | N |
| Suero: 35 | | | | | | | |
| 0 | NR | | | | NR | np | N |
| 30 | NR | | | | NR | np | N |
| 60 | NR | | | | NR | np | N |
| 90 | NR | | | | NR | np | N |
| 120 | NR | | | | NR | np | N |
| 150 | NR | | | | NR | np | N |
| 180 | NR | | | | NR | np | N |
| 30* | NR | | | | NR | np | N |
| Suero: 36 | | | | | | | |
| 0 | NR | | | | NR | np | N |
| 30 | NR | | | | NR | np | N |
| 60 | NR | | | | NR | np | N |
| 90 | NR | | | | NR | np | N |
| 120 | NR | | | | NR | np | N |
| 150 | I | | | | NR | np | N |
| 180 | NR | | | | NR | np | N |
| 30* | NR | | | | NR | np | N |
| Suero: 37 | | | | | | | |
| 0 | I | | | | NR | np | N |
| 30 | NR | | | | NR | np | N |
| 60 | NR | | | | NR | np | N |
| 90 | I | | | | NR | np | N |
| 120 | NR | | | | NR | np | N |
| 150 | NR | | | | NR | np | N |
| 180 | NR | | | | NR | np | N |
| 30* | NR | | | | NR | np | N |
| Suero: 38 | | | | | | | |
| 0 | NR | | | | NR | np | N |
| 30 | R | | | | NR | np | N |
| 60 | NR | | | | NR | np | N |
| 90 | NR | | | | NR | np | N |
| 120 | NR | | | | NR | np | N |
| 150 | NR | | | | NR | np | N |
| 180 | NR | | | | NR | np | N |
| 30* | NR | | | | NR | np | N |

**GROUP OF CATTLE GREATER THAN 2 Y VACCINATED AGAINST FMDV BLED MONTHLY**

**FARM: LA PAZ**
**TOWN: ARBOLETES**
**STATE: ANTIOQUIA.**

\* = After booster days

This group of bovines was bled at 0 and then 30, 60, 90, 120, 150, 180 d. After revaccination they were bled at 30 d.

FIG. 22. Examination of vaccinated and repeat vaccinated cattle.

| PVD | CPFA | | 3A 3B | 2C 3D 3ABC | UBI | P/B | IDGA |
|---|---|---|---|---|---|---|---|
| Serol 1/4 | | | | | | | |
| Mix A | | | | | | | |
| Mix D | | | | | | | |
| Mix F | | | | | | | |
| **Suero: 39** | | | | | | | |
| 0 | NR | | | | NR | np | N |
| 30 | NR | | | | NR | np | N |
| 60 | I | | | | NR | np | N |
| 90 | NR | | | | NR | np | N |
| 120 | NR | | | | NR | np | N |
| 150 | NR | | | | NR | np | N |
| 180 | NR | | | | NR | np | N |
| 30* | NR | | | | NR | np | N |
| **Suero: 40** | | | | | | | |
| 0 | NR | | | | NR | np | N |
| 30 | NR | | | | NR | np | N |
| 60 | NR | | | | NR | np | N |
| 90 | NR | | | | NR | np | N |
| 120 | NR | | | | NR | np | N |
| 150 | NR | | | | NR | np | N |
| 180 | NR | | | | NR | np | N |
| 30* | NR | | | | NR | np | N |
| **Suero: 41** | | | | | | | |
| 0 | NR | | | | NR | np | N |
| 30 | NR | | | | NR | np | N |
| 60 | I | | | | NR | np | N |
| 90 | NR | | | | NR | np | N |
| 120 | NR | | | | NR | np | N |
| 150 | NR | | | | NR | np | N |
| 180 | NR | | | | NR | np | N |
| 30* | NR | | | | NR | np | N |
| **Suero: 42** | | | | | | | |
| 0 | NR | | | | NR | np | N |
| 30 | NR | | | | NR | np | N |
| 60 | NR | | | | NR | np | N |
| 90 | NR | | | | NR | np | N |
| 120 | I | | | | NR | np | N |
| 150 | NR | | | | NR | np | N |
| 180 | I | | | | NR | np | N |
| 30* | R | | | | NR | np | N |
| **Suero: 43** | | | | | | | |
| 0 | I | | | | NR | np | N |
| 30 | I | | | | R | np | N |
| 60 | I | | | | NR | np | N |
| 90 | R | | | | NR | np | N |
| 120 | I | | | | NR | np | N |
| 150 | NR | | | | NR | np | N |
| 180 | NR | | | | NR | np | N |
| 30* | R | | | | R | np | N |

**GROUP OF BOVINES GREATER THAN 2 YEARS VACCINATED AGAINST FMDV AND BLEEDED MONTHLY**

**FARM: LA PAZ**
**TOWN: ARBOLETES**
**STATE: ANTIOQUIA.**

PVD = post-vaccination days
* = After booster days

This group of cattle was bled at 0 and then 30, 60, 90, 120, 150, 180 d. After revaccination they were bled at 30 d.

*FIG. 23. Examination of vaccinated and repeat vaccinated cattle.*

GROUP OF CATTLE GREATER THAN 2 Y VACCINATED AGAINST FMDV AND BLED MONTHLY

FARM: LA PAZ
TOWN: ARBOLETES
STATE: ANTIOQUIA.

| PVD | CPFA | 3A | 3B | 2C | 3D | 3ABC | UBI | P/B | IDGA |
|---|---|---|---|---|---|---|---|---|---|
| Serol 1/4 | | | | | | | | | |
| Mix A | | | | | | | | | |
| Mix D | | | | | | | | | |
| Mix F | | | | | | | | | |

Suero: 44

| PVD | CPFA | UBI | P/B | IDGA |
|---|---|---|---|---|
| 0 | NR | NR | np | N |
| 30 | NR | NR | np | P |
| 60 | NR | NR | np | N |
| 90 | NR | NR | np | N |
| 120 | NR | NR | np | N |
| 150 | I | NR | np | N |
| 180 | NR | NR | np | N |
| 30* | NR | NR | np | P |

← AGID pos after boost

Suero: 45

| PVD | CPFA | UBI | P/B | IDGA |
|---|---|---|---|---|
| 0 | R | R | np | N |
| 30 | R | R | np | N |
| 60 | R | R | np | N |
| 90 | R | R | np | N |
| 120 | R | R | np | N |
| 150 | R | R | np | N |
| 180 | R | R | np | N |
| 30* | R | R | np | P |

Blot and ELISA correlate

← AGID pos after boost

Suero: 46

| PVD | CPFA | UBI | P/B | IDGA |
|---|---|---|---|---|
| 0 | NR | NR | np | N |
| 30 | NR | NR | np | N |
| 60 | NR | NR | np | N |
| 90 | I | NR | np | N |
| 120 | NR | NR | np | N |
| 150 | I | NR | np | N |
| 180 | NR | NR | np | N |
| 30* | NR | NR | np | N |

Suero: 47

| PVD | CPFA | UBI | P/B | IDGA |
|---|---|---|---|---|
| 0 | NR | NR | np | N |
| 30 | I | NR | np | P |
| 60 | I | NR | np | N |
| 90 | NR | NR | np | N |
| 120 | I | NR | np | N |
| 150 | NR | NR | np | N |
| 180 | NR | NR | np | N |
| 30* | I | NR | np | N |

Suero: 48

| PVD | CPFA | UBI | P/B | IDGA |
|---|---|---|---|---|
| 0 | NR | NR | np | N |
| 30 | NR | NR | np | N |
| 60 | NR | NR | np | N |
| 90 | NR | NR | np | N |
| 120 | NR | NR | np | N |
| 150 | I | NR | np | N |
| 180 | NR | NR | np | N |
| 30* | NR | NR | np | N |

PVD = post-vaccination days
* = After booster days

This group of cattle was bled at 0 and then 30, 60, 90, 120, 150, 180 d. After revaccination they were bled at 30 d. Note serum 45 which was positive or reactive in several tests. This animal was 10 years old and show positive at 0 d.

FIG. 24. Examination of vaccinated and repeat vaccinated cattle.

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| PVD | CPFA | 3A | 3B | 2C | 3D | 3ABC | UBI | P/B | IDGA |
|-----|------|----|----|----|----|------|-----|-----|------|
| Serol 1/4 | | | | | | | | | |
| Mezcla A | | | | | | | | | |
| Mezcla D | | | | | | | | | |
| Mezcla F | | | | | | | | | |
| Suero: | 49 | | | | | | | | |
| 0 | NR | | | | | | NR | np | N |
| 30 | NR | | | | | | NR | np | N |
| 60 | NR | | | | | | NR | np | N |
| 90 | I | | | | | | NR | np | N |
| 120 | NR | | | | | | NR | np | N |
| 150 | NR | | | | | | NR | np | N |
| 180 | NR | | | | | | NR | np | N |
| 30* | NR | | | | | | NR | np | N |
| Suero: | 50 | | | | | | | | |
| 0 | NR | | | | | | NR | np | N |
| 30 | NR | | | | | | NR | np | N |
| 60 | NR | | | | | | NR | np | N |
| 90 | NR | | | | | | NR | np | N |
| 120 | NR | | | | | | NR | np | N |
| 150 | I | | | | | | NR | np | N |
| 180 | NR | | | | | | NR | np | N |
| 30* | R | | | | | | NR | np | N |

**GROUP OF CATTLE GREATER THAN 2Y VACCINATED AGAINST FMDV BLED MONTHLY**

FARM: LA PAZ
TOWN: ARBOLETES
STATE: ANTIOQUIA.

PVD         = post-vaccination days
*               = After booster days

This group of bovines was bled at
0 and then 30, 60, 90, 120, 150, 180 d.
After revaccination they were bled at
30 d.

*FIG. 25. Examination of vaccinated and repeat vaccinated cattle.*

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SHEEP EXPERIMENTALLY INFECTED WITH FMDV TYPE O

This sheep was inoculated with FMDV type O in the tongue at 10.000 DICC. It was sampled daily from 0d up to 30d and then 60, 90, 120, 150, 180, 210, 240, 270 and 280d post-inoculation. Note strong positivity by blotting to 280 d Unfiortunayel the UBI test could not be made on later sera, but it is tyo be expected from previous results that they would be positive (taking blots as being strongly correlated to UBI). The agar gel shows positivity from 10 d to 280 d.

*FIG. 26. Examination of a sheep infected with FMD.*

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**GOAT EXPERIMENTALLY INFECTED WITH FMDV TYPE O**

| DPI | 3A 3B | 2C | 3D 3ABC | UBI | P/B | IDGA |
|---|---|---|---|---|---|---|
| 0 | | | | NR | np | N |
| 1 | | | | NR | np | N |
| 2 | | | | NR | np | N |
| 3 | | | | NR | np | N |
| 4 | | | | NR | np | N |
| 5 | | | | NR | np | N |
| 6 | | | | NR | np | N |
| 7 | | | | R | np | N |
| 8 | | | | R | np | N |
| 9 | | | | R | np | N |
| 10 | | | | R | np | P |
| 11 | | | | R | np | P |
| 12 | | | | R | np | P |
| 13 | | | | R | np | P |
| 14 | | | | R | np | P |
| 15 | | | | R | np | P |
| 16 | | | | R | np | P |
| 17 | | | | R | np | P |
| 18 | | | | R | np | P |
| 19 | | | | np | np | P |
| 20 | | | | np | np | P |
| 21 | | | | np | np | P |
| 22 | | | | np | np | P |
| 23 | | | | np | np | P |
| 24 | | | | np | np | P |
| 25 | | | | np | np | P |
| 26 | | | | np | np | P |
| 27 | | | | np | np | P |
| 28 | | | | np | np | P |
| 29 | | | | np | np | P |
| 30 | | | | np | np | P |
| 60 | | | | np | np | P |
| 90 | | | | np | np | P |
| 120 | | | | np | np | P |
| 150 | | | | np | np | P |
| 180 | | | | np | np | P |
| 210 | | | | np | np | P |
| 240 | | | | np | np | P |
| 270 | | | | np | np | P |
| 280 | | | | np | np | P |

The goat was inoculated with FMDV type O in the tongue at 10.000 DICC and sampled daily from 0 - 30d, then at 60, 90, 120, 150, 180, 210, 240, 270 and 280d post-inoculation (DPI). Note that agar gel gives positives from 10 d to 280 d.

*FIG. 27. Examination of a goat infected with FMD.*

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| DPI | 3A | 3B | 2C | 3D | 3ABC | UBI | P/B | IDGA |
|-----|----|----|----|----|------|-----|-----|------|
| 0 | | | | | | NR | np | N |
| 1 | | | | | | NR | np | N |
| 2 | | | | | | NR | np | N |
| 3 | | | | | | NR | np | N |
| 4 | | | | | | NR | np | N |
| 5 | | | | | | NR | np | N |
| 6 | | | | | | NR | np | N |
| 7 | | | | | | NR | np | N |
| 8 | | | | | | R | np | P |
| 9 | | | | | | R | np | P |
| 10 | | | | | | R | np | P |
| 11 | | | | | | R | np | P |
| 12 | | | | | | R | np | P |
| 13 | | | | | | R | np | P |
| 14 | | | | | | R | np | P |
| 15 | | | | | | R | np | P |
| 16 | | | | | | R | np | P |
| 17 | | | | | | R | np | P |
| 18 | | | | | | R | np | P |
| 19 | | | | | | np | np | P |
| 20 | | | | | | np | np | P |
| 21 | | | | | | np | np | P |
| 22 | | | | | | np | np | P |
| 23 | | | | | | np | np | P |
| 24 | | | | | | np | np | P |
| 25 | | | | | | np | np | P |
| 26 | | | | | | np | np | P |
| 27 | | | | | | np | np | P |
| 28 | | | | | | np | np | P |
| 29 | | | | | | np | np | P |
| 30 | | | | | | np | np | P |
| 60 | | | | | | np | np | P |
| 90 | | | | | | np | np | P |
| 120 | | | | | | np | np | P |
| 150 | | | | | | np | np | P |
| 180 | | | | | | np | np | P |
| 210 | | | | | | np | np | P |
| 240 | | | | | | np | np | P |
| 270 | | | | | | np | np | P |
| 280 | | | | | | np | np | P |

**GOAT EXPERIMENTALLY INFECTED WITH FMDV TYPE O**

The goat was inoculated with FMDV type O in the tongue at 10.000 DICC and sampled daily from 0 - 30d, then at 60, 90, 120, 150, 180, 210, 240, 270 and 280d post-inoculation (DPI).

*FIG. 28. Examination of a goat infected with FMD.*

3.      CONCLUSIONS

The blotting technique proved highly successful in determining/confirming the antibody status of sheep, goats, pigs and cattle. The UBI ELISA was successful for the same animals and although having a lower diagnostic sensitivity as compared to the blotting technique, is highly useful for screening at the herd level. The originally developed Pirbright/Brescia test (now superseded by an Indirect ELISA from IDEXX) was not ideal. The agar gel test is variable in diagnostic usefulness particularly for infected pigs and cattle. The ideal system is to use ELISAs for screening herds then use of the blotting technique to confirm negativity, or ascribe positivity to a low or dubious positive by ELISA. This is the strategy given by PANAFTOSA where the immunoblot is used in combination with their Indirect ELISA.

3.     CONCLUSIONS

The blotting technique proved highly successful in determining/confirming the antibody status of sheep, goats, pigs and cattle. The UBI ELISA was successful for the same animals and although having a lower diagnostic sensitivity as compared to the blotting technique, is highly useful for screening at the herd level. The originally developed Pirbright/Brescia test (now superseded by an Indirect ELISA from IDEXX) was not ideal. The agar gel test is variable in diagnostic usefulness particularly for infected pigs and cattle. The ideal system is to use ELISAs for screening herds then use of the blotting technique to confirm negativity, or ascribe positivity to a low or dubious positive by ELISA. This is the strategy given by PANAFTOSA where the immunoblot is used in combination with their Indirect ELISA.

# USE OF NON-STRUCTURAL PROTEINS TO FMDV TO ASSESS ANTIBODIES IN VACCINATED AND INFECTED CATTLE IN PERU

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**Abstract**

The identification of animals that have been infected with FMDV is very important for the control measures of the disease, because the animals frequently become carriers and can be the cause of new outbreaks. In countries were vaccination is applied it is recognised that there is the necessity to use tests that can differentiate the antibodies resulted from the vaccine and the antibodies from infection. In recent years tests have been developed that identify antibodies against virus-specific proteins present only in infected animals, being the non-structural protein 2C and non-structural 3ABC polyprotein the most studied. The work made under the CRP can be divided into three phases. (1) Examination of a set of tests provided up to 2002; (2) Examination of tests provided from 2002 to present and; (3) Studies on post vaccinated animal to examine whether antibodies to NSP are produced.

1.     PHASE 1

## 1.1.     Methods

Kits to measure antibodies against FMD NSP used were: UBI; Brescia; PANAFTOSA; EITB. The VIAA (AGID) and PCR were also examined. Relative analytical sensitivities were calculated by titrating full dilution ranges of seven post infection positive sera in each kit.

## 1.2.     Sera

The sera used were:

Cattle infected, non-vaccinated,1–2 w post infection
Cattle infected, non-vaccinated, 2–3 m post infection
Cattle infected then vaccinated, 5 m post infection (1 dose of trivalent vaccine)
Cattle infected then vacinated, 11 m post infection (1 dose of trivalent vaccine)
Cattle vaccinated, non infected, one dose of vaccine
Cattle non vaccinated, non infected, 3 doses of vaccine
Oesophageal material from cattle 11 m after infection

## 2.    RESULTS

### 2.1.    Sera from cattle in acute phase of infection

Three animals had vesicles and ulcers on sampling and another 3 were in the healing phase. The results are shown in Table I.

TABLE I. SERA FROM CATTLE, <1 TO 2 WEEKS POST INFECTION. YAUYOS 2000

| Identity | Age (y) | VIA | UBI | Pir/Br | PA | EITB | Observation |
|----------|---------|-----|-----|--------|-----|------|-------------|
| 9 | 7 | - | - | - | - | - | vesicles |
| 10 | 1 | + | + | + | + | + | healed |
| 11 | 1 | - | - | - | - | - | vesicle |
| 12 | 6 | + | - | + | + | + | healed |
| 13 | 3 | + | + | + | + | + | healed |
| 14 | 6 | - | - | - | - | - | vesicles |
| Total | 6 | 3/6 | 2/6 | 3/6 | 3/6 | 3/6 | |

\* positives/total

### 2.2.    Sera from cattle after 2 to 3 m post infection

Eight animals were sampled from one outbreak. The results are shown in the Table II.

TABLE II. SERA FROM CATTLE, 2-3 MONTHS POST INFECTION. YAUYOS 2000

| Identity | Age   (y) | VIA | UBI | Pir/Br | PA | EITB |
|----------|-----------|-----|-----|--------|-----|------|
| 1 | 4 | + | + | + | + | + |
| 2 | 5 | + | + | + | + | + |
| 3 | 7 | - | - | - | - | - |
| 4 | 3 | + | + | + | + | + |
| 5 | 3 | + | + | + | + | + |
| 6 | 3 | - | - | - | - | - |
| 7 | 4 | + | - | + | + | + |
| 8 | 2 | + | + | + | + | + |
| Total | 8 | 6/9 | 5/9 | 6/9 | 6/9 | 6/9 |

\* positives/total

The animals were vaccinated two m after the infection with a trivalent oil vaccine. The results are shown in the Table III.

TABLE III. SERA FROM CATTLE 2-3 MONTHS POST INFECTION. LURIN, 1999

| Identity | Age(y) | VIA | UBI | Pir/Br | PA | EITB |
|----------|--------|-----|-----|--------|-----|------|
| 103-T | 5 | + | + | + | + | + |
| P-99 | 4 | - | + | + | + | + |
| P-886 | 5 | - | + | + | + | + |
| P-17x | 4 | + | + | + | + | + |
| L-1 | 2 | + | + | + | + | + |
| L-2 | 2 | + | + | + | + | + |
| L-3 | 2 | + | + | + | + | + |
| H-211 | 2 | + | + | + | + | + |
| L-177 | 3 | + | + | + | + | + |
| Total: | 9 | 7/9 | 9/9 | 9/9 | 9/9 | 9/9 |

\* positives/total

TABLE IV. SERA FROM CATTLE 5 MONTHS POST INFECTION. YAUYOS 2000

| Identity | Age (y) | VIA | UBI | Pir/Br | PA | EITB |
|----------|---------|-----|-----|--------|-----|------|
| 45 | 4 | - | - | + | + | + |
| 46 | 11 | - | - | - | + | + |
| 47 | 4 | + | + | + | + | + |
| 48 | 4 | - | + | + | + | + |
| 49 | 5 | - | + | + | + | + |
| 50 | 3 | - | - | - | - | - |
| 51 | 6 | + | + | + | + | + |
| 52 | 5 | + | + | + | + | + |
| 58 | 2 | - | | | | |
| Total | 9 | 2/9* | 6/9 | 7/9 | 8/9 | 8/9 |

\* positives/total

## 2.3. Sera from cattle eleven months after infection

The animals were vaccinated five months after the infection. Samples from O/P fluids were collected and examined by PCR (polymerase gene primers). The samples were not studied with the UBI kit because we did not have reagents. The results are shown in the Table V.

TABLE V. SERA FROM CATTLE, 11 MONTHS POST INFECTION. YAUYOS 2000

| Identity | Age (y) | VIA | UBI | Pir/Br | PA | EITB | PCR |
|----------|---------|-----|-----|--------|-----|------|-----|
| 71 | 4 | + | ND | + | + | + | + |
| 72 | 5 | | ND | + | + | + | - |
| 73 | 8 | | ND | - | - | - | - |
| 74 | 5 | | ND | + | + | + | - |
| 75 | 4 | | ND | + | + | + | + |
| 76 | 3 | | ND | - | - | - | - |
| 77 | 3 | | ND | + | + | + | - |
| 78 | 4 | | ND | + | + | + | - |
| 79 | 3 | | ND | - | - | - | - |
| Total | | *3/9 | ND | 6/9 | 6/9 | 6/9 | 2/9 |

\* positives/total

### 2.4. Sera from vaccinated cattle

Samples were taken from animals of the same region that had been vaccinated with one dose of trivalent oil vaccine. The animals had no history of FMD. The results are shown in the Table VI

TABLE VI. SERA FROM CATTLE VACCINATED (1 DOSE)

| Number of animals | Age (y) | VIA | UBI | Pir/Br | PA | EITB |
|---|---|---|---|---|---|---|
| 24 | Greater than 2 | - | - | - | - | - |
| 16 | Less than 2 | - | - | - | - | - |

### 2.5. Sera from cattle with multiple vaccines

Animals older than five years were selected to test the specificity of the ELISA kits. The results are shown in Table VII.

TABLE VII. SERA FROM CATTLE > 5 YEAR WITH /MULTIPLE VACCINATIONS

| Number of animals | Age (y) | VIA | UBI | Pir/Br | PA | EITB |
|---|---|---|---|---|---|---|
| 20 | Greater than 5 | 3 | 0 | 1 | 1 | 0 |

### 3. CONCLUSIONS ON WORK UP TO 2002 PHASE 1

All three ELISA were able to identify animals at early stage of infection (no less than one week) and at least for 11 m post infection.

The AGID test showed the lowest sensitivity. The kits from Pirbright and PANAFTOSA showed similar diagnostic sensitivities, that were both higher than the VIA and UBI kit. The ELISA kits showed very high specificity (highest for the UBI kit). Some false positives were detected in old animals with multiple vaccinations. The EITB was useful as a confirmatory test. NSP tests were very useful for determining the immune status of herds, and are important tools for the national control programmes establishing areas at risk. The profiles indicate different epidemiological status, differentiating infected and vaccinated animals. Carrier animals were detected by PCR at least after 11 m post infection.

In the comparison of titration of sera from infected animals, the UBI kit showed lowest end points. The Pirbright/Brescia kit had the highest end points (up to 20 times higher than UBI kit, and 10 times higher than PANAFTOSA kit).

In general the findings agreed with other researchers in the CRP looking at the same tests with different sera. The UBI kit cut off was set to favour specificity and hence lost sensitivity.

4.    WORK FROM 2002 PHASE 2

## 4.1.    Methods

A summary of the sera used is shown in Table VIII.

TABLE VIII. SERA USED IN STUDIES

| (1) Natural infected cattle post outbreak | 1-2 w | 2-3 m | 5 m | 11 m | | Total |
|---|---|---|---|---|---|---|
| **Number** | **6** | **18** | **9** | **9** | | **43** |
| (2)Experimentally infected post infection | 7 d | 8-9 d | 10 -12d | 13-15 d | 19-30 d | |
| **Number** | **34** | **18** | **28** | **11** | **7** | **98** |
| | | | | | | |
| (3) Field samples | - | - | - | - | - | 48 |
| | | | | | | |
| (4) Primo-vaccinated | - | - | - | - | - | 65 |
| (5) Multiple vaccinated | 2 doses | 3 doses | 4 doses | 5 doses | | |
| **Number in groups** | **27** | **27** | **17** | **17** | | **88** |
| (6) Naive animals | - | - | - | - | - | 65 |

## 4.2.    Post experimental infection study

TABLE IX. RESULTS OF ASSESSING POSITIVITY OF SAMPLES TAKEN AT DIFFERENT TIMES FOLLOWING INFECTION (SERIES 2 IN TABLE I) BY DIFFERENT KITS

| | Groups Days post infection | | | | |
|---|---|---|---|---|---|
| Test | 7 d | 8-9 d | 10-12 d | 13-15 d | 19-30 d |
| Bommeli% | 41 | 78 | 71 | 91 | 100 |
| CEDI% | 41 | 67 | 78 | 82 | 100 |
| PANAFTOSA% | 67 | 72 | 83 | 100 | 100 |
| Svanova% | 40 | 59 | 82 | 100 | 100 |
| UBI% | 0 | 40 | 40 | 64 | 86 |

Grey boxes indicate maximal diagnostic sensitivity. The PANAFTOSA and Svanova tests were similar in profile. The low number of samples affects this study for some groups.

## 4.3.    Natural infection results

TABLE X. DATA FROM NATURALLY INFECTED CATTLE AT 1-2 W, 2-3 M AND 11 M AFTER INFECTION

| Test | 1–2 w (n = 6) | 2–3 m (n = 18) | 11 m (n = 9) |
|------|---------------|----------------|--------------|
| Bommeli% | 33 | 89 | 23 |
| CEDI% | 50 | 94 | 78 |
| PANAFTOSA% | 50 | 94 | 78 |
| Svanova% | 50 | 94 | 78 |
| UBI% | 34 | 78 | 56 |

Grey boxes indicate maximum sensitivity seen.

Tables IX and X indicate that all tests have a sufficiently high DSn and DSp to be useful in determining recent infection. All tests performed well on sera taken 2-3 m after infection. The drop in detection rate at 11 m animals using Bommeli is interesting since it is has essentially the same reagents as the PANAFTOSA and Svanova tests. The difference in detection therefore, must be due to the different ant bovine conjugates used in the three tests. The detection of anti-NSP antibodies in a large proportion of the naturally infected animals is useful information when trying to analyse populations following infection.

### 4.4. Profiles of the samples from natural infection

The test results for all the naturally infected sera have been analysed in terms of the profile of the sera according to the individual test data. Fig. 1 shows the plots of the distribution of data from the tests.
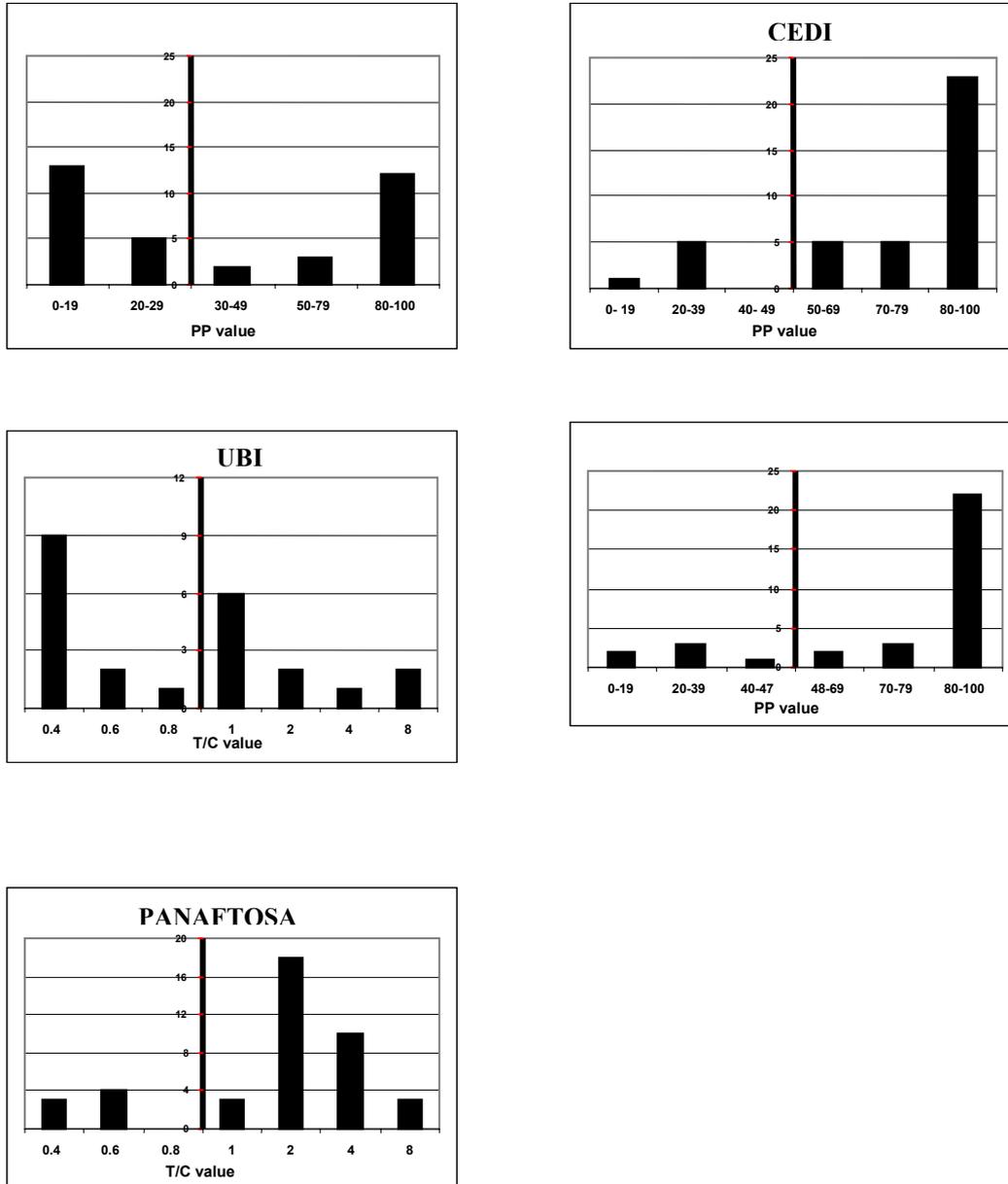


*FIG. 1. All sera from naturally infected animals profiles of numbers in particular reactivity groups for each assay.*

Provider given cut off is shown by vertical line.

## 4.5. Primo vaccinated cattle sampled at 0 and 30d post vaccination

The values obtained for all the sera before vaccination for three kits are shown below.
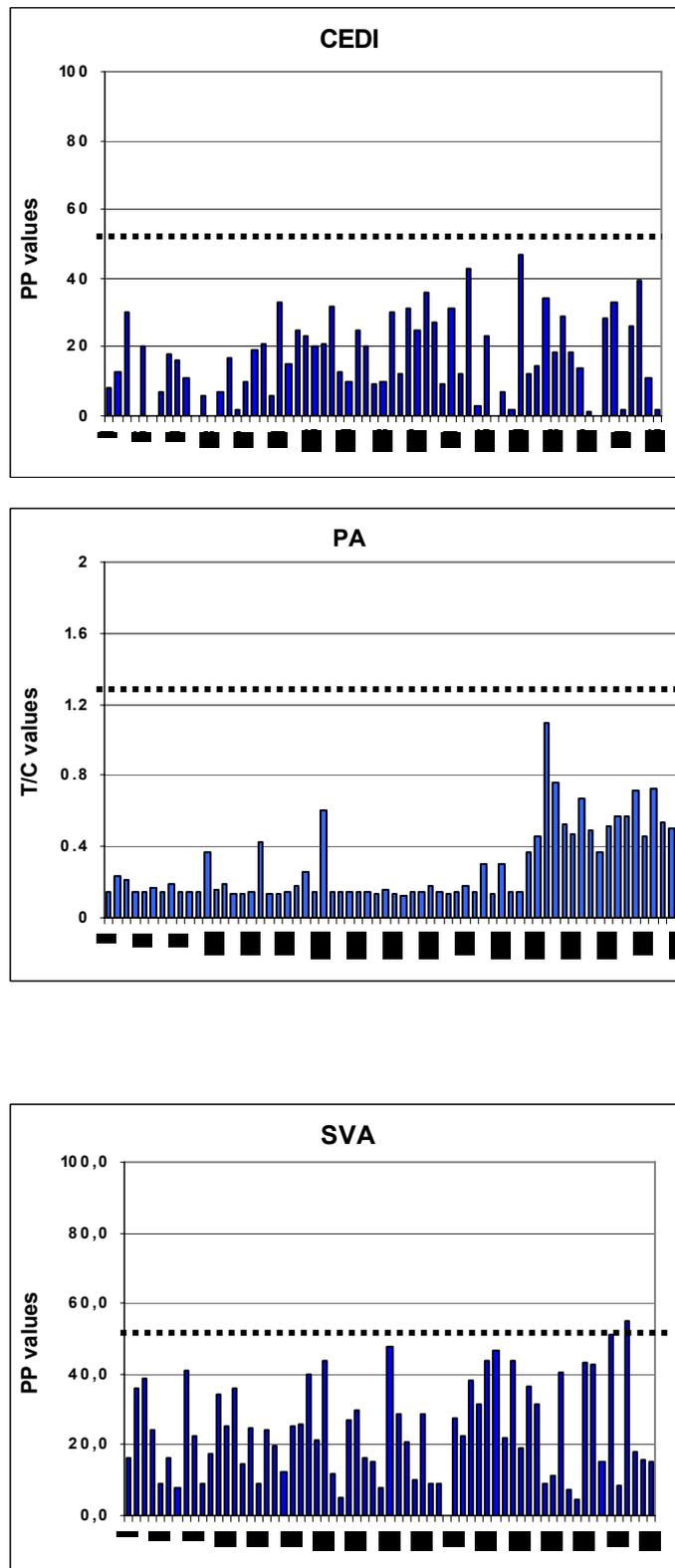


*FIG. 2. Individual non-vaccinated sera analysed by different tests.*

LPBE
titre

Positivity cut-off for test

PANAFTOSA data

LPBE
titre

3

2.5

2

1.5

1

0.5

0

Positivity cut-off for test
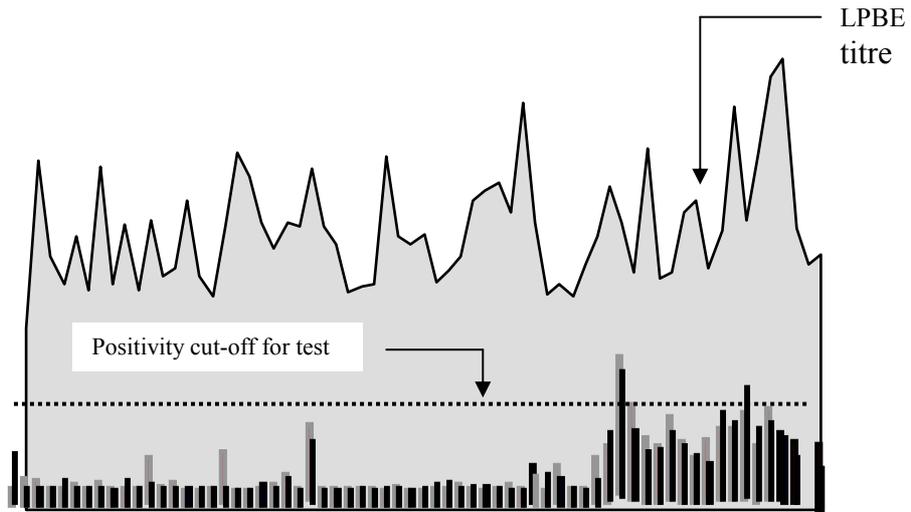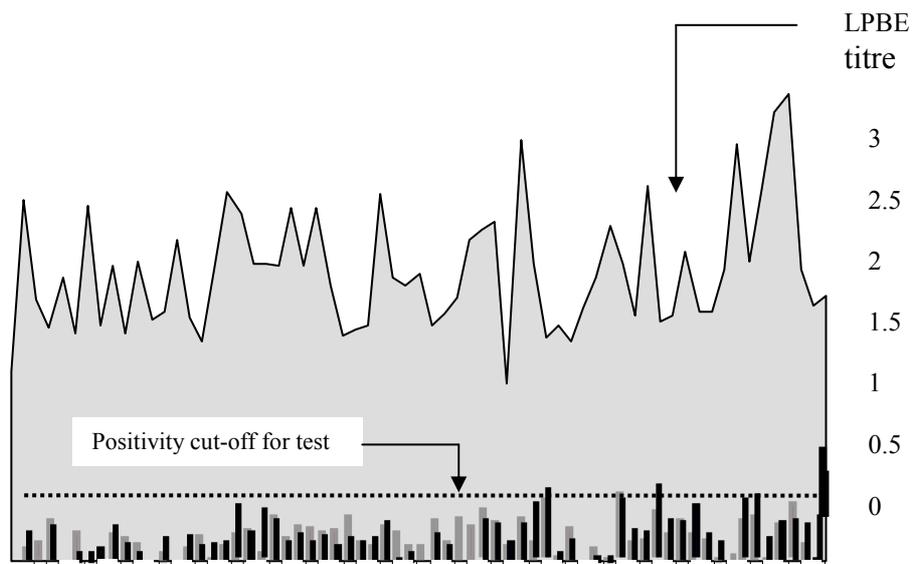
CEDI data

*FIG. 3. Profiles of individual sera before and 30d after vaccination.*

# STUDIES USING MULTIPLY VACCINATED ANIMALS

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**Abstract**

The serological responses in vaccinated and multiple vaccinated cattle against non-structural proteins (NSP) of foot-and-mouth disease (FMD) virus were measured using four commercially available assays. Vaccines were concentrated using polyethylene glycol to contain higher antigenic payloads that those routinely used. Animals received up to five doses of polyvalent oil vaccines over six months, administered by the intra-muscular route on d 0, 90, 130, 160 and 200. Serum samples were taken 30-40 d after each vaccination. At 60 d post vaccination the antibody response to each of the vaccine strains showed high levels of antibodies against structural proteins that correlated with protection against challenge above 81%. The detection of antibodies against NSP was made with two ELISAs using expressed 3ABC as antigen; one ELISA using peptides from 3B and an enzyme-immunotransfer blot assay (EITB). Locally produced ELISA-3ABC reagents and agar gel immunodiffusion using VIAA, were also evaluated. After four doses of vaccine, animals were negative in all the assays. After the fifth immunization, two of seventeen animals were reactive in one ELISA kit, but these samples proved negative by confirmatory tests. Antibodies against NSP were not detected in primo-vaccinated cattle used for potency tests using three batches of standard vaccine. The principle of the NSP ELISA as screening test for large sero surveys in South America is established and this paper emphasises the importance using vaccines that have no demonstrated interference with NSP ELISAs and the advantages of reducing the number of false-positives that would require further confirmation by other assays.

## 1. INTRODUCTION

Programmes for the control and eradication of FMD in South America are based on active epidemiological surveillance, systematic vaccination of the bovine population in risk areas, control of animal movement, slaughter of infected animals and contacts and large-scale sero surveys to evaluate herd immunity levels and evidence for viral activity irrespective of vaccination status.

During viral replication antibodies against both the virion and the non-structural proteins (NSP) are produced This led to the development of tests for the detection of the antibodies against NSP having the potential to differentiate vaccinated from infected animals with the added advantage of detecting antibodies independent of the need to use as antigen the infecting virus serotype [1–7]. Vaccines ideally should induce antibodies mainly against structural antigens since the virus used is inactivated and preparations should include low amounts or no NSP. During the manufacturing process for FMD vaccines, different methods are used for the concentration and purification of the antigens (ultrafiltration, precipitation with polyethylene glycol, chromatography, etc.) that mostly reduce or possibly eliminate the NSP present in viral suspensions. These methods allow the formulation of vaccines with high antigenic payloads but that may also concentrate any NSP that may induce antibodies that interfere with the tests currently used for viral activity assessment.

For many years, the detection of antibodies against the virus infection associated antigen (VIAA) was used as indicator of infection [8, 9]. The principle proved useful in the evaluation of viral activity in primo-vaccinated or non-vaccinated cattle, and in small ruminant population [10, 11]. Since 1990, studies showed that antibodies against various other NSPs are better indicators of infection than antibodies to polymerase 3D (the major component of VIAA) against which certain vaccines induce antibodies in vaccinated cattle [12, 13]. Various ELISA-based assays for detecting antibodies against *E. Coli* and baculo expressed 3ABC and synthesised peptide 3B have been reported to be sensitive, specific and reliable [14]. In addition, an enzyme-linked immunotransfer blot (EITB) for the simultaneous detection of antibodies to five NSP in a single test has shown its value for confirmation of infection in animals reactive for antibodies to 3ABC by ELISA [14]. Currently, three ELISA-based kits and one EITB are commercially available [15] and other assays are being developed.

The importance of the use of vaccines free of contaminating NSP is considered as a prerequisite for vaccine authorisation and has been discussed in recent FMD meetings [16, 17]. No official procedures are available for vaccine quality control in this respect that assure that the vaccines for systematic vaccination or for emergency use, after single or multiple immunisations, do not induce NSP antibodies; however some proposals are being reviewed for further approval [17].

For FMD vaccine manufacturers it is important to demonstrate that the production of high potency vaccines is not accompanied by the presence of NSP that may induce antibodies detected by specific tests. In previous reports it has been shown that high concentrated vaccines manufactured in Argentina do not induce antibodies to NSP as detectable by an ELISA 3ABC after single vaccination [18]. To provide more information, we have studied primo-vaccinated or repeatedly immunised cattle with concentrated commercial vaccines containing several FMD virus (FMDV) strains to examine sera for antibodies against NSP. This is the first report where three recognised NSP ELISA and EITB have been used to demonstrate that antibodies to NSP are not detectable after multiple immunizations of cattle with high concentrated vaccines. The use of NSP testing is demonstrated in an interesting study examining anti NSP responses in vaccines.


2.    MATERIALS AND METHODS

The serological responses in vaccinated and multiple vaccinated cattle against FMDV were measured using four commercially available assays. The detection of antibodies against NSP was made with two ELISAs using expressed 3ABC as antigen; one ELISA using peptides from 3B and an enzyme-immunotransfer blot assay (EITB). Locally produced ELISA-3ABC reagents and agar gel immunodiffusion using VIAA, were also evaluated as shown below.

ELISA 3ABC PANAFOTSA
EITB, PANAFTOSA
FMD- 3ABC bo-ov CHEKIT, Bommeli/Intervet (now IDEXX)
UBI FMDV NSP 3B EIA (Ruminant) – UBI FMDV NSP 3A EIA (Confirmatory test)
ELISA 3ABC, Centro de Virología Animal, CONICET, Argentina

Vaccines were concentrated using polyethylene glycol so contained higher antigenic payloads that those normally used. Animals received up to five doses of polyvalent oil

vaccines over 6 m, administered by the intra-muscular route on d 0, 90, 130, 160 and 200. Serum samples were taken 30–40 d after each vaccination. Three groups of 17 animals were used for this study. Each group received one vaccine formulation by the intra-muscular route. Three batches of polyvalent commercial vaccines composed of O1 Campos, A24 Cruzeiro, A Arg 2000, and A Arg 2001 FMDV strains, used in the systematic vaccination campaign in Argentina, were evaluated. The vaccines (C, D, E) contained 17, 20.8 and 21.3 μg of 140S /2mLcattle dose, respectively. Serum samples were obtained at 0 and at 30 dpv and antibodies against FMDV structural and non-structural proteins were analysed.

## 3.    RESULTS

At 60 d post vaccination the antibody response to each of the vaccine strains showed high levels of antibodies against structural proteins that correlated with protection against challenge above 81%. After four doses of vaccine, animals were negative in all the assays.

After the fifth immunization, two of seventeen animals were reactive in one ELISA kit, but these samples proved negative by confirmatory tests. Antibodies against NSP were not detected in primo-vaccinated cattle used for potency tests using three batches of standard vaccine. Results are shown in Table I.

TABLE I. ASSESSMENT OF SERA AFTER VACCINATION BY NSP TESTS

| Days post vaccination | Kit A | Kit B | Kit C | EITB |
|---|---|---|---|---|
| 0 (first vaccination) | 1*/17 | 1/17** | 0/17 | 0/17 |
| 30 | 1/17 | 0/17 | 0/17 | 0/17 |
| 90 (second vaccination) | 0/17 | 0/17 | 0/17 | |
| 130 (third vaccination) | 0/17 | 0/17 | 0/17 | |
| 160 (fourth vaccination) | 0/17 | 0/17 | 0/17 | |
| 200 (fifth vaccination) | 0/17 | 0/17 | 0/17 | |
| 230 | 0/17 | 0/17 | 2/17*** | 0/17 |

Results for different NSP Kits and EITB obtained after vaccination
* number of positives/ total
** one sample recorded as ambiguous
*** Samples were all negative when confirmed by kit C- confirmatory ELISA
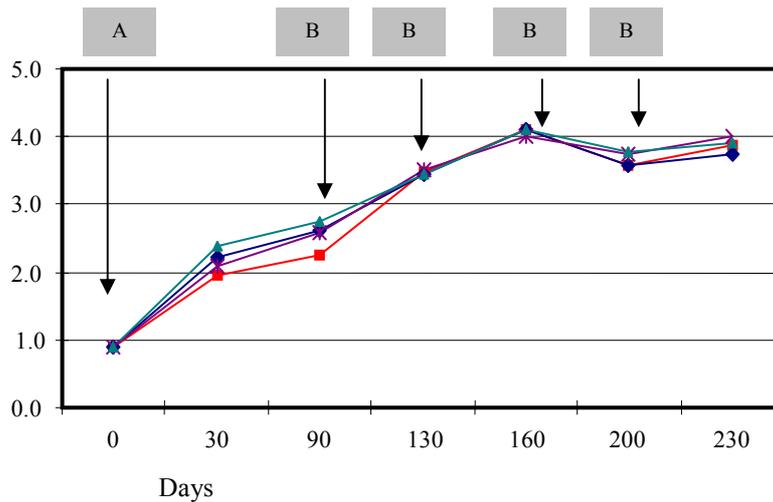
*FIG. 1. Post vaccination responses measured by LPBE.*
Vaccines administered as shown by down arrows.

The data for the sera tested by different NSP assays is shown in Fig. 2A to 2E.

Frequency distribution charts of reactivity to NSP ELISAs of samples taken from vaccinated and the same sequentially vaccinated cattle. The $y$ axis is the number of animals, each line represents one animal; the $x$ axis: T/C values or PP values. The cut-off value for each kit is indicated by a dotted line.

(a) Bovine No. 38651 was reactive at 0 and 30 dpv by ELISA kit A. Bovine No. 38650 was recorded as ambiguous at day 0 by ELISA kit B. Samples were all negative at 0 and 30 dpv by ELISA kit C. Positive and negative controls of each kit were within the limits established by manufacturers. All the samples were negative when analysed by EITB.

Frequency distribution charts of NSP ELISAs reactivity of cattle samples taken after the second, third, fourth and fifth vaccination. Bovines No. 38660 and 38662 were reactive by kit C at 30 d post fifth vaccination. All samples were negative by EITB.

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A. PANAFTOSA                    B. BOMMELI

**90 d 2nd**
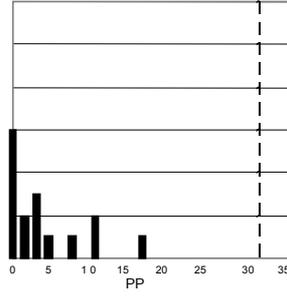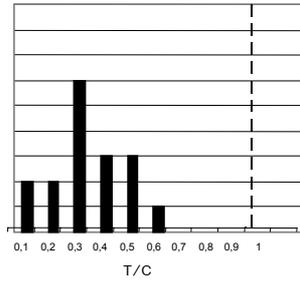


**130 d 3rd**
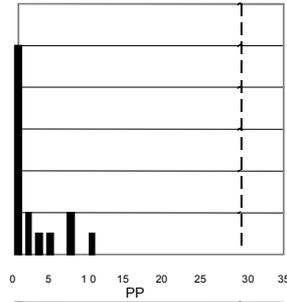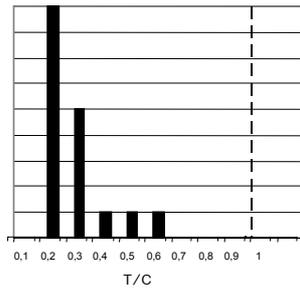


**160 d 4th**
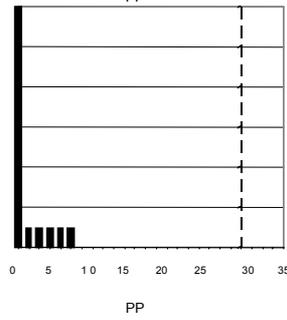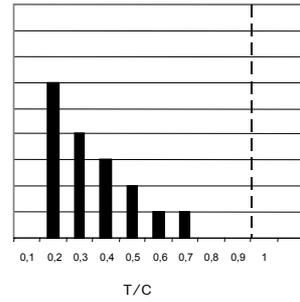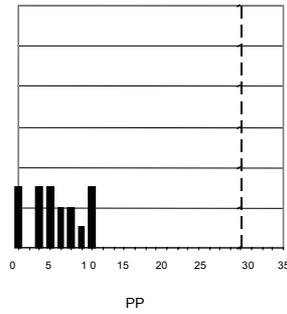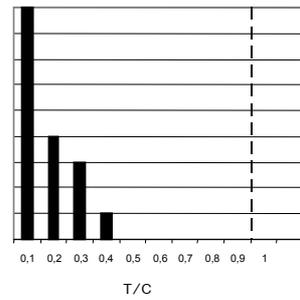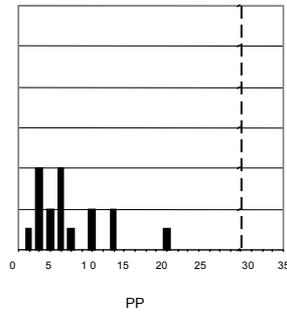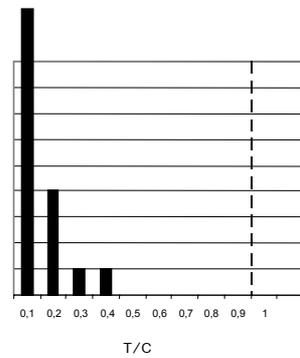


**200d 5th**



**230 d**

|  | C. UBI | D. SVANOVA |
|---|---|---|
| 90 d 2nd | | |
| 130 d 3rd | | |
| 160 d 4th | | |
| 200 d 5th | | |
| 230 d | | |
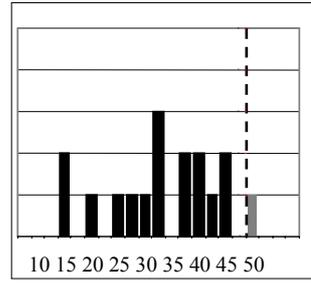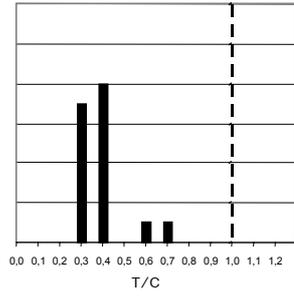
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E. CEDI

90 d 2nd



% Competiton

130 d 3rd



% Competiton

160 d 4th



% Competiton

200 d 5th



% Competiton

230d



% Competiton
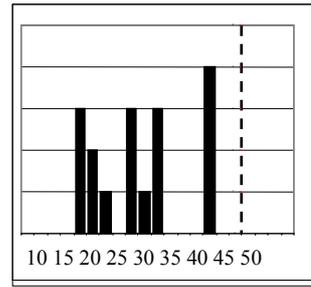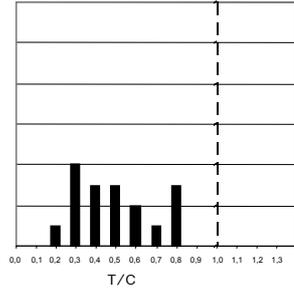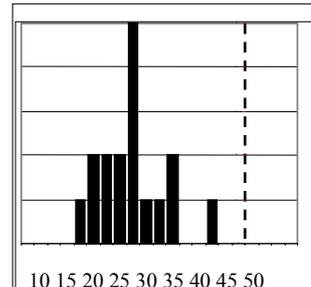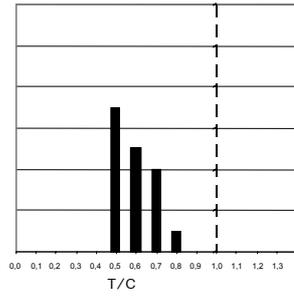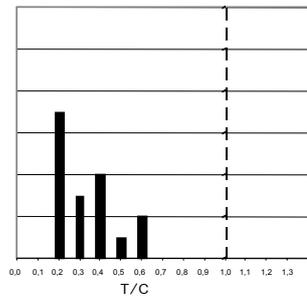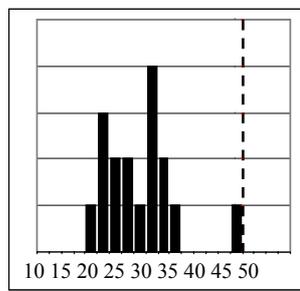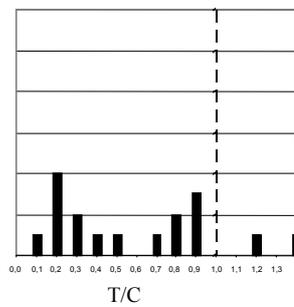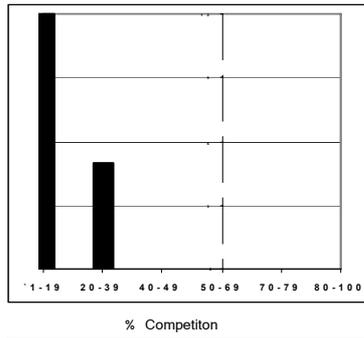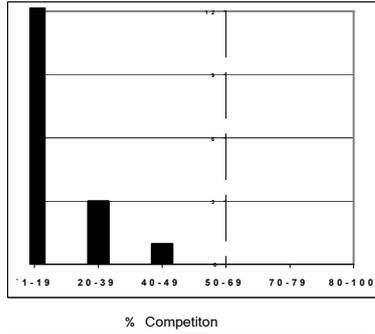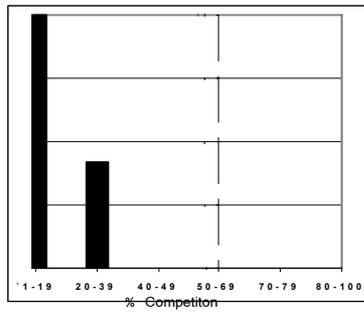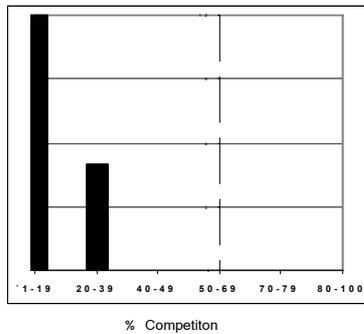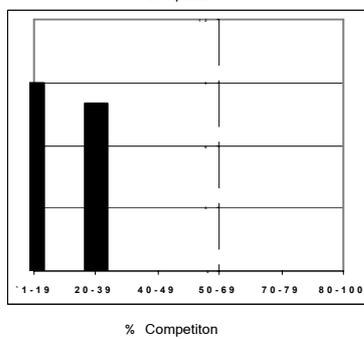
FIG. 2. Results of different NSP tests in analyzing sera from multiply-vaccinated animals.

# 4. DISCUSSION

It is generally accepted that modern FMD vaccines, containing partially purified antigens in which have been excluded the majority of NSP, induce little if any antibodies to NSP [14, 16]. In areas under systematic vaccination, where detection to NSP antibodies is used for certification of free areas of FMDV infection, it is of particular importance to use of vaccines which have been demonstrated as not eliciting responses to NSP. The proposals made from international organizations [17] consider that manufacturers shall present data showing that repeated immunization with vaccines formulated with the maximum permitted amount and number of antigens does not result in sero-conversion to NSP. Currently, there is not a reference assay for NSP antibody detection.

The vaccines used in this study all gave a satisfactory potency (EPP>81% at 60 dpv, data not shown). Cattle vaccinated four times were conclusively non-reactive in three NSP ELISA kits and by EITB. In previous work [19] the response to NSP antibodies in repeatedly vaccinated cattle was evaluated using only EITB. Our results indicate that low T/C or PP values were recorded by the NSP ELISAs used. However, there were overall quantitative differences observed for the NSP ELISA kits when examining individual sera, i.e. a serum with some reactivity in one kit does not necessarily reacted similarly with a different kit. This observation has already been made [20] and it can be the result of the different proteins used as antigens or on the assembly of the kits. Some differences were also found in the detection of reactive samples and can be interpreted as non-specific reactions affecting the specificity profile of the tests. In agreement with observations [21] in which very low percentage of animals (0.4%) from free areas reacted to NSP 3ABC.

After the fifth and final immunization, 2/17 were reactive only in kit C. This result was not confirmed although one sample had a borderline result. These results do reflect the differences in the sensitivity and specificity of the NSP ELISAs, based on the given cut-off values from the manufacturers. It is worth noting that the fitness for use of assays implies that a test performance has to be judged with regard to its sensitivity or specificity criteria dependant on the disease status of the population [22]. In our study, the criteria of specificity and sensitivity of the tests was not altered from that given in the test protocols. Considering that the animals used in this study originated from FMD free areas and demonstrated no sero conversion to structural and NSP in the non-vaccinated controls, viral activity during this study can be excluded as a source of eliciting antibodies

The frequency distribution of reactivity data has been used to characterize epidemiological situations [23] and such reactivity profiles were obtained in this study after each vaccination. Even though most of the samples were non-reactive as judged by the cut off criteria given for the assays, examination of the population statistics revealed that, on increasing the number of vaccinations to three, there was a distinct increase in the mean of the values closer to the cut off value given. After the fourth dose however, significantly lower reactivity (mean of populations) were shown in accordance with a significant decrease in antibody titres to structural proteins (p< 0,001). This reduction of antibodies and consequent chances of cross-reaction or non specific binding, could be explained by interaction and elimination of excess administered vaccine antigen by the high levels of circulating antibodies [27].All primo vaccinated cattle induced satisfactory level of antibodies against each vaccine strain and did not produce antibodies against NSP was shown using any system (Fig. 3 and 3a). In Argentina, commercial batches of vaccines are routinely checked for structural proteins antibodies level for potency testing and for NSP antibodies by ELISA kit

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A, reactive or doubtful results being confirmed by EITB. In order to increase the validation of a locally produced kit (E), we evaluated its performance in primo vaccinated animals. Previous data obtained with kit E when analysing sera from infected and naïve cattle, sheep and pigs, revealed satisfactory sensitivity and specificity comparable with other kits (non published data).

Previous reports [19], [25] have shown that other concentration and purification methods, different from the polyethylene glycol evaluated in this study, reduced the NSP content of the antigens and antibodies were not detected after three vaccinations. In the present report the vaccines applied contained four and five viral strains and very high antigenic payloads, thus an excess of virus and possible contaminants as compared with conventionally used vaccines. An important fact to stress is that animals received five doses of vaccine in a period of six months, which is far more than in any vaccination programme. The absence of antibodies to NSP is also supported by field data where cattle in vaccination programmes, using vaccine manufactured according to the above mentioned process, showed very low prevalence of NSP antibodies [26].

In this work we compared three NSP ELISAs and the EITB for the evaluation of antibodies against NSP in repeatedly vaccinated cattle. The NSP ELISAs are used in South America as a screening test for large scale sero surveys and only any reactive samples are examined using the confirmatory EITB. This test is more complex to run and more expensive than the ELISA, thus it is most desirable the demonstration of no occurrence of false positives due to vaccination in the ELISA. We conclude that FMD vaccine manufactured under appropriate antigen purification even with high antigenic payload allow differentiation of infected animals when evaluated by the current available NSP antibody detection tests.

The principle of the NSP ELISA as screening test for large sero surveys in South America is established and this paper emphasises the importance using vaccines that have no demonstrated interference with NSP ELISAs and the advantages of reducing the number of false-positives that would require further confirmation by other assays.

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# DIFFERENTIATION OF FMD INFECTED ANIMALS FROM VACCINATED ANIMALS BY THE USE OF NON-STRUCTURAL PROTEINS OF FOOT AND MOUTH DISEASE VIRUS IN MYANMAR

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**Abstract**

Four non-structural proteins (NSP) foot and mouth disease virus (FMDV) ELISA kits were received through the FAO/IAEA Coordinated Research Project (CRP). From 1999–2004, 10 States and Divisions out of 14 in Myanmar were visited by the staff of National FMD Laboratory and a total of 4704 sera from cattle, buffaloes, goats and pigs were collected. Sera were investigated for FMD serotype prevalence using the Liquid Phase Blocking ELISA (LPBE) and then any positive sera in this system were tested to differentiate infected animals from vaccinated by using four commercial FMDV NSP ELISA kits. The negative and positive results were evaluated to compare the sensitivity and the specificity of various FMDV NSP ELISA kits.

## 1. INTRODUCTION

According to outbreak reports and epidemiological data on infectious animal diseases in Myanmar, foot and mouth disease (FMD) is endemic; is the most serious and occurs every year. In 2003–2004 the FMD susceptible livestock population was approximately 11.7 million cattle, 2.6 million buffaloes, 4.8 million pigs and 2.2 million sheep and goats. Livestock production is mainly based on small scale holdings with traditional livestock farming system in rural areas. This makes it is difficult to implement FMD control plans systematically in Myanmar. The highest incidence usually occurs at the onset of the monsoon (May–August) and at the end of the monsoon (October–December). The first coincides with the cultivation season and the second at the time of harvesting hence FMD outbreaks correlate with the highest activities of draft animals in the field. Among the 7 States and 7 Divisions in Myanmar, FMD outbreaks are common where there is a large concentration of livestock, as found in the low lying plains from the central part to delta coast. These regions include Yangon, Ayeyarwady, Bago, Magway, Mandalay and Sagaing Divison. An epidemiological investigation to evaluate the immune status of animals in these regions is an essential precursor to an effective control and eradication programme for FMD. From 1999 to 2004, virus specimens from FMD outbreaks were investigated by ELISA and the serotypes were O, A and Asia 1.

## 2. MATERIALS AND METHODS

### 2.1. Serum collection and submission

A total of 4704 serum samples from cattle, buffaloes, pigs and goats from 10 of the States and Divisions (regions) out of 14 in Myanmar were collected for investigation of antibodies to structural and non-structural proteins of FMDV. These include:

- 1672 sera from Yangon Division, Magway, Ayeyarwady , Mandalay, Sagaing and Rakhine regions in 1999.
- 908 sera from Yangon, Ayeyarwady, Bago, Mandalay, Rakhine and Kachin regions in 2000.
- 425 sera from Sagaing, Mandalay, Yangon, Bago,Kachin and Rakhine regions in 2001.
- 581 sera from Sagaing, Mandalay, Yangon, Bago and Tanintharyi regions in 2002.
- 612 sera from Mandalay, Tanintharyi, Yangon and Shan regions in 2003.
- 506 sera from Yangon, Kachin, Tanintharyi, Ayeyarwady, Bago, Shan and Sagaing regions in 2004

The majority of serum samples were collected by staff of the National FMD Laboratory, Livestock Breeding and Veterinary Department (LBVD) with the assistance of local LBVD staff. Blood samples were taken by vein puncture into 15 mL vacutainer brand evacuated blood collection tubes. The vacutainer systems (0.9 x 38 mm x 20G-1.5 inches) and (0.8 x 25 mm x 21G-1 inch) were used. After leaving the tubes overnight, sera were decanted into sterile 2 mL cryo vials on the morning of the next day. The sera were placed in the aluminium racks with drawers and dividers and stored at $-20^{o}C$.

TABLE I. PERCENTAGE OF SERUM SAMPLE COLLECTION IN STATES AND DIVISIONS IN MYANMAR DURING 1999-2004

| State/ Divisions | Cattle and buffaloes | Sample size | % collection |
|---|---|---|---|
| Yangon | 528231 | 680 | 0.128 |
| Rakhine | 806395 | 1525 | 0.189 |
| Magway | 1523820 | 290 | 0.019 |
| Ayeyarwady | 1309802 | 295 | 0.023 |
| Mandalay | 1623348 | 445 | 0.027 |
| Sagaing | 2041898 | 212 | 0.010 |
| Bago | 1311106 | 205 | 0.016 |
| Kachin | 386047 | 81 | 0.028 |
| Tanintharyi | 243686 | 556 | 0.228 |
| Shan | 1473392 | 415 | 0.028 |
| Total | 11 247707 | 4704 | 0.041 |

## 2.2. Antigen detection in field FMD outbreaks

FMDV antigen detection was based on the standard sandwich ELISA to investigate for the presence of FMD viral antigens in the specimen. ELISA virus typing results from1999 to 2004 in State and Divisions are shown in Table II.

TABLE II. VIRUS TYPING FMD OUTBREAKS OF STATE (1999-2004)

| State/ Division | Virus types of FMD outbreak | | | | | |
|---|---|---|---|---|---|---|
| | 1999 | 2000 | 2001 | 2002 | 2003 | 2004 |
| Kachin | - | - | O | - | - | - |
| Kayah | - | O | - | - | - | - |
| Kayin | - | - | - | - | - | - |
| Chin | - | - | - | - | - | - |
| Mon | - | - | - | - | - | - |
| Rakhine | O | - | O | - | - | - |
| Shan | - | - | Asia1 | - | - | - |
| Sagaing | - | - | - | O | O | - |
| Tanintharyi | A | - | O | - | - | - |
| Bago | O | Asia1 | - | O | O | O |
| Magway | O | O | O | O | - | - |
| Mandalay | O | O | - | - | - | - |
| Yangon | O | O, Asia1 | O | O | - | O |
| Ayeyarwady | Asia1 | - | O | O | - | - |

ELISA kits for antigen detection were provided by WRL, Pirbright through the IAEA TC project MYA/5/009 and able to detect virus types: O, A, C and Asia1.

The bench protocol instructed by WRL was strictly followed to ensure a standard level of assay performance

## 2.3. Immunization with FMD vaccine

The States and Divisions that were sampled and tested have a high animal density and many cases of suspected FMD are reported every year. The National FMD Laboratory can produce about 100 000 doses of monovalent vaccine type O or Asia 1.

The vaccine is prepared with aluminium gel precipitation of double BEI treated preparations to inactivate the virus. During an FMD outbreak, animal movement control and ring vaccination was made.

## 2.4. Antibody ELISA kits

Liquid Phase Blocking ELISA kits were obtained from the WRL, Pirbright. FMDV NSP kits were from different producers:

- 3ABC Monoclonal Antibody Trapping ELISA (3ABC MAT ELISA) prepared by Pirbright.
- FMDV NSP 3ABC ELISA for  bovine-ovine prepared by Bommeli Diagnostics, Liebefeld-Bern, Switzerland.
- FMDV NSP 3B ELISA prepared by United Biomedical Incorporation ,USA.

- CEDI test FMDV-NSP kit prepared by CEDI Diagnostics B.V, Lelysatd, The Netherlands.

The sera were tested for FMD antibody in the LPBE and retested in the NSP ELISAs. In 2000-01 only the 3ABC MAT ELISA was used for NSP tests.

3. RESULTS

**3.1. Surveillance samples**

Table III shows the data after examining cattle samples from sero surveillance sampling from different states in 2000-2001.

TABLE III. RESULTS OBTAINED SEROSURVEILLANCE (2000–2001)

| **State/Division** | **Sera tested** | **LPBELISA** | | **NSP** | |
|---|---|---|---|---|---|
| | | Pos | Neg | Pos | Neg |
| Rakhine | 321 | 305 | 16 | 217 | 104 |
| Magway | 16 | 16 | - | 3 | 13 |
| Ayeyarwady | 63 | 63 | - | 37 | 26 |
| Yangon | 20 | 20 | - | 15 | 5 |
| Mandalay | 75 | 73 | 2 | 54 | 21 |
| Tanintharyi | 110 | 78 | 32 | 4 | 106 |
| QC(FMD Lab) | 22 | 18 | 4 | 1 | 21 |
| Total | 627 | 573 | 54 | 335 | 292 |

Table IV shows the data after examining cattle samples from sero surveillance sampling from different states in 2002.

TABLE IV. RESULTS OBTAINED SEROSURVEILLANCE (2002)

| **State/Division** | **Sera collected/tested** | | | | **Type O%** | **Type A%** | **Type Asia1%** | **All serotypes %** |
|---|---|---|---|---|---|---|---|---|
| | C&B | Pig | goat | Total | | | | |
| Ayeyarwady | 5 | 7 | - | 12 | 41.66 | 16.7 | 33.3 | 16.7 |
| Bago | 18 | 23 | - | 41 | 36.58 | 4.9 | 7.3 | - |
| Mandalay | 5 | - | 38 | 43 | 23.25 | - | 9.3 | 4.6 |
| Rakhine | 44 | - | - | 44 | 31.81 | - | - | - |
| Sagaing | 18 | - | - | 18 | 50 | - | 27.8 | 22.2 |
| Tanintharyi | 214 | - | - | 214 | 8.4 | 3.3 | 1.4 | - |
| Yangon | 101 | 182 | - | 283 | 52.29 | 6.7 | 28.6 | 24.0 |
| Total / overall mean% | 405 | 212 | 38 | 655 | 34.8 | 4.5 | 15.3 | 9.6 |

Table V shows the data after examining samples from cattle, buffalo and pigs in 2003

TABLE V. RESULTS OF TESTS ON CATTLE, BUFFALO AND PIGS (2003–2004)

| State/Division | Sample collected | | | Sera tested | Type O % | Type A % | Type Asia1 % | All serotypes % |
|---|---|---|---|---|---|---|---|---|
| | C&B | Pig | Total | | | | | |
| Bago | 23 | - | 23 | - | NT | NT | NT | NT |
| Kachin | 54 | - | 54 | 54 | 29.6 | - | - | - |
| Mandalay | 79 | - | 79 | 66 | 75.7 | 31.8 | 24.2 | 3.0 |
| Shan | 261 | - | 261 | 240 | 20.0 | 12.9 | 7.1 | 0.4 |
| Tanintharyi | 306 | - | 306 | 306 | 13.1 | 7.2 | 0.9 | 0.3 |
| Yangon | 38 | 10 | 48 | 48 | 58.3 | - | 10.4 | - |
| Total/overall mean% | 761 | 10 | 771 | 771 | 39.3 | 10.4 | 8.5 | 0.7 |

NT= Not tested
(LPBELISA, Positive=>PI 50%)
Cattle, buffaloes, pigs & goats examined from different State and Divisions

## 3.2. Comparative test data

Table VI compares NSP ELISA data after examining samples from cattle, buffalo and goats.

TABLE VI. ANTI NSP ANTIBODY RESULTS CATTLE, BUFFALOES AND GOATS

| State/Divisions | UBI | | | Bommeli | | | CEDI | | |
|---|---|---|---|---|---|---|---|---|---|
| | Sera tested | No. of Pos: | Pos % | Sera tested | No. of Pos: | Pos % | Sera tested | No. of Pos: | Pos % |
| Ayeyarwady | 4 | 4 | 100 | 4 | 4 | 100 | 1 | 1 | 100 |
| Bago | 20 | 11 | 55 | 20 | 16 | 80 | 20 | 18 | 90 |
| Mandalay | 70 | 35 | 50 | 70 | 49 | 70 | 36 | 35 | 97 |
| Rakhine | 31 | 19 | 61 | 31 | 18 | 58 | 11 | 11 | 100 |
| Shan | 146 | 26 | 18 | 125 | 23 | 18 | 125 | 55 | 44 |
| Yangon | 117 | 38 | 33 | 117 | 44 | 38 | 27 | 25 | 93 |
| Experimental (vaccinate) | 10 | 1 | 10 | 10 | 2 | 20 | NT | NT | NT |
| Total/ percentage | 630 | 151 | 23.9 | 540 | 159 | 29.4 | 360 | 192 | 53.3 |

NT= Not tested

Table VII shows comparative test data after examining samples from pigs.

TABLE VII. RESULTS ASSESSING PIGS UNDER SEROSURVEILLANCE (SWINE)

| State/Divisions | UBI | | | Bommeli | | | CEDI | | |
|---|---|---|---|---|---|---|---|---|---|
| | Sera tested | No. of Pos | Pos % | Sera tested | No. of Pos: | Pos % | Sera tested | No. of Pos: | Pos % |
| Bago | 50 | 7 | 14.0 | NT | - | - | 50 | 0 | - |
| Yangon | 40 | 22 | 55.0 | NT | - | - | 40 | 24 | 60.0 |
| Total/ percentage | 90 | 29 | 32.2 | NT | - | - | 90 | 24 | 26.6 |

NT= Not tested

(c) Analysis of control values in the test plates

### 3.3. Plate control data

Table VIII shows the data analysing plate control data obtained in the UBI NSP ELISA

TABLE VIII. UBI-NSP TESTS PLATE CONTROL DATA

| Plate No | NRCs | RCs | Cut-off value | Species/No.of sera tested | Acceptance |
|---|---|---|---|---|---|
| 1 | 0.058 | 0.751 | 0.173 | Bov/45 | Valid |
| 2 | 0.066 | 0.733 | 0.168 | Bov/45 | Valid |
| 3 | 0.056 | 1.133 | 0.260 | Bov/45 | Valid |
| 4 | 0.071 | 0.862 | 0.198 | Bov/45 | Valid |
| 5 | 0.063 | 1.520 | 0.349 | Bov/90 | Valid |
| 6 | 0.063 | 1.320 | 0.305 | Bov/90 | Valid |
| 7 | 0.047 | 1.070 | 0.246 | Bov/90 | Valid |
| 8 | 0.040 | 0.905 | 0.208 | Bov/90 | Valid |
| 9 | 0.051 | 1.700 | 0.391 | Bov/90 | Valid |
| 10 | 0.047 | 0.815 | 0.187 | Swine/90 | Valid |

NRCs=non-reactive control serum

RCs = reactive control serum

Table IX shows the data analysing plate control data obtained in the BOMMELI NSP ELISA

TABLE IX. BOMMELI-NSP TEST PLATE CONTROL DATA

| Plate No | NCs | PCs | Cut-off value | Species/No.of sera tested | Acceptance |
|---|---|---|---|---|---|
| 1 | 0.116 | 1.614 | Value% | Bov/45 | Valid |
| 2 | 0.128 | 1.485 | must be | Bov/45 | Valid |
| 3 | 0.151 | 1.565 | >30% | Bov/45 | Valid |
| 4 | 0.150 | 1.446 | Difference | Bov/45 | Valid |
| 5 | 0.164 | 1.286 | OD between | Bov/90 | Valid |
| 6 | 0.202 | 1.209 | PC&NC | Bov/90 | Valid |
| 7 | 0.162 | 1.228 | Must be | Bov/90 | Valid |
| 8 | 0.180 | 1.388 | >0.4 | Bov/90 | Valid |

NCs=Negative control serum
PCs= Positive control serum

Table X shows the data analysing plate control data obtained in the CEDI NSP ELISA

TABLE X. CEDI -NSP TESTS PLATES CONTROL DATA

| Plate No | Mean OD of Blank | Mean PI Ref; serum 1 | Mean PI Ref; serum 2 | Cut-off PI | Species/ No. of sera tested | Acceptance |
|---|---|---|---|---|---|---|
| 1 | 1.55 | 91 | 12 | Pos=>50PI | Bov/90 | Valid |
| 2 | 1.78 | 94 | 4 | Blank OD | Bov/90 | Valid |
| 3 | 1.81 | 87 | 21 | must be > 1.0 | Bov/90 | Valid |
| 4 | 1.97 | 83 | 13 | | Bov/90 | Valid |
| 5 | 1.38 | 86 | 13 | | Swine/90 | Valid |

## 3.4 Comparison of NSP kits

A total of 311 serum samples found positive in the LPBE were examined using three NSP ELISA kits. Some of the sera were collected from outbreak areas and hence likely to be infected. Some of the sera were collected from the clinically free areas showing positive results. The following tables show the region-wide results of three commercial NSP kits for post infection, vaccinated and negative animals.

*3.4.1. Cattle, buffalo and pigs tested for specificity*

TABLE XI. RESULTS COMPARING COMMERCIAL ELISA AND LPBE DATA

| State/Division | No. | LPBE | | UBI | | Bommeli | | CEDI | |
|---|---|---|---|---|---|---|---|---|---|
| | | N/ST | % | N/ST | % | N/ST | % | N/ST | % |
| Rakhine | 3 | 3/3 | 100 | 2/3 | 66.6 | 2/3 | 66.6 | NT | - |
| Shan | 83 | 83/83 | 100 | 80/83 | 96.4 | 80/83 | 96.4 | 65/83 | 78.3 |
| Tanintharyi | 25 | 25/25 | 100 | 25/25 | 100. | 25/25 | 100 | NT | - |
| Tanintharyi | 32 | 32/32 | 100 | 29/31 | 93.5 | 31/31 | 100 | 25/32 | 78.1 |
| Tanintharyi | 50 | 50/50 | 100 | 48/50 | 96.0 | 49/50 | 98.0 | 30/41 | 73.2 |
| | 50 | 50/50 | 100 | 43/50 | 86.0 | NT | - | 50/50 | 100 |
| Bago (Swine) Experimental | 1 | 1/1 | 100 | 1/1 | 100. | 1/1 | 100. | NT | - |
| Total/percent | 244 | 244/244 | 100 | 228/243 | 93.8 | 188/193 | 97.4 | 170/206 | 82.5 |

N= Negative
ST= Sera tested
NT=Not tested

*3.4.2. Positivity sera compared by different tests*

Sera collected and tested from outbreak areas and likely to be carriers

TABLE XII. POSSIBLY POSITIVE SAMPLES TESTED FOR SENSITIVITY

| State/Division | No. | LPBE | | UBI | | Bommeli | | CEDI | |
|---|---|---|---|---|---|---|---|---|---|
| | | P/ST | % | P/ST | % | P/ST | % | P/ST | % |
| Ayeyarwady | 4 | 4/4 | 100 | 4/4 | 100. | 4/4 | 100 | 1/1 | 100 |
| Bago | 20 | 15/15 | 100 | 11/20 | 55.0 | 16/20 | 80.0 | 18/20 | 90.0 |
| Mandalay | 36 | 35/35 | 100 | 27/36 | 75.0 | 31/36 | 86.1 | 35/35 | 100 |
| Rakhine | 12 | 12/12 | 100 | 12/12 | 100 | 12/12 | 100. | 11/11 | 100 |
| Rakhine | 4 | 3/4 | 75.0 | 4/4 | 100 | 2/4 | 50.0 | NT | - |
| Shan(North) | 33 | 33/33 | 100 | 22/33 | 66.7 | 18/33 | 54.5 | 30/33 | 90.9 |

| State/Division | No. | LPBE | | UBI | | Bommeli | | CEDI | |
|---|---|---|---|---|---|---|---|---|---|
| | | P/ST | % | P/ST | % | P/ST | % | P/ST | % |
| Shan(East) | 9 | 9/9 | 100 | 1/9 | 11.1 | 2/9 | 22.2 | 7/9 | 77.8 |
| Tanintharyi | 32 | 32/32 | 100 | 11/32 | 34.4 | 2/32 | 6.2 | 27/32 | 84.4 |
| Yangon | 21 | 21/21 | 100 | 21/21 | 100 | 21/21 | 100. | 17/17 | 100. |
| Total/percent | 171 | 164/165 | 99.4 | 113/171 | 66.1 | 108/171 | 63.1 | 146/158 | 92.4 |

N=Negative
P= Positive
ST= Sera tested
NT=Not tested

TABLE XIII. RESULTS ON VACCINATED CATTLE AND BUFFALOES BY NSP ELISA

| State/Division | No. | LPBE | | UBI | | Bommeli | | CEDI | |
|---|---|---|---|---|---|---|---|---|---|
| | | P/ST | % | N/ST | % | N/ST | % | N/ST | % |
| Bago | 1 | 1/1 | 100. | 1/1 | 100. | 1/1 | 100. | 1/1 | 100 |
| Mandalay | 1 | 1/1 | 100 | 1/1 | 100. | 1/1 | 100. | 1/1 | 100 |
| Mandalay | 32 | 32/32 | 100 | 25/32 | 21.8 | 15/32 | 46.9 | NT | - |
| Rakhine | 13 | 13/13 | 100 | 10/13 | 23.1 | 9/13 | 69.2 | NT | - |
| Tanintharyi | 17 | 17/17 | 100 | 17/17 | 100. | 17/17 | 100. | 13/13 | 100 |
| Yangon | 95 | 95/95 | 100 | 78/95 | 82.1 | 72/95 | 75.8 | 2/10 | 20.0 |
| Experimental | 9 | 9/9 | 100 | 8/9 | 88.9 | 7/9 | 77.8 | NT | - |
| Total/percent | 168 | 168 | 100 | 140 | 83.3 | 122 | 72.6 | 17/25 | 68.0 |

N=Negative
ST= Sera tested
P= Positive
NT=Not tested

## 4. DISCUSSION

Myanmar is not a FMD free country and sera suitable for testing infected, vaccinated and negative animals were collected from FMD outbreak areas; clinically free areas for some years and from vaccinated livestock (using locally produced FMD monovalent vaccine). Generally all the NSP ELISA kits worked very well. Antibodies in sera were firstly detected by the LPBE and confirmation was made by NSP tests as to whether animals were of an

infected, vaccinated or negative status. Therefore, the diagnositic sensitivity of LPBE is taken as 100% when comparing the test performances of the NSP ELISA kits. The diagnostic sensitivity (DSn) of the CEDI test kit was highest. The specificity of the UBI and Bommeli are the same and higher than the CEDI test kit. When working with the majority of plates until the expiry date mentioned on the kit box, there was no significant difference in relation to the controls. All the plates tested in three NSP kits passed the validity criteria determined by the kit instructions of the manufacturers. Therefore, robustness of the reagents in all three kits is acceptable. Test plate incubation at 37$^{\circ}$C is necessary in the procedure of UBI and Bommeli kits, but CEDI needs only room temperature incubation thus is more suitable to make the tests in the field situation, where electricity and incubators are not available. The CEDI kit takes two days for one testing run.

The NSP results from the three kits for testing serum samples collected during and after FMD outbreaks in Myanmar are excellent for determining sensitivity of the test kits. But in the region with no FMD outbreaks, some results may be confused with the real FMD situation in that area such as Tanintharyi Division of MTM area. In that area a small percentage of sera showed NSP positive mostly in CEDI test. Officials from OIE Regional Coordination Unit for Southeast Asia FMD Control Programme and FMD laboratory staff of the Livestock Breeding and Veterinary Department (LBVD) of Myanmar visited villages in the Buffer zone and Control Zone of MTM FMD campaign area of Myanmar to survey the real situation of FMD in that area. The team studied the outbreak history of cattle and buffaloes and collected the serum samples to be tested for the evidence of FMD in this Division. Those sera were tested recently and shown in the result tables of this report. It will be necessary to study more serological investigations for more precise results in the MTM FMD campaign area. The FMD laboratory also receives technical know-how and able to detect more effectively between vaccinated and infected animals by the FAO/IAEA Coordinated research project.

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# THE USE OF NON-STRUCTURAL PROTEINS OF FMDV TO DIFFERENTIATE BETWEEN VACCINATED AND INFECTED ANIMALS IN THAILAND

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**Abstract**

The detection of antibody to non-structural proteins (NSP) of foot and mouth disease virus (FMDV) using various types of NSP kits produced from World Reference Laboratory (WRL), United Biomedical Inc.(UBI), Bommeli/Intervet, CEDI and other research institutes, has been studied. The Liquid Phase blocking ELISA (LPBE) for measurement of antibody to FMDV type O, A and Asia1 was used in parallel with the NSP tests to study the specificity and sensitivity of each NSP test kit. Three thousand nine hundred and twenty two serum samples from cattle, buffaloes and pigs were grouped according to known history comprising: non vaccinated, single vaccinated, multiple vaccinated and field infected animals. The NSP tests all gave showed high specificity in non vaccinated cattle of 100%, 100%, 99% and 100% respectively. In pigs both the WRL and UBI kits gave 100%. In the single vaccination group, the specificities were WRL = 99.5% and UBI = 98.7%. The NSP test in multiple vaccinated cattle and buffaloes gave specificities of WRL = 97.73%, and UBI = 100%. In the FMD field outbreak area, clinically distinguishable infected cattle gave sensitivity results by NSP tests of WRL, 98.3%; UBI, 88.9%; Bommeli/Intervet, 84.6%; CEDI, 90.1%; Inoue/Japan, 93.8%; USDA, 88.3%; and Pen Side/Korea, 84.6%. This study indicates that the NSP kits from WRL, UBI, Bommeli/ Intervet, and CEDI had a high specificity for cattle and pigs from 99-100% and a high sensitivity from 84.6-93.8% in cattle and 73.33-80% in pigs. In addition, the NSP tests have been applied to FMD sero surveillance in animals being quarantined at the international quarantine station in a multilateral Malaysia Thailand Myanmar (MTM) project and also used in sero-monitoring of field animals in the Thailand national plan for the FMD vaccination campaign programme in the country. Hence, the use of NSP test to differentiate between vaccinated and infected animals would be used as a standard diagnostic test for the control and eradication of FMD in the region.

## 1. INTRODUCTION

Foot and mouth disease (FMD), an acute, highly contagious disease of cloven-hoofed animal, is an important economic disease of livestock in Thailand. There are three serotypes of O, A and Asia1 that are considered endemic in the country and cause economic losses due to decreased production and export trade restrictions. Rapid and accurate diagnosis of the disease is very important to prevent spread and assist with the selection of the appropriate vaccines. The Regional Reference Laboratory for FMD in South East Asia (RRL) at Pakchong Nakhonratchasima Province, Thailand, provides the FMD diagnosis service to the region.

The standard ELISA typing test [1] is used to assay field samples submitted for type identification. Other serological tests include the virus neutralization (VN) test [2] and the liquid phase blocking ELISA (LPBE) [3] have been used for disease surveillance and sero-monitoring of vaccinated animals in parallel with the virus infection associated agar gel immunodiffusion (VIA-AGID) test [4]. Cowan and Graves [5] proposed this test for differentiating infected from infected animals. The VIA antigen has been identified as RNA-

dependent RNA polymerase which is presented in virus preparations used for vaccine production and is also a constituent of the 146S viral particles which is a major immunogenicity of viral harvests [6].

Recently an advanced biotechnology application of developed 2B, 3B, 3AB, 3ABC and the 3D non-structural protein of FMDV have been used to replace the VIA-AGID test [7], [8], [9]. This test uses the non-structural proteins of FMDV in an indirect ELISA [10] or competitive ELISA [11] and tests have demonstrated as showing a higher specificity and sensitivity as compared to existing methods for differentiating the vaccinated from naturally infected animals [12].

The Department of Livestock Development (DLD) has been established the national FMD vaccination campaign over the country. A trivalent vaccine FMD type O, A and Asia1 has been used for vaccinating field animals, twice a year. The purified vaccine is produced by the Bureau of Veterinary Biologics (BVB) and contains FMDV type O, A and Asia1. The seed virus vaccine strains were selected from local strains causing outbreaks in Thailand. The serum samples were collected one month after vaccination in each round, to determine the antibody titre against FMDV type O, A and Asia1 using the LPBE and also to determine the antibodies to FMD NSP.

The IAEA Coordinated Research Project (CRP) provided NSP kits from World Reference Laboratory (WRL), Pirbright and various other commercial NSP kits that were used to detect antibodies against 2B, 3B, 3AB and 3ABC in animal sera from many sources. In addition the assays were used to examine sera sampled from sero monitoring and disease surveillance campaigns at the national level during the FMD vaccination campaign programme. The tests were also compared in surveillance of animals along the border or being at quarantine stations supporting the bilateral and multilateral project on the establishment of disease free zone in Malaysia Thailand Myanmar (MTM), and the Upper Mekong and Lower Mekong projects in SEAFMD countries.

The objectives were:

- To study the specificity of various NSP reagent kits in difference animal status.
- To study the sensitivity of various NSP reagent kits in nationally infection and experimental animals.
- Application of the NSP test in sero-monitoring and surveillance in field animals at national and regional level.

## 2.    MATERIALS AND METHODS

### 2.1.    Serum samples

A total of 3922 serum samples from various groups of animal were studied including non vaccination, single vaccination, multiple vaccinations, experimental infection and nationally infected with vaccination. Field sera from FMD vaccination campaign and regional project were also tested. Those samples were used to detect antibodies to structural protein of FMDV type O, A and Asia1 by the LBPE and to detect antibody to NSP of FMDV by indirect ELISA and competitive ELISA.

## 2.2. Purified vaccine

FMD trivalent vaccine containing type O, A and Asia1 was produced by the Bureau of Veterinary Biologics (BVB), Department of Livestock Development, and Thailand. The cattle Al(OH)$_3$ adjuvant vaccine and pig oil adjuvant vaccine were prepared by the suspension method using 146S antigen as the major antigenic component.

## 2.3. Liquid phase blocking ELISA (LPBE)

The LPBE measuring total antibodies against FMDV O, A and Asia1 was performed in duplicate, using two-fold dilution series method described by [3] and 13].

## 2.4. Non-structural protein (NS) test

NSP test by using various type of NSP kits were listed as this following:

### 2.4.1. WRL NSP reagents

A NSP reagent set produced by World Reference Laboratory (WRL)/Brescia, Pirbright Laboratory, United Kingdom was used to detect antibody to 3ABC NSP of FMDV by indirect ELISA, the test procedure was described in the instruction manual of WRL.

### 2.4.2. UBI$^®$ FMDV NS EIA (UBI)

A commercial NSP kit produced by United Biochemical Inc. USA was used to detect antibodies to FMDV 3B by indirect ELISA, the test procedure was described in the instruction manual of the manufacturer.

### 2.4.3. CHEKIT FMDV-3ABC Bommeli

A commercial NSP kit produced by Bommeli Switzerland, was used to detect antibodies to FMDV 3ABC by indirect ELISA, the test procedure was described in the instruction manual of the manufacturer.

### 2.4.4. CEDI Test$^®$ FMDV-NS (CEDI)

A commercial NSP kit from CEDI Diagnostic was used to detect antibody to 3ABC NSP of FMDV by competitive ELISA method, as described by instruction manual.

## 2.5. Other NSP reagents

### 2.5.1. 3AB NS reagent.

Developed by USDA, Plum Island Animal Disease Center, USA, the test procedure was described by protocol of USDA.

### 2.5.2. 2B NS reagent

Developed by Dr. Toru Inoue, NIAH, Japan, the test procedure was described in the instruction manual of NIAH.

*2.5.3. ABC rapid pen-side test*

A commercial NSP kit produced by Korea, the test procedure was described in the instruction manual of the manufacturer.

TABLE I. SERUM SAMPLES USED FOR TESTING OF NSP KITS AND PEN-SIDE TEST. (TOTAL SAMPLE = 3922 )

| Immunization status | Species | No. of sample |
|---|---|---|
| Non vaccination and non infection | Cattle/pig | 380 |
| Single vaccination and non infection | Cattle/pig | 395 |
| Multiple vaccination and non infection | Cattle/buffalo | 220 |
| Nationally infection and vaccination | Cattle/pig | 360 |
| 1 month post vaccination  from the national FMD control programme | Cattle/buffalo | 1515 |
| Animal movement at quarantine station | Cattle/buffalo | 1052 |

3.    RESULTS

## 3.1.    Data from cattle and pigs

In order to illustrate the extent of testing, the data summarising a variety of tests is show in the next series of tables. A = agreement of all tests was positive. D = disagreement in ELISA results. A = agreement between ELISA test data as negative, but AGID positive.

## TABLE II. DATA FROM CATTLE COMPARING LPBE, VIA AGID, WRL AND UBI TESTS

| Exp.no | Serum history | LP ELISA | | | VIA | | | |
|--------|---------------|------|------|------|------|------|------|------|
| | | O | A | AS1 | Test | WRL | UBI | |
| 1110 | Field sera | 2560 | 160 | 640 | + | + | + | A |
| 1111 | 2 weeks post infection | 2560 | 320 | 640 | + | + | + | A |
| 1112 | from field outbreak of | 5120 | 2560 | 640 | + | + | + | A |
| 1113 | FMDV | 5120 | 2560 | 2560 | + | + | + | A |
| 1114 | " | 2560 | 2560 | 2560 | + | - | - | A- |
| 1115 | " | 5120 | 2560 | 2560 | + | + | + | A |
| 1116 | " | 5120 | 2560 | 640 | + | + | + | A |
| 1117 | " | 5120 | 640 | 640 | + | + | + | A |
| 1118 | " | 5120 | 640 | 2560 | + | + | + | A |
| 1119 | " | 5120 | 640 | 640 | + | + | + | A |
| 1120 | " | 5120 | 640 | 2560 | + | + | + | A |
| 1121 | " | 5120 | 160 | 320 | + | + | + | A |
| 1122 | " | 5120 | 2560 | 2560 | + | + | + | A |
| 1123 | " | 5120 | 2560 | 5120 | + | + | + | A |
| 1124 | " | 5120 | 2560 | 1280 | + | + | + | A |
| 1125 | " | 5120 | 2560 | 2560 | + | - | - | A- |
| 1126 | " | 5120 | 1280 | 640 | + | + | + | A |
| 1127 | " | 5120 | 5120 | 5120 | + | - | - | A- |
| 1128 | " | 5120 | 2560 | 2560 | + | - | - | A- |
| *1129* | *"* | *2560* | *2560* | *640* | *+* | *+* | *-* | *D* |
| 1130 | " | 5120 | 2560 | 5120 | + | - | - | A- |
| Number | | O | A | Asia1 | VIA | WRL | UBI | |
| 1131 | " | 5120 | 640 | 2560 | + | + | + | A |
| 1132 | " | 5120 | 5120 | 5120 | + | + | + | A |
| 1133 | " | 5120 | 640 | 640 | + | + | + | A |
| 1134 | " | 5120 | 5120 | 2560 | + | + | + | A |
| 1135 | " | 5120 | 2560 | 2560 | + | + | + | A |
| 1136 | " | 5120 | 320 | 640 | + | + | + | A |
| 1137 | " | 5120 | 320 | 320 | + | + | + | A |
| 1138 | " | 5120 | 2560 | 320 | + | + | + | A |
| 1139 | " | 5120 | 160 | 160 | + | + | + | A |
| 1140 | " | 5120 | 1280 | 1280 | + | + | + | A |
| 1141 | " | 5120 | 2560 | 2560 | + | + | + | A |
| 1142 | " | 5120 | 2560 | 1280 | + | + | + | A |
| 1143 | 2 weeks post infection from | 5120 | 1280 | 1280 | + | + | + | A |
| 1144 | field outbreak of FMDV | 5120 | 1280 | 1280 | + | + | + | A |
| 1145 | Type O | 5120 | 5120 | 5120 | + | + | + | A |
| 1146 | " | 5120 | 5120 | 2560 | + | + | + | A |
| 1147 | " | 5120 | 5120 | 2560 | + | + | + | A |
| 1148 | " | 5120 | 5120 | 5120 | + | + | + | A |
| 1149 | " | 5120 | 5120 | 2560 | + | + | + | A |
| 1150 | " | 5120 | 2560 | 1280 | + | + | + | A |
| *1151* | *"* | *5120* | *5120* | *5120* | *+* | *+* | *-* | *D* |
| 1152 | " | 5120 | 5120 | 5120 | + | + | + | A |
| *1153* | *"* | *2560* | *5120* | *5120* | *+* | *+* | *-* | *D* |
| 1154 | " | 5120 | 5120 | 1280 | + | - | - | A- |
| 1155 | | 5120 | 5120 | 5120 | + | + | + | A |
| 1156 | | 5120 | 5120 | 5120 | + | + | + | A |

| Exp.no | Serum history | LP ELISA | | | VIA | | | |
|---|---|---|---|---|---|---|---|---|
| | | O | A | AS1 | Test | WRL | UBI | |
| 1157 | | 5120 | 5120 | 5120 | + | + | + | A |
| 1158 | | 5120 | 2560 | 2560 | + | + | + | A |
| 1159 | | 5120 | 5120 | 5120 | + | + | + | A |
| *1160* | " | *5120* | *5120* | *5120* | + | + | - | *D* |
| 1161 | " | 5120 | 5120 | 5120 | + | - | - | **A-** |
| 1162 | " | 5120 | 5120 | 2560 | + | + | + | A |
| 1163 | " | 5120 | 5120 | 5120 | + | + | + | A |
| 1164 | " | 5120 | 5120 | 5120 | + | + | + | A |
| 1165 | " | 5120 | 5120 | 2560 | + | + | + | A |
| 1166 | " | 5120 | 640 | 640 | + | + | + | A |
| 1167 | " | 5120 | 5120 | 5120 | + | + | + | A |
| 1168 | " | 5120 | 1280 | 1280 | + | + | + | A |
| 1169 | " | 5120 | 5120 | 5120 | + | + | + | A |
| 1170 | " | 5120 | 1280 | 5120 | + | + | + | A |
| 1171 | " | 5120 | 2560 | 5120 | + | + | + | A |
| 1172 | " | 2560 | 640 | 1280 | + | + | + | A |
| 1173 | " | 5120 | 5120 | 5120 | + | + | + | A |
| 1174 | " | 5120 | 320 | 320 | + | + | + | A |
| 1175 | " | 5120 | 2560 | 2560 | + | + | + | A |
| 1176 | " | 5120 | 5120 | 5120 | + | + | + | A |
| Number | | O | A | Asia1 | VIA | WRL | UBI | |
| 1177 | " | 5120 | 640 | 1280 | + | + | + | A |
| 1178 | 2 weeks post infection from | 1280 | 640 | 320 | + | + | + | A |
| 1179 | field outbreak of FMDV | 5120 | 320 | 1280 | + | + | + | A |
| 1180 | Type O | 5120 | 5120 | 1280 | + | + | + | A |
| 1181 | " | 2560 | 640 | 1280 | + | + | + | A |
| 1182 | " | 5120 | 2560 | 1280 | + | + | + | A |
| 1183 | " | 2560 | 640 | 640 | + | + | + | A |
| 1184 | " | 5120 | 320 | 640 | + | + | + | A |
| 1185 | " | 5120 | 1280 | 2560 | + | + | + | A |
| 1186 | " | 5120 | 1280 | 2560 | + | + | + | A |
| 1187 | " | 5120 | 1280 | 2560 | + | + | + | A |
| 1188 | " | 5120 | 5120 | 5120 | + | + | + | A |
| 1189 | " | 5120 | 1280 | 5120 | + | + | + | A |
| 1190 | " | 5120 | 2560 | 2560 | + | + | + | A |
| 1191 | " | 5120 | 1280 | 640 | + | + | + | A |
| 1192 | " | 640 | 1280 | 640 | + | + | + | A |
| *1193* | " | *5120* | *5120* | *5120* | + | + | - | *D* |
| 1194 | " | 5120 | 1280 | 2560 | + | + | + | A |
| 1195 | " | 5120 | 5120 | 2560 | + | + | + | A |
| 1196 | " | 5120 | 2560 | 5120 | + | - | - | **A-** |
| *1197* | " | *5120* | *320* | *640* | - | + | - | *D* |
| 1198 | " | 5120 | 1280 | 5120 | + | + | + | A |
| 1199 | " | 2560 | 320 | 1280 | + | + | + | A |
| 1200 | " | 5120 | 2560 | 2560 | + | + | + | A |
| 1201 | " | 5120 | 5120 | 5120 | + | + | + | A |
| 1202 | " | 5120 | 640 | 2560 | + | + | + | A |

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| Exp.no | Serum history | LP ELISA | | | VIA | | | |
|---|---|---|---|---|---|---|---|---|
| | | O | A | AS1 | Test | WRL | UBI | |
| 1203 | " | 5120 | 1280 | 5120 | + | + | + | A |
| 1204 | " | 5120 | 1280 | 5120 | + | + | + | A |
| 1205 | 2 weeks post infection from | 1280 | 320 | 320 | + | + | + | A |
| 1206 | field outbreak of FMDV | 5120 | 640 | 640 | + | + | + | A |
| 1207 | Type O | 5120 | 320 | 1280 | + | + | + | A |
| 1208 | " | 5120 | 640 | 1280 | + | + | + | A |
| 1209 | " | 5120 | 5120 | 5120 | + | + | + | A |
| 1210 | " | 80 | 320 | 320 | + | + | + | A |
| 1211 | " | 320 | 5120 | 5120 | + | + | + | A |
| 1212 | " | 80 | 2560 | 2560 | + | + | + | A |
| 1213 | " | 160 | 640 | 1280 | + | + | + | A |
| 1214 | " | 80 | 5120 | 5120 | + | + | + | A |
| 1215 | " | 5120 | 320 | 640 | + | + | + | A |
| 1216 | " | 5120 | 320 | 2560 | + | + | + | A |
| 1217 | " | 5120 | 5120 | 5120 | + | + | + | A |
| 1218 | " | 5120 | 320 | 640 | + | + | + | A |
| 1219 | " | 5120 | 640 | 640 | + | + | + | A |
| 1220 | " | 5120 | 5120 | 5120 | + | + | + | A |
| 1221 | " | 5120 | 5120 | 2560 | + | + | + | A |
| 1222 | " | 5120 | 1280 | 320 | + | + | + | A |
| 1223 | " | 5120 | 5120 | 1280 | + | + | + | A |
| 1224 | " | 2560 | 2560 | 1280 | + | + | + | A |
| 1225 | " | 5120 | 320 | 320 | + | + | + | A |
| 1226 | " | 5120 | 5120 | 640 | + | + | + | A |
| 1227 | " | 5120 | 5120 | 640 | + | + | + | A |
| 1228 | " | 5120 | 5120 | 2560 | + | + | + | A |
| **1229** | " | 2560 | 2560 | 2560 | + | - | - | **A-** |
| 1230 | " | 2560 | 1280 | 2560 | + | + | + | A |
| 1231 | " | 5120 | 2560 | 1280 | + | + | + | A |
| 1232 | " | 5120 | 640 | 1280 | + | + | + | A |
| *1233* | " | *640* | *640* | *2560* | + | + | *-* | *D* |
| 1234 | " | 2560 | 160 | 640 | + | + | + | A |
| *1235* | '" | *640* | *640* | *640* | + | + | *-* | *D* |
| 1236 | " | 5120 | 1280 | 2560 | + | + | + | A |
| 1237 | " | 5120 | 5120 | 5120 | + | + | + | A |
| 1238 | " | 2560 | 160 | 640 | + | + | + | A |
| 1239 | " | 640 | 80 | 320 | + | + | + | A |
| 1240 | " | 2560 | 2560 | 1280 | + | + | + | A |
| 1241 | " | 1280 | 320 | 640 | + | + | + | A |
| 1242 | " | 5120 | 2560 | 2560 | + | + | + | A |
| 1243 | " | 5120 | 320 | 640 | + | + | + | A |
| 1244 | " | 2560 | 640 | 2560 | + | + | + | A |
| 1245 | " | 2560 | 160 | 640 | + | + | + | A |
| 1246 | " | 5120 | 80 | 320 | + | + | + | A |
| 1247 | " | 2560 | 160 | 320 | + | + | + | A |
| 1248 | " | 5120 | 160 | 640 | + | + | + | A |

| Exp.no | Serum history | LP ELISA | | | VIA | | | |
|--------|---------------|----------|----------|----------|------|-----|-----|---|
| | | O | A | AS1 | Test | WRL | UBI | |
| 1249 | " | 2560 | 160 | 640 | + | + | + | A |
| 1250 | " | 5120 | 2560 | 5120 | + | + | + | A |
| *1251* | *"* | *5120* | *5120* | *5120* | + | + | *-* | *D* |
| 1252 | " | 5120 | 2560 | 2560 | + | + | + | A |
| 1253 | " | 5120 | 640 | 1280 | + | + | + | A |
| 1254 | " | 5120 | 160 | 640 | + | + | + | A |
| *1255* | *"* | *5120* | *2560* | *2560* | + | + | - | *D* |
| 1256 | " | 5120 | 160 | 320 | + | + | + | A |
| 1257 | " | 5120 | 160 | 640 | + | + | + | A |
| *1258* | *"* | *5120* | *1280* | *1280* | + | + | *-* | *D* |
| 1259 | " | 5120 | 160 | 640 | + | + | + | A |
| 1260 | " | 5120 | 320 | 320 | + | + | + | A |
| 1261 | " | 5120 | 5120 | 5120 | + | + | + | A |
| 1262 | " | 5120 | 640 | 320 | + | + | + | A |
| 1263 | " | 5120 | 5120 | 5120 | + | + | + | A |
| 1264 | " | 5120 | 5120 | 5120 | + | + | + | A |
| 1265 | " | 5120 | 5120 | 1280 | + | + | + | A |
| 1266 | " | 1280 | 640 | 640 | + | + | + | A |
| 1267 | " | 2560 | 640 | 640 | + | + | + | A |
| 1268 | " | 5120 | 5120 | 640 | + | + | + | A |
| 1269 | " | 5120 | 1280 | 160 | + | + | + | A |
| 1270 | " | 5120 | 640 | 320 | + | + | + | A |
| 1271 | " | 5120 | 1280 | 2560 | + | + | + | A |
| 1272 | 1 month post infection from | 2560 | 640 | 1280 | + | + | + | A |
| 1273 | field outbreak of FMDV | 2560 | 160 | 320 | + | + | + | A |
| 1274 | Type O | 5120 | 1280 | 640 | + | + | + | A |
| 1275 | Field serum | 5120 | 160 | 320 | + | + | + | A |
| 1276 | | 2560 | 640 | 160 | + | + | + | A |
| 1277 | " | 5120 | 5120 | 2560 | + | + | + | A |
| 1278 | " | 5120 | 2560 | 5120 | + | + | + | A |
| 1279 | " | 5120 | 1280 | 2560 | + | + | + | A |
| 1280 | " | 5120 | 320 | 320 | + | + | + | A |
| 1279 | " | 5120 | 1280 | 2560 | + | + | + | A |
| 1280 | " | 5120 | 320 | 320 | + | + | + | A |
| 1281 | " | 2560 | 320 | 160 | + | + | + | A |
| 1282 | " | 2560 | 640 | 320 | + | + | + | A |
| 1283 | " | 5120 | 2560 | 640 | + | + | + | A |
| 1284 | " | 640 | 160 | 160 | + | + | + | A |
| 1285 | " | 2560 | 160 | 160 | + | + | + | A |
| 1286 | " | 1280 | 80 | 160 | + | + | + | A |
| 1287 | " | 640 | 80 | 80 | + | + | + | A |
| 1288 | " | 2560 | 160 | 320 | + | + | + | A |
| 1289 | " | 1280 | 1280 | 640 | + | + | + | A |
| 1290 | " | 2560 | 160 | 160 | + | + | + | A |
| 1291 | " | 1280 | 160 | 320 | + | + | + | A |
| *1292* | *"* | *640* | *160* | *40* | + | + | *-* | *D* |

| Exp.no | Serum history | LP ELISA | | | VIA | | | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | | O | A | AS1 | Test | WRL | UBI | |
| 1293 | " | 1280 | 640 | 640 | + | + | + | A |
| 1294 | " | 5120 | 1280 | 640 | + | + | + | A |
| 1295 | " | 1280 | 640 | 320 | + | + | + | A |
| 1296 | " | 5120 | 640 | 1280 | + | + | + | A |
| 1297 | " | 5120 | 320 | 320 | + | + | + | A |
| 1298 | " | 2560 | 640 | 640 | + | + | + | A |
| 1299 | " | 1280 | 320 | 320 | + | + | + | A |
| 1300 | " | 2560 | 1280 | 1280 | + | + | + | A |
| 1301 | " | 2560 | 640 | 1280 | + | + | + | A |
| 1302 | " | 1280 | 640 | 640 | + | + | + | A |
| 1303 | " | 5120 | 1280 | 640 | + | + | + | A |
| *1304* | *"* | *2560* | *640* | *640* | + | + | - | *D* |
| 1305 | " | 5120 | 640 | 640 | + | + | + | A |
| 1306 | " | 2560 | 320 | 160 | + | + | + | A |
| *1307* | *"* | *160* | *80* | *80* | + | + | - | *D* |
| 1308 | " | 5120 | 640 | 1280 | + | + | + | A |
| 1309 | " | 2560 | 640 | 320 | + | + | + | A |
| 1310 | " | 2560 | 1280 | 1280 | + | - | - | A- |
| *1311* | *"* | *640* | *640* | *1280* | + | + | - | *D* |
| 1312 | " | 2560 | 640 | 1280 | + | - | - | A- |
| 1313 | " | 640 | 160 | 80 | + | + | + | A |
| 1314 | " | 2560 | 640 | 640 | + | + | + | A |
| 1315 | " | 2560 | 640 | 640 | + | + | + | A |
| *1316* | *"* | *2560* | *640* | *1280* | + | + | - | *D* |
| 1317 | " | 640 | 160 | 160 | + | + | + | A |
| *1318* | *"* | *2560* | *160* | *160* | + | + | - | *D* |
| *1319* | *"* | *2560* | *160* | *640* | + | + | - | *D* |
| *1320* | *"* | *640* | *80* | *640* | + | - | + | *D* |
| 1321 | " | 1280 | 640 | 1280 | + | - | - | A- |
| 1322 | " | 320 | 320 | 640 | + | + | + | A |
| 1323 | " | 2560 | 160 | 640 | + | + | + | A |

# TABLE III. DATA FROM PIGS COMPARING LPBE, VIA AGID, WRL AND UBI TESTS

| PIGS | | O | A | AS1A 1 | VIA | WRL | UBI | Compare |
|---|---|---|---|---|---|---|---|---|
| 678 | 3W Vacc+ challenge O | 320 | 320 | 320 | - | - | - | A- |
| 679 | 3W Vacc+ challenge O | 80 | 80 | 160 | - | - | - | A- |
| 708 | 3W Vacc+ challenge O | 2560 | 320 | 320 | - | - | - | A- |
| 709 | 3W Vacc+ challenge O | 640 | 320 | 640 | - | - | - | A- |
| 710 | 3W Vacc+ challenge O | 640 | 640 | 320 | - | - | - | A- |
| 711 | 3W Vacc+ challenge O | 2560 | 640 | 2560 | - | - | - | A- |
| 712 | 3W Vacc+ challenge O | 160 | 160 | 160 | - | - | - | A- |
| | | | | | | | | |
| 686 | 3W Vacc+ challenge A | 1280 | 5120 | 2560 | - | + | - | D |
| 687 | 3W Vacc+ challenge A | 1280 | 5120 | 2560 | + | + | + | A+ |
| 688 | 3W Vacc+ challenge A | 1280 | 5120 | 2560 | + | + | - | D |
| 689 | 3W Vacc+ challenge A | 2560 | 5120 | 2560 | + | + | - | D |
| 690 | 3W Vacc+ challenge A | 2560 | 5120 | 2560 | + | - | - | A- |
| | | | | | | | | |
| 697 | 3W Vacc+ challenge As1 | 320 | 160 | 1280 | - | - | - | A- |
| 698 | 3W Vacc+ challenge As1 | 1280 | 320 | 2560 | - | - | - | A- |
| 699 | 3W Vacc+ challenge As1 | 640 | 320 | 1280 | - | - | - | A- |
| 700 | 3W Vacc+ challenge As1 | 640 | 1280 | 1280 | - | - | - | A- |
| 701 | 3W Vacc+ challenge As1 | 1280 | 1280 | 1280 | - | - | - | A- |
| | | | | | | | | |
| 754 | 3W vacc+ 1 w PC As1 | 320 | 320 | 640 | - | + | - | D |
| 755 | 3W vacc+ 1 w PC As1 | 640 | 640 | 1280 | - | - | - | A- |
| 756 | 3W vacc+ 1 w PC As1 | 320 | 320 | 2560 | - | - | - | A- |
| 757 | 3W vacc+ 1 w PC As1 | 640 | 1280 | 1280 | - | - | - | A- |
| 758 | 3W vacc+ 1 w PC As1 | 320 | 320 | 640 | + | - | - | A- |
| | | | | | | | | |
| 764 | 3W vacc+ 2 w PC As1 | 1280 | 320 | 1280 | - | - | + | D |
| 765 | 3W vacc+ 2 w PC As1 | 1280 | 320 | 2560 | - | - | - | A- |
| 766 | 3W vacc+ 2 w PC As1 | 1280 | 320 | 1280 | - | - | - | A- |
| 767 | 3W vacc+ 2 w PC As1 | 1280 | 640 | 1280 | - | - | - | A- |
| 768 | 3W vacc+ 2 w PC As1 | 320 | 320 | 640 | + | + | - | D |
| | | | | | | | | |
| 789 | 3W vacc+ 3 w PC As1 | 80 | 160 | 320 | - | + | + | A+ |
| 790 | 3W vacc+ 3 w PC As1 | 320 | 160 | 1280 | - | - | - | A- |
| 791 | 3W vacc+ 3 w PC As1 | 160 | 160 | 640 | - | + | + | A+ |
| 792 | 3W vacc+ 3 w PC As1 | 320 | 320 | 640 | - | - | + | D |
| 793 | 3W vacc+ 3 w PC As1 | 160 | 160 | 320 | + | + | - | D |
| | | | | | | | | |
| 799 | 3W vacc+ 4 w PC As1 | 160 | 160 | 320 | - | + | + | A+ |
| 800 | 3W vacc+ 4 w PC As1 | 320 | 160 | 640 | - | - | - | A- |
| 801 | 3W vacc+ 4 w PC As1 | 640 | 160 | 640 | - | + | + | A+ |
| 802 | 3W vacc+ 4 w PC As1 | 640 | 80 | 640 | - | - | + | D |
| 803 | 3W vacc+ 4 w PC As1 | 320 | 160 | 320 | - | - | - | A- |
| | | | | | | | | |
| 809 | 3 W vacc+ 5 w PC As1 | 160 | 80 | 320 | - | - | + | D |
| 810 | 3 W vacc+ 5 w PC As1 | 640 | 640 | 640 | - | - | - | A- |
| 811 | 3 W vacc+ 5 w PC As1 | 320 | 320 | 640 | - | - | + | D |
| 812 | 3 W vacc+ 5 w PC As1 | 640 | 640 | 640 | - | - | + | D |
| 813 | 3 W vacc+ 5 w PC As1 | 320 | 160 | 640 | - | + | - | D |
| | | | | | | | | |
| 684 | 1W postchallenge O | <40 | <40 | <40 | - | + | + | A+ |
| 685 | 1W postchallenge O | <40 | <40 | <40 | - | - | - | A- |
| 713 | 1W postchallenge O | 1280 | 160 | 40 | - | - | - | A- |
| 714 | 1W postchallenge O | 320 | 160 | 80 | - | - | - | A- |
| 715 | 1W postchallenge O | 320 | 80 | 40 | - | - | - | A- |
| 716 | 1W postchallenge O | 640 | 320 | 80 | + | - | - | A- |
| 717 | 1W postchallenge O | 1280 | 320 | 160 | - | + | - | D |
| 718 | 1W postchallenge O | 40 | 40 | <40 | - | - | - | A- |
| | | | | | | | | |
| 691 | 1W postchallenge A | 160 | 640 | 320 | - | + | - | D |
| 692 | 1W postchallenge A | 160 | 640 | 640 | - | + | - | D |
| 693 | 1W postchallenge A | 40 | 640 | 80 | + | + | - | D |
| 694 | 1W postchallenge A | <40 | 320 | 40 | - | - | - | A- |
| 695 | 1W postchallenge A | 40 | 1280 | 80 | - | - | - | A- |
| 696 | 1W postchallenge A | 80 | 640 | 160 | - | + | - | D |

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| Exp | | | O | A | AS1 | VIA | WRL | UBI | Compare |
|-----|---|---|---|---|-----|-----|-----|-----|---------|
| 719 | 710-733 Infected area | | 80 | 40 | 40 | + | - | - | A- |
| 720 | 2 weeks post infection with | | 40 | <40 | <40 | - | - | - | A- |
| 721 | In field outbreak | | 160 | 80 | 80 | - | - | - | A- |
| 722 | of FMDV type O | | 5120 | 2560 | 640 | + | - | - | A- |
| 723 | | | 5120 | 5120 | 5120 | + | + | - | D |
| 724 | | | 40 | 40 | <40 | - | - | - | A- |
| 725 | | | 1280 | 1280 | 1280 | - | - | - | A- |
| 726 | | | 160 | 320 | 160 | - | - | - | A- |
| 727 | | | 2560 | 1280 | 2560 | + | + | - | D |
| 728 | | | 1280 | 1280 | 1280 | + | - | - | A- |
| 729 | | | 5120 | 1280 | 1280 | - | + | - | D |
| 730 | | | 5120 | 5120 | 5120 | - | - | - | A- |
| 731 | | | 5120 | 5120 | 5120 | + | - | - | A- |
| 732 | | | 2560 | 5120 | 5120 | - | + | - | D |
| 733 | | | 320 | 320 | 640 | + | - | - | A- |
| | | | | | | | | | |
| 734 | Field sample serum 734-743 | | ND | ND | ND | - | - | - | A- |
| 735 | Post O outbreak | | 2560 | 640 | 1280 | - | - | - | A- |
| 736 | | | 2560 | 1280 | 320 | - | - | - | A- |
| 737 | | | 640 | 320 | 640 | - | - | - | A- |
| 738 | | | 3940 | 320 | 960 | - | - | - | A- |
| 739 | | | 5120 | 2560 | 1280 | + | + | - | D |
| 740 | | | 5120 | 2560 | 1280 | + | - | - | A- |
| 741 | | | 5120 | 2560 | 2560 | - | - | - | A- |
| 742 | | | 5120 | 2560 | 2560 | + | + | + | A+ |
| 743 | | | 5120 | 2560 | 1280 | + | + | - | A+ |

### 3.2. Vaccine/challenge on selected data

TABLE IV. VACCINATED PIGS AT DIFFERENT DAYS AFTER CHALLENGE WITH ASIA 1

| Pig | Treatment | O | A | ASIA1 | VIA | WRL | UBI | Compare | |
|-----|-----------|-----|------|-------|-----|-----|-----|---------|---|
| 91 | 3W vacc+ 1 w PC | 320 | 320 | 640 | - | + | - | D | |
| 91 | 3W vacc+ 2 w PC | 1280 | 320 | 1280 | - | - | + | | D |
| 91 | 3W vacc+ 3 w PC | 80 | 160 | 320 | - | + | + | A+ | |
| 91 | 3W vacc+ 4 w PC | 160 | 160 | 320 | - | + | + | A+ | |
| 91 | 3 W vacc+ 5 w PC | 160 | 80 | 320 | - | - | + | | D |
| | | | | | | | | | |
| 92 | 3W vacc+ 1 w PC | 640 | 640 | 1280 | - | - | - | A- | |
| 92 | 3W vacc+ 2 w PC | 1280 | 320 | 2560 | - | - | - | A- | |
| 92 | 3W vacc+ 3 w PC | 320 | 160 | 1280 | - | - | - | A- | |
| 92 | 3W vacc+ 4 w PC | 320 | 160 | 640 | - | - | - | A- | |
| 92 | 3 W vacc+ 5 w PC | 640 | 640 | 640 | - | - | - | A- | |
| | | | | | | | | | |
| 93 | 3W vacc+ 1 w PC | 320 | 320 | 2560 | - | - | - | A- | |
| 93 | 3W vacc+ 2 w PC | 1280 | 320 | 1280 | - | - | - | A- | |
| 93 | 3W vacc+ 3 w PC | 160 | 160 | 640 | - | + | + | A+ | |
| 93 | 3W vacc+ 4 w PC | 640 | 160 | 640 | - | + | + | A+ | |
| 93 | 3 W vacc+ 5 w PC | 320 | 320 | 640 | - | - | + | | D |
| | | | | | | | | | |
| 94 | 3W vacc+ 1 w PC | 640 | 1280 | 1280 | - | - | - | A- | |
| 94 | 3W vacc+ 2 w PC | 1280 | 640 | 1280 | - | - | - | A- | |
| 94 | 3W vacc+ 3 w PC | 320 | 320 | 640 | - | - | + | | D |
| 94 | 3W vacc+ 4 w PC | 640 | 80 | 640 | - | - | + | | D |
| 94 | 3 W vacc+ 5 w PC | 640 | 640 | 640 | - | - | + | | D |
| | | | | | | | | | |
| 95 | 3W vacc+ 1 w PC | 320 | 320 | 640 | + | - | - | A- | |
| 95 | 3W vacc+ 2 w PC | 320 | 320 | 640 | + | + | - | D | |
| 95 | 3W vacc+ 3 w PC | 160 | 160 | 320 | + | + | - | D | |
| 95 | 3W vacc+ 4 w PC | 320 | 160 | 320 | - | - | - | A- | |
| 95 | 3 W vacc+ 5 w PC | 320 | 160 | 640 | - | + | - | D | |

Pigs vaccinated with trivalent O, A and Asia 1 vaccine.
At three weeks the pigs were challenged with Asia 1 virus.
Animals were sampled (serum) at 1 w, 2 w, 3 w, 4 w and 5 w after challenge (PC)
Sera were tested by L PBE (O, A, Asia1); NSP ELISAs and VIA AGID.

### 3.3. Comparisons of data

The results of NSP test using various NSP kits were shown in the Table IV.

TABLE V. THE NSP TEST AND LP ELISA TITER IN NON-VACCINATED CATTLE AND PIGS. NSP REAGENT KITS WERE USED FROM WRL AND UBI

| Species | Total sample | NSP Negative | | | | LP ELISA titer | | |
|---------|--------------|------|------|------|------|------|------|------|
| | | WRL | UBI | Bomm | CEDI | O | A | Asia1 |
| Cattle | 279 | 0/272 100% | 0/272 100% | 1/100 99% | 0/100 100% | 1.348± 0.265 | 1.602± 0.307 | 1.381± 0.311 |
| Pigs | 101 | 0/101 | 0/56 | | | 1.165± 0.237 | 1.025±0.244 | 1.126± 0.263 |
| Specificity | 380 | 100% | 100% | 99% | 100% | | | |

TABLE VI. RESULT OF NSP TEST AND LP ELISA TITER IN CATTLE AND PIGS RECEIVING SINGLE VACCINATION

| Species | Total sample | NSP negative | | LP ELISA titre | | |
|---------|--------------|------|------|------|------|------|
| | | WRL | UBI | O | A | Asia1 |
| Cattle | 272 | 270/272 99/26% | 0/112 100% | 1.813±0.269 | 1.99±0.367 | 1.756±0.321 |
| Pig | 123 | 0/123 | 5/123 | 2.178±0.445 | 2.064±0.451 | 2.011±0.476 |
| Specificity | 395 | 99.5% | 98.7% | | | |

TABLE VII. NSP TEST AND LP ELISA TITERS IN CATTLE AND BUFFALOS RECEIVING MULTIPLE VACCINATION 5 TIMES OR MORE

| Species/history | Total sample | NSP negative | | LP ELISA titer | | |
|-----------------|--------------|------|------|------|------|------|
| | | WRL | UBI | O | A | Asia1 |
| Cattle | 180 | 175/180 97.22% | 180/180 100% | 2.399 ± 0.246 | 2.315 ± 0.456 | 2.415 ± 0.456 |
| Buffalo | 40 | 0/40 100% | 0/40 100% | 2.685 ± 0.554 | 2.226 ± 0.353 | 2.35 ± 0.389 |
| Specificity | 220 | 97.73% | 100% | | | |

Status: non infected , multiple vaccination animals from bleeding station, receiving vaccination every 6 m.

TABLE VIII. RESULT OF NSP TEST AND LP ELISA TEST IN CATTLE AND PIG SERA FROM FIELD OUTBREAK. (TOTAL SAMPLE = 360)

| Serum history | NSP test (positive/total) | | | | | | |
|---|---|---|---|---|---|---|---|
| | WRL | UBI | Bommeli/ Intervet | CEDI | Inoue (Japan) | USDA | Pen-side (Korea) |
| 1 M PI + vaccinated, cattle | 177/180 (98.3%) | 160/180 (88.9%) | 126/149 (84.6%) | 163/180 (90.1%) | 61/65 (93.8%) | 159/180 (88.3%) | 55/65 (84.6%) |
| *2 M PI + vaccinated, cattle | ND | 123/165 (74.5%) | 121/165 (73.3%) | 136/165 (82.4%) | 135/165 (81.8%) | ND | ND |
| Infected pig | 10/15 (73.33%) | 12/15 (80%) | ND | ND | ND | ND | ND |

1 M = 1 m post infected and vaccinated cattle with trivalent vaccine , field outbreak type O in 2000
2 M = 2 m post infected and vaccinated cattle with trivalent vaccine, field outbreak type A in 2004

WRL = 3ABC NSP World Reference Laboratory, UK; UBI = 3B NSP United Biomedical Inc., USA;
USDA =3AB NSP Plum Island Animal Disease center, USA; Bomm = 3ABC NSP Bommeli;
Inoue/Japan = 2B NSP T. Inoue, NIAH, Japan; CEDI = 3ABC NSP CEDI Diagnostic, Holland;
Pen-Side = 3ABC NSP rapid test from Korea; ND = Not done

TABLE IX. RESULTS ON ANNUAL VACCINATED ANIMALS TWICE A YEAR

| Province | Total sample | NSP positive | LP ELISA positive (> 1:80) | | |
|---|---|---|---|---|---|
| | | | O | A | Asia1 |
| Burirum | 231 | 11/231 (4.8%) | 170/231(73.6%) | 172/231 (74.5%) | 181/231 (78.4%) |
| Ubonratchathani | 259 | 24/259 (9.3%) | 181/259 (69.9%) | 181/259 (69.9%) | 192/259 (74.1%) |
| Umnadchareon | 349 | 5/349 (1.4%) | 268/349 (76.8%) | 273/349 (78.2%) | 263/349 (75.4%) |
| Srisaked | 120 | 7/120 (5.83%) | 80/120 (66.7%) | 84/120 (70%) | 97/129 (80.83%) |
| Khonkhaen | 76 | 1/76 (1.3%) | 74/76 (97.36%) | 70/76 (92.1%) | 73/76 (96.05%) |
| Yasothon | 120 | 3/120 (2.5%) | 77/120 (60.2%) | 67/120 (55.8%) | 81/120 (67.5%) |
| Chaiyaphum | 120 | 12/120 (10%) | 80/120 (66.67%) | 57/120 (47.5%) | 69/120 (57.5%) |
| Surin | 120 | 17/120 (14.2%) | 109/120 (90.83%) | 100/120 (83.3%) | 111/120 (92.5%) |
| Roi et | 120 | 4/120 (3.33%) | 61/120 (50.8%) | 63/120 (53%) | 61/120 (50.8%) |
| Total | 1515 | 84 (5.5%) | 1100 (72.6%) | 1067 (70.4%) | 1128 (74.5%) |

TABLE X. RESULTS ON CATTLE AND BUFFALOES IN BUFFER ZONE IN MTM PROJECT

**NS test in animals being quarantied at Prachoubkirikhan animal quarantine station during 2004-2005**

| | Aprl/o4 | May/04 | Jun/04 | Nov/04 | Feb/05 | Mar/05 | Apr/05 |
|---|---|---|---|---|---|---|---|
| □ % NS negative | 95.1 | 94.8 | 98.53 | 95.2 | 97.08 | 94.97 | 100 |
| ■ %NS positive | 4.9 | 5.2 | 1.47 | 4.8 | 2.92 | 5.03 | 0 |
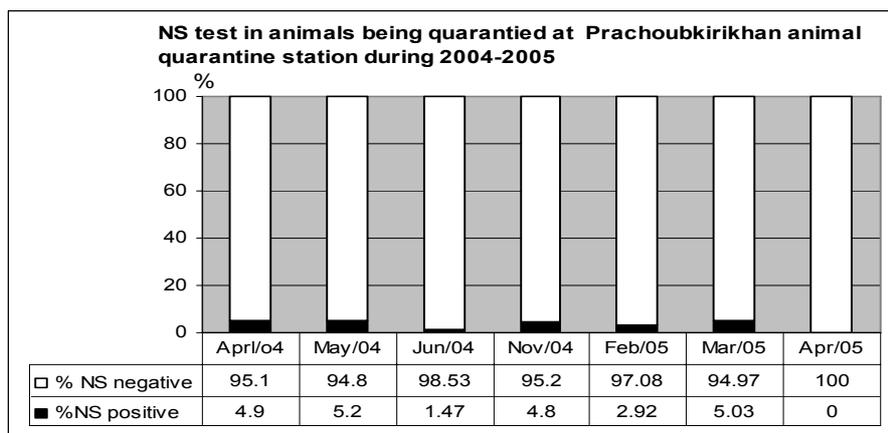
Blood samples were collected at the international quarantine station in Prachubkirikhan Province during year 2004–2005. (Total sample =1052)

## 3.4. Comparison of analytical sensitivity data on selected cattle sera

Selected cattle and pig sera were titrated in twofold dilution ranges and kits used to detect the signal. Comparison of analytical sensitivities was made by comparing the titration curves and end points (dilution at which the sample became negative in tests).

### 3.4.1. Comparison of early UBI and IAH kits using cattle sera

Table X shows data for comparison of tests at two defined OD values for the Indirect ELISAs from IAH and UBI. The figures represent the values obtained for the recommended dilution in test; the reciprocal of the dilution to achieve the OD designated (0.6) and the endpoint (reciprocal of the last dilution positive). The ratios dividing the IAH test results by the UBI are also shown.

### 3.4.2. Comparison of later CEDI, Bommeli and UBI kits using cattle sera

Figs 1–3 show the twofold titration curves for selected cattle sera using different kits.

TABLE XI. UBI AND IAH TESTS TITRATING SELECTED CATTLE SERA

| | OD at Recommended dilution for test | Result at rec. dilution | 0.6 OD | E.P. (0.2) | Ratio IAH/UBI At 0.6OD | Ratio IAH/UBI E.P |
|---|---|---|---|---|---|---|
| IAH | OD (1/200) | | | | | |
| 6 weeks post challenge | 1.23 | + | 900 | 5000 | 900/25 = 36 | 5000/130 = 38 |
| 6 weeks post challenge | 2.97 | + | 9000 | 45,000 | 9000/220 = 45 | 45,000/900 = 50 |
| 1 week post challenge | 1.19 | + | 600 | 2000 | 600/20 = 30 | 2000/90 = 22 |
| 1 week post challenge | 2.78 | + | 6000 | 30,000 | 6000/10 60 | 30,000/700 = 43 |
| 1 week post challenge | 0.8 | - | 0 | 450 | 1/10 = 0.1 | 450/50 = 9 |
| vacc | 0.28 | | 0 | 0 | 0/0 | 0/0 |
| UBI | OD (1/20) | | | | | |
| 6 weeks post challenge | 0.71 | + | 25 | 130 | | |
| 6 weeks post challenge | 2.85 | + | 220 | 900 | | |
| 1 week post challenge | 0.67 | + | 20 | 90 | | |
| 1 week post challenge | 2.25 | + | 180 | 700 | | |
| 1 week post challenge | 0.3 | + | 10 | 50 | | |
| vacc | 0.08 | | 0 | 0 | | |
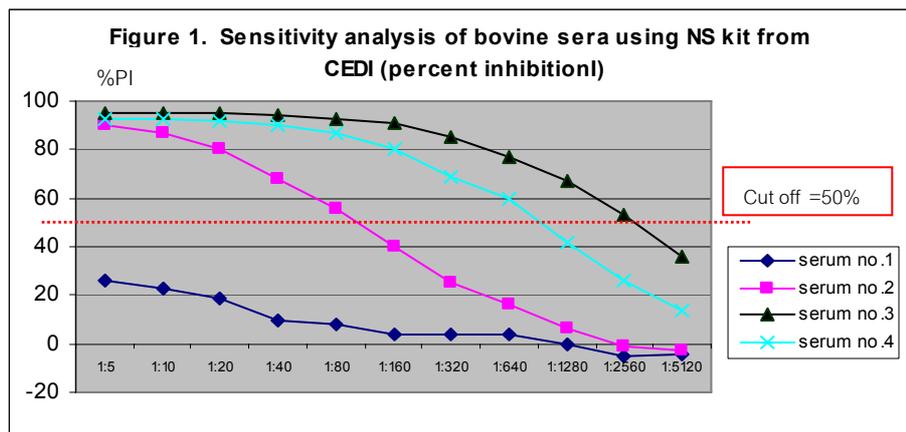


FIG. 1. Analysis of bovine serum titration curves usng CEDI kits.
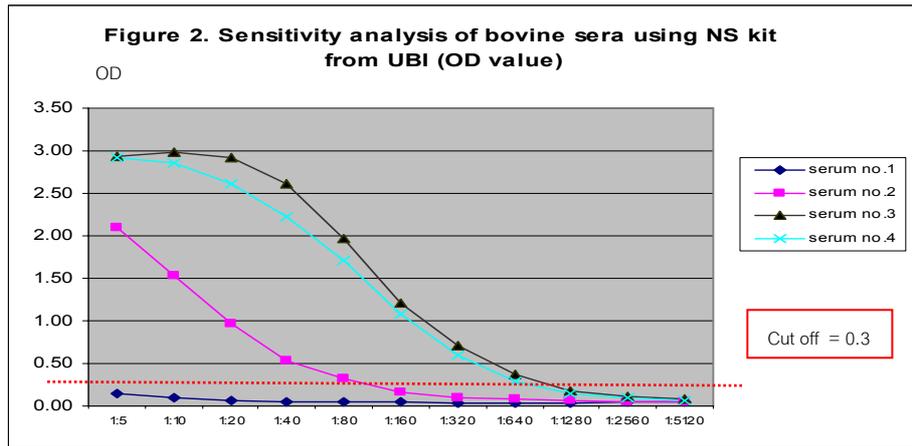
*FIG.2. Analysis of bovine serum titration curvesuisng UBI kit.*
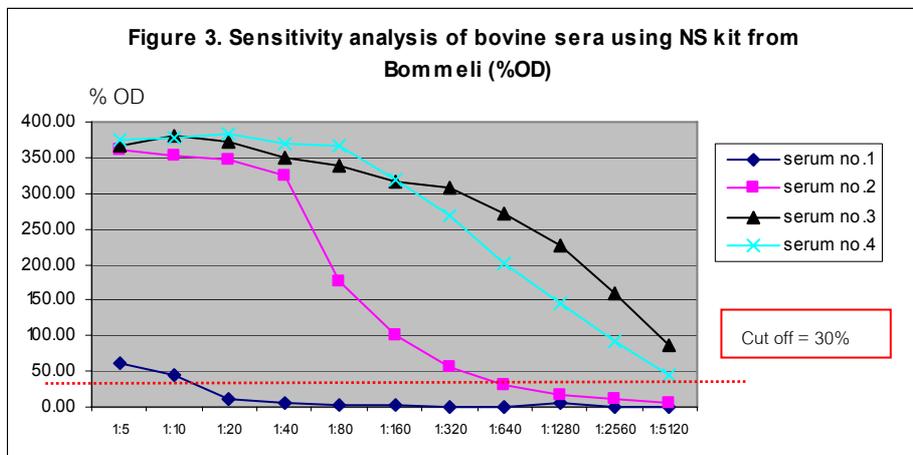


Serum 1 = post vaccinated cow, serum 2 = 6 weeks post challenged
Serum 3 = 6 weeks post challenged, serum 4 = 4 weeks post challenged

*FIG. 3. Analysis of bovine serum titration curves uisng Bommeli kit.*

| | CEDI (50% competition) | UBI (end point) | Bommeli (end point) |
|---|---|---|---|
| Serum | | | |
| 1 | 0 (0) | | |
| 2 | 100 (EP 2,500) | 100 | 640 |
| 3 | 3000 (EP 10,000) | 1000 | 7000 |
| 4 | 1000 (EP 16,000) | 800 | 5,500 |

## 3.5. Comparison of Analytical sensitivity data on selected pig sera

Table XI shows the values obtained for the recommended dilution in test; the reciprocal of the dilution to achieve the OD designated (0.6) and the endpoint (reciprocal of the last dilution positive).

TABLE XII. TITRATIONS OF PIG SERA USING DIFFERENT KITS

| | Recommended dilution for test | 0.6 OD | E.P. (0.2) | Ratio IAH/UBI at 0.6 OPD | Ratio IAH/UBI at EP |
|---|---|---|---|---|---|
| IAH | OD (1/200) | | | | |
| 1 w post challenge | 0.42 | 120 | 500 | 120/1 = 120 | 500/20 = 25 |
| 2 w post infection | 1.21 | 550 | 2000 | 550/0 = 550 | 2000/40 = 50 |
| 1 w post infection | 1.88 | 1500 | 6000 | 1500/120 = 12.5 | 6000/800 = 75 |
| 5 w post challenge | 0.71 | 250 | 800 | 250/20 = 12.5 | 800/100 = 8 |
| 5 post challenge | 0.68 | 250 | 800 | 250/20 = 12.5 | 800/100 = 8 |
| 3 w post vaccination vacc | 0.24 | 0 | 240 | 0 | 240/1 = 240 |
| UBI | OD (1/20) | | | | |
| 1 w post challenge | 0.25 | 0 | 20 | | |
| 2 w post infection | 0.31 | 0 | 40 | | |
| 1 w post infection | 1.58 | 120 | 800 | | |
| 5 w post challenge | 0.67 | 20 | 100 | | |
| 5 w post challenge | 0.73 | 20 | 100 | | |
| 3 w post vaccination vacc | 0.19 | 0 | 0 | | |

## 4.    CONCLUSION AND DISCUSSION

The detection of antibody to NSP of FMDV using various types of kit from WRL, UBI, Bommeli, and CEDI gave a high DSp when testing non vaccinated cattle and pig sera, in the range of 99-100% and 98.7-99.5% in single vaccinated animals groups. High DSps were also found for multiply vaccinated animals (range 97.7-100%) using the early WRL and UBI kits in the face of antibodies against structural proteins as assessed from the LPBE results.

The LP ELISA titres of such sera increased generally according to the increased number of vaccinations. Linchongsubongkoch [14] reported similar results, where the NSP tests in multiple vaccinated animals gave a high specificity by NSP test but low specificity by virus infection associated antigen agar gel immunodiffusion (VIA-AGID) test. This is not surprising since VIA is a part of the vaccine and induces antibodies in a good proportion of cattle even after a single vaccination, and this percentage rises on subsequent vaccination. Tests using the same cattle and buffaloes sera showed that there was a 65% specificity and 35% false positivity using the VIA test [8]; [9]. The current work confirms that the VIA-AGID test is not appropriate for countries that use vaccination.

The measurement of antibodies to structural protein of FMDV type O, A and Asia1 by LP ELISA [4]; [6] found that a low LP ELISA titre was detected in pig sera possibly from young animals presenting maternal immunity.

The DSn studies indicated a range of 84.6–93.8% in 1 m post infected cattle, and range 73.3–82.4% at 2 m post infection. Cattle from these groups may not all have been infected (which would affect the DSn figures) as a result of lack of contact or protection

through vaccination. However, it was shown that all the field sera showing clinical signs of FMD type O, contained high titres of antibodies to FMDV type O, A and Asia1 by LPBE. Cattle were vaccinated with trivalent vaccine after an outbreak and gave titres of were 3.216 + 0.310, 2.496 + 0.415 and 2.637 + 0.314, respectively.

The NSP test proved highly effective when used to survey cattle and buffalos moving in the FMD sero surveillance buffer in the multilateral project on "Tristates Commission on the Establishment of Malaysia Thailand Myanmar Peninsular Campaign of FMD Freedom" or MTM project.

The Department of Livestock Development (DLD) has established a national plan for FMD control and eradication programme with the two main objectives, (1) to strengthen the vaccination campaign to increase herd immunity in the animal population and 2) to restrict both domestic and international animal movement.

An FMD trivalent vaccine has been used to vaccinate animal twice a year, countrywide. Serum samples are collected regionally. The LPBE is used to determine the titres of antibodies to FMDV type O, A and Asia, in order to estimate the likely herd immunity. The NSP ELISAs are also being used to differentiate between vaccinated and infected animals. Results demonstrated a low incidence of viral replication in field animals in the face of vaccination with trivalent vaccine (e.g. 1 m post vaccination where a small number of NSP positive sera were found by UBI kit in range 1.3–14.2%). This indicates that there is a low incidence of FMD outbreaks in the face of vaccination and a high herd immunity against FMDV types O, A, and Asia1 (72.6%, 70.4% and 74.5%) respectively.

The situation in pigs is interesting and there seems to be a circulation of virus in heavily vaccinated farms where there is no apparent clinical signs. The results of the trivalent protected pigs producing antibodies to NSP some weeks after challenge, without clinical signs, need further investigation.

In conclusion, the use of NSP ELISAs to differentiate between vaccinated and infected animals has become an important assay for disease control. Although there was a demonstrable variation, all the later kits give a useable DSn and Dsp for use in sero-monitoring and surveillance of FMD at national and regional level. This form of assay as a standardized test in the control and eradication of FMD in the region is necessary to meet the target of establishment of FMD free zone in the S. E. Asian countries under the OIE regulations and also would be useful in international trade of livestock in FMD free countries.

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**OBSERVATIONS ON THE USE OF THE VARIOUS ELISA KITS FOR THE DETECTION OF ANTIBODIES AGAINST NON-STRUCTURAL PROTEINS OF FOOT AND MOUTH DISEASE IN PIGS IN HONG KONG**

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**Abstract**
Data is reviewed from results using different ELISA kits for the determination of antibodies to FMD NSP tests on samples from pigs. Kits had similar proficiencies for detecting antibodies. The circulation of FMD virus in vaccinated pigs in the absence of clinical disease is indicated, and this could account for the repeated infections seen in various farms in Hong Kong.

## 1. INTRODUCTION

In June 2004 the population of pigs in Hong Kong was approximately 240 000 on 354 farms in northern part of the New Territories. Farm sizes vary from 20–30 to over 1000 sows. Most are housed indoors on slatted or concrete floors and fed imported commercial products. Pigs supply about 20% of local demand. FMD is endemic with only FMDV type O present until Asia 1 infected farms in 2005. Apparent disease is controlled by vaccination, decontamination and through increased biosecurity measure and there is very variable effectiveness of on-farm control. FMD occurs in local abattoirs through sub-optimal disinfection. Diagnosis is helped though taking skin samples to confirm FMD infection (tested using Pirbright antigen capture ELISA). Applied research centres on understanding FMD outbreak farms (age affected, husbandry practices, other endemic diseases) and improving FMD vaccination protocols through monitoring protective antibodies against FMDV structural proteins using LPBE and understanding the local FMD prevalence using the NSP ELISA to monitor likely viral replication. The recommendations for vaccination are 2 doses (Merial or Intervet) at 12 w and 16 w; a booster 2 w before marketing (~24 w). In practice single doses are used; non-registered vaccine(s) are used; mixed brands of vaccines are used; vaccination is made when animals are too young and vaccination is made only during the winter. A serum bank is available with the serum source and history in multiple aliquots of individual serum stored in freezer at -20oC. The serum categories are abattoir (4-monthly, collected during slaughtering by abattoir staff and local farms (irregular frequency depends on other work duties).

## 2. METHODS

### 2.1 Kits examined

Throughout the time of the studies various formulations were used. Although some of the data is redundant it serves to show developmental trends and relates data to more modern kits.

UBI® FMDV NSP 3B EIA (Swine)-2000/2003/2004
Sorensen (3AB) blocking ELISA- 2000/2001 (original and modified version)
Brescia (3ABC)-2001 (capture 3ABC then indirect ELISA-3ABC *E. coli* antigen)
Checkit® FMD-3ABC po-2003/2004 (I- ELISA) (Bommeli)
CEDItest® FMD-NSP-2004 (Competition ELISA)

## 2.2. Samples for tests-relative diagnostic sensitivity

A panel of 148 pig sera from 8 confirmed FMD outbreak farms in 1999 was available for testing. Samples collected at different farms and at different time points post infection (dpi). Results are shown in Table I.

## 2.3. Samples for tests-relative diagnostic specificity

A panel of 273 porcine sera from 7 farms with no FMD for at least 6 m in 1999 were examined. Results are shown in Table II.

## 3. RESULTS

## 3.1. Comparison of pig sera

TABLE I. RELATIVE ANTI NSP POSITIVE PIG SERA FROM VARIOUS TESTS

| Farm | dpi | UBI | Sorensen 1 | Sorensen 2 | Brescia | CEDI |
|------|-----|-----|------------|------------|---------|------|
| A | 19 | 6/8 | 2/8 | 4/8 | 6/8 | 7/8 |
| C | 23 | 15/15 | 2/15 | 8/15 | 6/15 | 9/10 |
| E | 58 | 13/15 | 14/15 | 14/15 | 8/15 | 13/14 |
| F | 60 | 18/20 | 17/20 | 13/17 | 9/20 | 16/18 |
| H | 74 | 13/24 | 21/24 | 19/23 | 5/18 | 18/21 |
| I | 86 | 2/10 | 4/10 | 6/10 | 0/10 | 3/10 |
| J | 100 | 4/24 | 6/24 | 6/24 | 2/24 | 5/23 |
| K | 138 | 6/32 | 14/32 | 1/28 | 5/29 | 8/19 |

## 3.2. Comparison of diagnostic sensitivities with pig sera

TABLE II. RELATIVE DIAGNOSTIC SPECIFICITY EXAMINATION

| Age(week) | Vacc | Samples | UBI | Soren1 | Soren2 | Brescia | CEDI | LPBE |
|-----------|------|---------|-----|--------|--------|---------|------|------|
| <12 | 0 | 144 | 0/144 | 2/144 | 1/144 | 0/144 | 0/41 | 87/144 |
| 14-16 | 1 | 48 | 1/48 | 0/48 | 1/48 | 0/48 | 0/24 | 32/48 |
| 16-32 | 2 | 81 | 0/58 | 0/81 | 1/58 | 0/81 | 0/39 | 64/81 |
| Total positive | 273 | 1/250 | 2/273 | 1/250 | 0/273 | 0/273 | 0/114 | 183/273 |
| Specificity % | | | 99.6 | 99.3 | 99.6 | 100 | 100 | - |

### 3.3. Evidence for virus circulation

TABLE III. EXAMINATION OF VACCINATED PIGS WITH NO CLINICAL SIGNS

| Farm | Sample* | Brescia 2001 | Checkit 2003 | UBI 2003 | LPBE 1999 |
|------|---------|--------------|--------------|----------|-----------|
| 1 | 31 | 2D | 2D | 1 | 74% |
| 2 | 26 | 1 | 1 | 4 | 100% |
| 3 | 23 | 0 | 0 | 1 | 96% |
| 4 | 25 | 5 + 1D | 5 + 1D | 1 | 60% |
| 5 | 25 | 1 | 0 | 0 | 56% |
| 6 | 13 | 1 | 1 | 0 | 52% |
| 7 | 25 | 1 + 1D | 0 | 1 | 76% |
| 8 | 12 | NT | 1 | 2 | 100% |

**\*** Samples >4 m old pigs, two vaccinations with no clinical disease in last 3 m

### 3.4. Abattoir surveillance

TABLE IV. EXAMINATION OF IMPORTED PIGS FROM MAINLAND (I)

| Year | UBI (3B) 2004* | Checkit 2004 | LPBE 2004 |
|------|----------------|--------------|-----------|
| 2001 Apr | 7/115 | 0/115 | 53% |
| Sept | 7/100 | 2/100 | 56% |
| Dec | 5/85 | 0/85 | 55% |
| 2002 Mar | 2/120 | 0/120 | 69% |
| Aug | 4/80 | 0/80 | 59% |
| Dec | 3/100 | 0/100 | 92% |
| 2003 Apr | 7/96 | 0/96 | 65% |
| Aug | 5/102 | 3/102 | 60% |
| Nov | 1/101 | 0/102 | 64% |

TABLE V. EXAMINATION OF IMPORTED PIGS FROM MAINLAND (II)

| | UBI (3B) 2004* | Checkit 2004 | LPBE 2004 |
|------|----------------|--------------|-----------|
| 2001 Apr | 6/129 | 1/129 | 39% |
| Sept | 6/112 | 1/112 | 62% |
| Dec | 3/95 | 1/95 | 64% |
| 2002 Mar | 7/120 | 5/120 | 83% |
| Aug | 11/80 | 1/80 | 40% |
| Dec | 1/100 | 0/100 | 72% |
| 2003 Apr | 6/98 | 0/98 | 74% |
| Aug | 8/102 | 0/102 | 52% |
| Nov | 2/101 | 0/101 | 45% |

\* UBI (3B) NEUTRALIZATION: 4/15 POSITIVE

## 4. DISCUSSION

Questions to answer in formulating strategic FMD control programmes in Hong Kong include

- Does FMD circulate subclinically in vaccinated pig farms in summer?
- What is the FMD sero-prevalence in local abattoirs?
- Do feral cattle/buffalos have roles in FMD in Hong Kong?

Circulation in pigs is very important in the light of the mass vaccination campaigns particularly in S.E Asian countries. The continued presence of antibodies against NSP in vaccinated pig herds in the absence of clinical signs is strongly indicative of circulating virus. The mechanism for this is not elucidated since few studies have examined where virus is maintained but episodes of infection with newly introduced non-immunised or recently immunised pigs, are common.

Pigs are not thought to be carriers. It is much more likely that virus is amplified in the throats of pigs genuinely receiving aerosolized virus [1]. Here, experimentally vaccinated pigs were shown to produce a large amplification of infectious virus on aerosol infection that was excreted into the atmosphere. The measured titres of virus were almost as high as those in a full infection. The window for this was small. No clinical manifestations of the aerosol infection were observed. Virus infecting vaccinated pigs via the blood through needle and presumably the most common on route in the field through scratches in the snout and feet did not cause the production of any virus presumably because the virus was bound and neutralised effectively by the high titres of post vaccinal antibodies in the blood. This process of vaccinated pigs being able to amplify virus and excrete it into the atmosphere would be enough to maintain virus in herds. Re-infection of surrounding vaccinated pigs would occur and re-amplification of virus. The replication events in the throat elicited the production of anti NSP proteins in a large proportion of the pigs studied (replicative events were stimulative).

This circulating virus scenario in pigs is not popular, partly because it goes against previously published data on aerosol work in pigs held as dogma and because the use of ring vaccination of pigs is a strong recommendation in the case of an outbreak e.g. in Europe. The amplification of virus in ring might well allow virus to be disseminated over an even greater area than from a focus of disease. Recognising that this occurs is vital to disease management in pigs. Mass vaccination with circulating virus means that although there is no disease, there is virus which remains a threat to new animals brought into contact with infected hers, or to areas where there is transportation of such animals. Vaccination where there are large numbers of animals in relatively close contact will never eradicate FMD from pig herds and alternative strategies may have to be examined. The NSP tested allow identification of herds where virus circulation can be traced.

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# USE OF NON-STRUCTURAL PROTEIN OF FMD VIRUS TO DIFFERENTIATE BETWEEN VACCINATED AND INFECTED ANIMALS IN LAOS

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**Abstract**

This paper reviews the FMD situation in Laos. Different kits for the detection of antibodies to NSP of FMDV were used to evaluate field sera from pigs and cattle. Differences in diagnostic performance were observed, particularly for pigs.

## 1.     INTRODUCTION

Lao People's Democratic Republic (Lao PDR) is a land-locked country with extended borders on the west and east with Thailand and Vietnam respectively, and more limited borders with Myanmar and China in the north, and Cambodia in the south. Lao PDR has the lowest human population of the region, with approximately 5 million people (19 per square kilometres) who have an average annual GDP per capita of approximately US $300.

While rice is the major agricultural commodity in Lao PDR, livestock play a highly significant role in the predominantly smallholder farming system of the country. Cattle and buffalo populations are both approximately 1.1 million and the pig population approximately 1.3 million (see Table 1. below). Not only do buffalo and cattle make a very significant contribution to rice production itself, through paddy cultivation, harvesting and transport of rice, they also play a key role as units of investment, which have traditional values as assets for negotiation at times of weddings and other ceremonies. Cattle and buffalo together with other species, in particular pigs, poultry and goats play a key role in cash income generations.

TABLE I. LIVESTOCK POPULATIONS IN LAO PDR (1989–2001)

| Year | Buffalo | Cattle | Goat/Sheep | Pigs |
|------|---------|--------|------------|------|
| 1989 | 1,026,160 | 816,530 | 105,160 | 1,349,980 |
| 1995 | 1,191,410 | 1,145,870 | 152,930 | 1,723,590 |
| 1996 | 1,211,700 | 1,186,000 | 159,000 | 1,772,000 |
| 1997 | 1,223,800 | 1,227,500 | 165,000 | 1,813,000 |
| 1998 | 1,092,740 | 1,126,600 | 122,170 | 1,432,140 |
| 1999 | 1,008,000 | 1,080,000 | 112,000 | 1,250,000 |
| 2000 | 1,028,000 | 1,144,000 | 121,400 | 1,325,000 |

Being the only landlocked country in the region, Lao PDR finds itself in the position of having access to livestock markets in Thailand, Vietnam, southern China, Myanmar and Cambodia. As well as being a thoroughfare for transboundary animal movement and trade. With an ever-increasing regional human population, an increase in the demand for meat and fish has gone hand-in-hand with this population growth. Subsequently, a high demand for

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meat, together with the trading of live animals and animal products, animal movement to and from neighbouring countries is considerable. As previously mentioned, the agricultural sector of Lao PDR is mainly smallholder farming systems, with a slightly increasing number of commercial farming enterprises. To maintain production in both farming systems at a level of sustainability and economic viability, effective disease monitoring and control is of utmost importance. Of very high country and regional priority is the monitoring and control of foot and mouth disease (FMD).

FMD is a highly contagious viral disease of cloven-hoofed animals and is endemic in many South East Asian countries, including Lao PDR where extensive work has been undertaken to better understand the epidemiology of the disease. Because large-scale vaccination is not routinely practised, the livestock population of Lao PDR remains largely susceptible to infection although residual immunity from previous infections remains. As Lao PDR is a major thoroughfare for transboundary animal movement, the threat of FMD epidemics with its largely susceptible population is constant and real. Since 1980 the level of FMD incidence in Lao PDR has been influenced to a large extent by the demands of the international animal trade.

## 1.1. Laboratory capabilities

FMD has played and continues to play, an important role in livestock production and the limitation of subsistence livelihood in Lao PDR. Outbreak awareness and control are a priority. An essential component of any countries ability to provide effective disease control and monitoring is the laboratory capability to carry out routine diagnosis and surveillance. As a result, The Department of Livestock and Fisheries (DLF) with the support of the IAEA developed the laboratory capability to diagnose and type suspected outbreaks of FMD in 1994 using WRL, Pirbright ELISA kits.

The DLF, through ACIAR project AS1/94/38 further expanded upon this capability by way of the development of an FMD diagnostic facility at the National Veterinary Diagnostic Laboratory, Vientiane in 1997, with support from the IAEA, FAO and WRL, Pirbright. The diagnostic capabilities now in place play a major role in the control of FMD by providing:

Prompt diagnosis and virus typing through the antigen typing ELISA test. With the capability of serotyping for O, A, C and Asia 1 serotypes, and monitoring of herd immunity through the FMD LPB-ELISA.

## 1.2. IAEA Contract objectives and methodology

Currently the laboratory is engaged in a new research contract with the IAEA (No. 10442/RBF) on "Use of non-structural protein of FMD virus to differentiate between vaccinated and infected animals". The capability of the diagnostic laboratory, mentioned above, will be further enhanced by the potential introduction of this new technology, which will enable differentiation of FMD infected from vaccinated animals in the screening programme.

The objective of the current contract between IAEA and the DLF was to evaluate three new ELISA technologies in their ability to differentiate between vaccinated and infected animals. The three test kits evaluated in this study are (i) WRL 3ABC non-structural protein ELISA, (ii) UBI 3ABC non-structural protein ELISA and (iii) Sorenson 3AB non-structural protein ELISA.

*1.2.1. Serum sample history*

Since the commencement of ACIAR project 9438 in 1997, serum samples have been collected and stored as part of an extensive serum bank. Samples have been collected for both active and passive (opportunistic) FMD surveillance, to determine pre- and post-vaccination herd immunity and as part of FMD vaccination trials in swine. Structured active surveillance, sampling to determine pre- and post-vaccination herd immunity and FMD vaccine trial samples were used for this study, with the vaccination history of the animals sampled well documented.

TABLE II. VACCINATION AND INFECTION HISTORY OF SERUM SAMPLES USED IN STUDY

| | Swine |
|---|---|
| 1 | Serum from FMD virus (type Asia 1) experimentally infected 3 month old piglets |
| 2 | Serum from vaccinated pigs using trivalent (O, A and Asia 1) vaccine on FMD virus free farm |
| 3 | Serum from 13-30 day old piglets with maternally derived antibodies to a trivalent FMD vaccine |
| | Ruminants |
| 1 | Serum from vaccinated cattle & buffalo in an FMD virus outbreak area (clinical history not known) |
| 2 | Serum from structured surveillance in FMD virus active area (vaccination history not known) |
| 3 | Serum from vaccinated cattle & buffalo with recent clinical infection |
| 4 | Serum from vaccinated cattle & buffalo with no recent clinical symptoms (outbreak area) |
| 5 | Serum from vaccinated cattle & buffalo in FMD free zone |
| 6 | Serum from non-vaccinated cattle & buffalo in FMD outbreak zone |
| 7 | Serum from non-vaccinated cattle & buffalo from FMD outbreak zone (animals with recent clinical infection) |
| 8 | Non-vaccinated goats from FMD outbreak zone |

2.     RESULTS

Before progressing to a somewhat more detailed description of the results obtained during the course of this study, the problems experienced with the test kits examined must be noted. The 3AB NSP-ELISA kit developed by Sorenson could not provide results to be analysed. The controls provided with the kit failed every time the ELISA was run. The reason for this occurrence cannot be determined. Likewise, the controls provided with the WRL 3ABC NSP-ELISA did not respond as described in the manual. There was a clear distinction between the controls; however, the OD readings were much lower than expected, making it more difficult to distinguish between negative and weakly positive samples. Once again, the reason for this occurrence can only be speculated at and further details and possible reasons will not be discussed in this report.

## 2.1. Pigs

Serum samples used to compare WRL 3ABC NSP-ELISA, UBI 3ABC NSP-ELISA and the LPB-ELISA came from animals used in FMD virus vaccination and infection trials. This provided samples with a definite vaccination and infection history.

### 2.1.1. Sensitivity

Eight 3 m old piglets were experimentally infected with type Asia 1 and serum collected weekly for 10 w, and again at 17 and 21 w post infection. The serum was tested for serological conversion by LPB-ELISA. The sensitivity of the UBI kit was very high up to 9 weeks post infection and dropped away at weeks 10, 17 and 21. The WRL kit on the other hand had very low sensitivity up to week 8, and zero sensitivity at 9, 10, 17 and 21 weeks post infection (*see* Table III and Fig. 1). No correlation could be made between a drop in mean titres by LPB-ELISA and a drop in sensitivity (results not shown) over the course of the experiment, the number of samples tested were however quite low.

### 2.1.2. Specificity

Over 100 pigs were vaccinated using a trivalent vaccine (O, A and Asia 1). For the purposes of this study, the serum from 114 vaccinated pigs (35 and 79 serologically negative and positive, respectively) was used to determine the specificity of the NSP-ELISA test kits. Two serologically positive serum samples returned a positive result for the UBI kit, with a specificity of 97%. No samples returned a positive result using the WRL kit, with a specificity of 100% (see Table IV). Serum samples collected from piglets with maternally derived antibodies to a trivalent vaccine were also tested, and both kits showed 100% specificity (see Table IV).

TABLE III. EXPERIMENTAL INFECTION OF SWINE WITH FMD SEROTYPE ASIA 1

| w.p.i | # Piglets Seropositive by LPB-ELISA | # Piglets Seropositive by WRL 3ABC NSP-ELISA | Sensitivity of WRL NSP-ELISA | # Piglets Seropositive by UBI 3ABC NSP-ELISA | Sensitivity of UBI NSP-ELISA |
|---|---|---|---|---|---|
| 1 | 4 | 0 | 0 | 4 | 100 |
| 2 | 6 | 5 | 83 | 6 | 100 |
| 3 | 7 | 4 | 57 | 7 | 100 |
| 4 | 7 | 5 | 71 | 6 | 86 |
| 5 | 6 | 3 | 50 | 6 | 100 |
| 6 | 7 | 4 | 57 | 7 | 100 |
| 7 | 7 | 4 | 57 | 7 | 100 |
| 8 | 8 | 2 | 25 | 7 | 88 |
| 9 | 8 | 0 | 0 | 8 | 100 |
| 10 | 8 | 0 | 0 | 6 | 75 |
| 17 | 8 | 0 | 0 | 4 | 50 |
| 21 | 7 | 0 | 0 | 5 | 71 |
| Total | 83 | 27 | | 73 | |
| Overall% sensitivity | 33 | | | 88 | |

Sensitivity expressed as percent positive in comparison to LPB-ELISA results
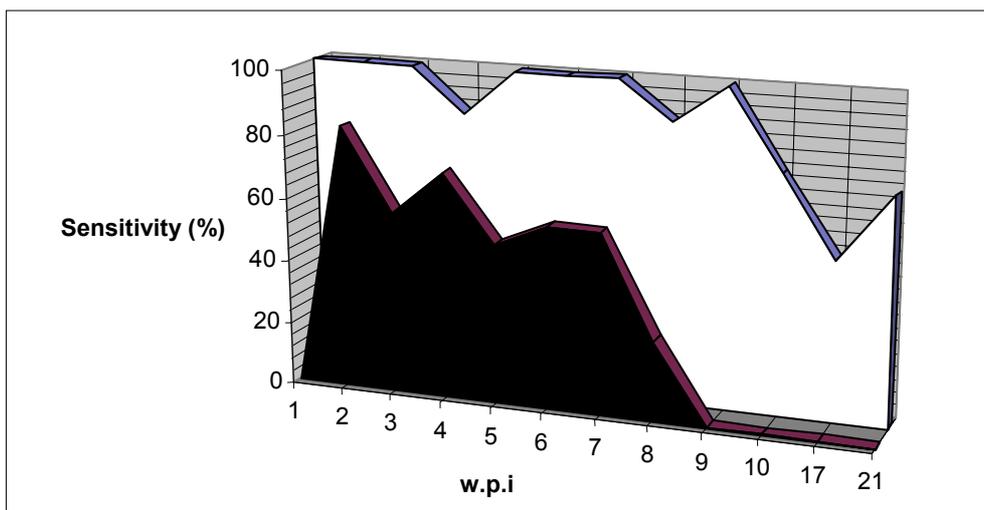
*FIG. 1. Relative sensitivity of test kits verses LPB-ELISA results for FMD challenge experiment (pig serum); w.p.i, weeks post infection.*

TABLE IV. NSP-ELISA TEST KIT RESULTS FOR SWINE

| Sample history a | | FMD Serological Status (LPB-ELISA) | | WRL 3ABC NSP-ELISA | | | UBI 3ABC NSP-ELISA | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Neg. | Pos. | Specificity (%) | Neg. | Pos. | Specificity (%) |
| Vaccinated pigs from FMD free farm | | Neg. | 35 | 35 | 0 | | 35 | 0 | |
| | | Pos. | 79 | 79 | 0 | 100 | 77 | 2 | 97 |
| Maternal Antibody transfer experiment | 13 d.o | Neg. | 0 | 0 | 0 | | 0 | 0 | |
| | | Pos. | 4 | 4 | 0 | 100 | 4 | 0 | 100 |
| | 18–30 d.o | Neg. | 15 | 15 | 0 | | 15 | 0 | |
| | | Pos. | 0 | 0 | 0 | | 0 | 0 | |

Vaccinated using trivalent vaccine (O, A & Asia 1)

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## TABLE V. NSP-ELISA RESULTS FOR RUMINANTS

| Sample History | FMD Seropositive by LPB-ELISA | WRL 3ABC NSP-ELISA | | | | UBI 3ABC NSP-ELISA | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Negative | Positive | Specificity (%) | Sensitivity (%) | Negative | Positive | Specificity (%) | Sensitivity (%) |
| (a) Vaccinated cattle & buffalo in outbreak area (clinical history not known) | 66 | 37 | 29 | -- | -- | 49 | 17 | -- | 43 |
| Structured surveillance in FMD active area (vaccination history unknown) | 322 | 174 | 148 | -- | -- | 166 | 156 | -- | -- |
| (b) Vaccinated cattle & buffalo and FMD recently clinically infected (outbreak area) | 52 | 15 | 37 | -- | 71 | 30 | 22 | -- | 42 |
| (c) Vaccinated cattle & buffalo with no clinical symptoms (outbreak area) | 20 | 15 | 5 | -- | -- | 18 | 2 | -- | -- |
| (d) Vaccinated cattle & buffalo from FMD free zone | 64 | 64 | 0 | 100 | -- | 64 | 0 | 100 | -- |
| (e) Non-vaccinated cattle & buffalo from FMD outbreak zone | 36 | 33 | 3 | -- | 9 | 36 | 0 | -- | 0 |
| (f) Non-vaccinated cattle & buffalo from animals having had a recent clinical infection | 38 | 2 | 36 | -- | 95 | 0 | 38 | -- | 100 |
| (g) Non-vaccinated goats from FMD outbreak zone | 18 | 8 | 10 | -- | 55 | 2 | 16 | -- | 89 |

(a) Serum samples collected 9 m after vaccination; (b) Serum samples collected 1–2 m post-vaccination; (c) Serum samples collected 6 m post-vaccination; (d) Serum samples collected 9 m post-vaccination; (e) Serum samples collected 12 m post-outbreak; (f) Serum samples collected 2 post-outbreak; (g) Serum samples collected 2 m post-outbreak

## 2.2. Ruminants

### 2.2.1. Specificity

Sixty-four serologically positive by LPBE samples were tested from cattle and buffalo that had been vaccinated using a bivalent (O and Asia 1) vaccine. The serum was collected approximately 9 m post-vaccination. All samples returned a negative result by both UBI and WRL NSP-ELISA, 100% specific (Table V).

### 2.2.2. Sensitivity

Four separate groups of sera were used to determine the sensitivity of the NSP-ELISA test kits with mixed results. (i) Thirty-eight serologically positive serum samples collected from non-vaccinated cattle and buffalo 2 m post infection returned very high sensitivities. Ninety-five and one hundred percent for WRL and UBI kits respectively. (ii) Interestingly, serum samples that were collected from animals 12 m post infection returned surprisingly low sensitivities, 9 and 0% for WRL and UBI respectively. (iv) Serum collected from cattle and buffalo 2 m post-vaccination and known to have had a recent clinical infection retuned a low level of sensitivity using the UBI kit, 42%, and a below optimal sensitivity using the WRL kit, 71%. (v) Sera from 18 serologically positive goats, 2 m post-outbreak (no goats showed signs of disease), were also examined to determine the sensitivity of each kit. The WRL NSP-ELISA kit returned 10 positives from 18; (55% sensitivity) and the UBI NSP-ELISA kit returned 16 positives from 18; (89% sensitivity) see Table V.

## 3. DISCUSSION

The ability to be able to effectively and quickly distinguish between vaccinated and infected animals has the potential to be a very valuable tool in epidemiological investigations and for the control of FMD. Only 2 out of 3 FMD NSP-ELISA kits were examined during this contract; however, the results of which show great promise. The result that most obviously stands out from this study is the very high specificity of both the 3ABC NSP-ELISA procedures examined. A total of 147 samples (from 83 swine and 64 cattle and buffalo) were examined for specificity, with the WRL kit 100% specific and the UBI kit 99% specific. The question of sensitivity is something that needs to be examined a little closer however. Both the WRL and UBI kits showed a high degree of sensitivity, 95 and 100% respectively, when samples collected from infected cattle approximately 2 m post infection/outbreak were assayed. When samples that had been collected 12 m post infection/outbreak were assayed, sensitivity was very low for the WRL kit, 9%, and non-existent, 0%, for the UBI kit. A closer look at a possible correlation between low antibody titres and a lack of sensitivity will need to be undertaken. Samples were also examined where serum was collected 1-2 m post-vaccination from cattle and buffalo who had recently recovered from a clinical infection. The sensitivities of both test kits were greatly reduced. Why?

A distinct difference also exists in sensitivities between the two 3ABC kits when goat and pig sera were assayed. The UBI kit proved to be a much more sensitive test when swine and goat serum was examined. Is there a species bias?

In terms of specificity, both kits are similar. In terms of sensitivities, questions have been raised that will possibly be answered by combining the data of all members participating in this study.

# USE OF NON-STRUCTURAL PROTEINS OF FMDV TO DIFFERENTIATE VACCINATED AND INFECTED ANIMALS IN ARGENTINA

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**Abstract**

In this paper, we present the results of the use of serological tests to detect antibodies to non-structural proteins (NSP) in cattle and pigs from different epidemiological situations in Argentina. The work was made under the sponsoring of the IAEA/FAO from 2000 to 2004, as part of the Coordinated Research Project. Serological tests are used at the National Reference Laboratory (DILAB) for sero-surveillance; monitoring; control of animal movement and as diagnostic tool in case of outbreaks. The control of vaccine purity in terms of NSP contents was examined using various assays.

## 1.    INTRODUCTION

Argentina has an animal population susceptible of FMD: 55 million cattle, 12 million sheep, 2.5 million pigs, 0.78 million South American camelids. Argentina's division according to OIE status is Patagonia Region (FMD free without vaccination) and northern region (free with vaccination).The FMD National Eradication Plan comprises:

- Regionalization based on the ecosystems of the disease

- All sectors participation (farmers, public and private agencies)

- Systematic vaccination of 100% cattle population with single-oil vaccine

- Continuous control on animal movement

- Epidemiological surveillance.

Sero epidemiological surveys are carried out by SENASA yearly. The survey includes the monitoring of viral activity and immunity level. Viral activity in cattle is determined by the detection of antibodies to NSP using the 3ABC-ELISA/EITB system developed by PANAFTOSA, being the 3ABC-ELISA used as screening and the EITB as confirmatory test. In the case of sheep, goats and pigs viral activity is determined by the detection of specific antibodies against FMD serotypes O, A and C by Liquid-Phase Blocking ELISA.

## 2.    MATERIAL AND METHODS

### 2.1.    Serum samples

Serum samples were collected from: free FMD region without vaccination, field outbreaks, sero epidemiological surveys, and vaccinated and revaccinated cattle from potency test of vaccines

### 2.2.    NSP assays

At the start of the project, different NSP reagents were received in order to determine sensitivity and specificity

The assays used were:

- 3ABC-ELISA reagents from Brescia

- UBI FMDV NSP ELISA (cattle)

- ELISA 3ABC-CEDI

- ELISA 3ABC-PANAFTOSA

- EITB (Electro-immuno transfer blot)

- ELISA 3ABC – SVANOVIR.

3.    RESULTS

### 3.1.    Specificity in naïve cattle from FMD free region (Table I)

A total of 456 sera from naïve cattle and 184 naïve sheep were evaluated by the UBI kit. Three out of the total were positive (0.46%). Specificity was 99.5%

A total of 103 naïve cattle were tested by 3ABC ELISA reagents from Brescia, 4 out of 103 were positive (3.88%) Specificity of 96.1%

A total of 106 sera from naïve cattle were tested by 3ABC-ELISA- PANAFTOSA and 3ABC-ELISA SVANOVIR. 1 serum was reactive in PANAFTOSA kit and in SVANOVIR Kit (0.95%) Specificity was 99.0%

TABLE I. SPECIFICITY OF DIFFERENT NSPELISA KITS

| Animal Species | Number of animals | Specificity / Kit |
|---|---|---|
| Bovine Sheep | 456 184 | 99.5% ELISA UBI kit |
| Bovine | 103 | 96.1% ELISA- Brescia reagents |
| Bovine | 106 | 99% ELISA PANAFTOSA kit |
| Bovine | 106 | 99% ELISA SVANOVIR kit |

These results indicated that the performance of the kits were much more consistent than the provision of reagents.

### 3.2.    Specificity in singly vaccinated cattle

A total of 202 serum samples from single vaccinated cattle of official potency tests at 60 d post vaccination were evaluated using 3ABC ELISA SVANOVIR and 3ABC ELISA PANAFTOSA. In both assays, 3 sera were reactive (1.47%), but were different samples. In the confirmatory test (EITB) samples reactive by ELISA PANAFTOSA, were negative. Both tests had a specificity of 98.5%.
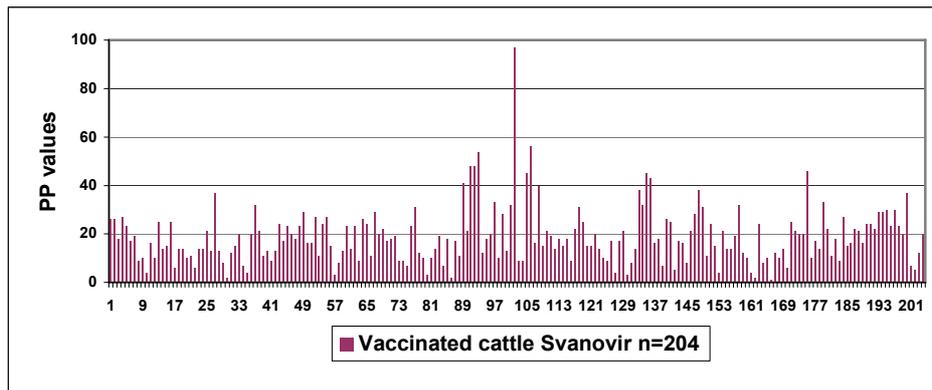
Cut-off: PP 50%

*FIG. 1. Specificity in single vaccinated cattle. Svanovir ELISA 3ABC kit.*
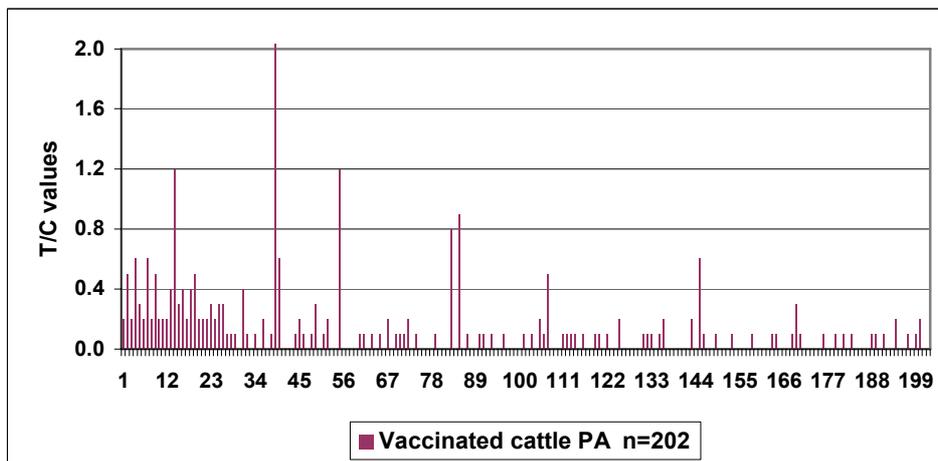


Cut-off: T/C value 1.0

*FIG. 2. Specificity in single vaccinated cattle. PANAFTOSA ELISA 3ABC kit.*

### 3.3. Sensitivity for cattle from field outbreak

A total of 19 sera collected from an outbreak 20 d post infection were tested using 3ABC-ELISA PANAFTOSA and SVANOVIR kits. Seventeen out of 19 samples were reactive in both kits. Sensitivity of 89.5% (Figs. 3 and 4).

A total of 28 samples collected from another outbreak were tested by 3ABC-ELISA reagents from Brescia. Twenty six out of 28 samples were positive. Sensitivity was 92.7%

These results are expected in case of a field outbreak. The sensitivity value is just as indicator and not the real sensitivity of the kits.
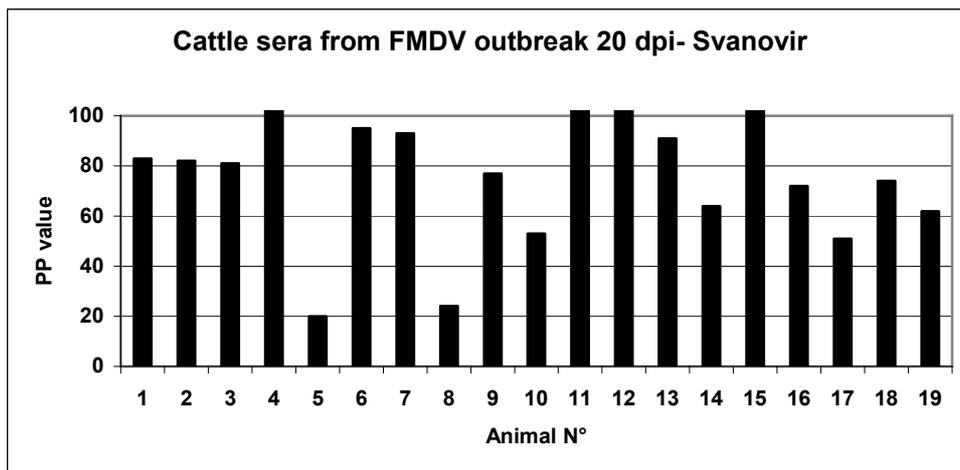
Cut-off value: PP 50%

*FIG. 3. Sensitivity of Svanovir ELISA 3ABC kit- Samples from outbreak.*
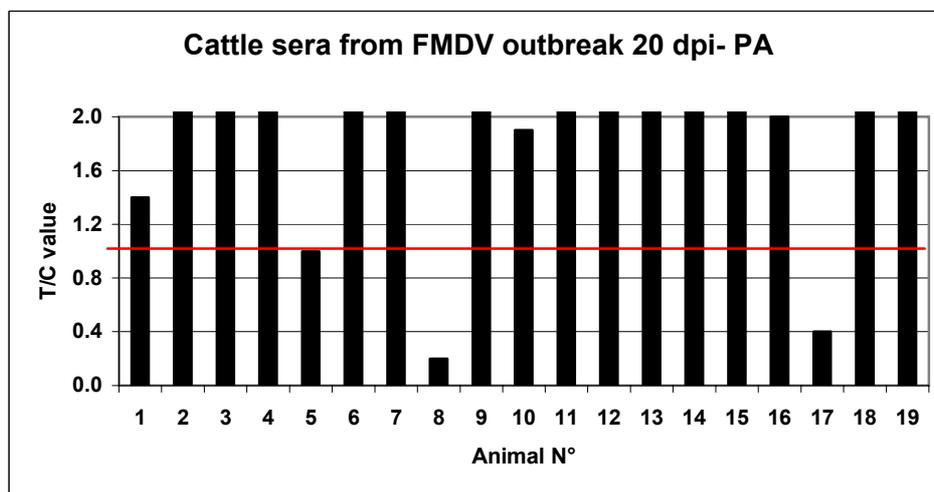


Cut-off value: T/C 1.0

*FIG. 4. Sensitivity of PANAFTOSA ELISA 3ABC. Samples from outbreak.*

## 3.4. Comparative results of four NSP assays using a sera reference panel from PANAFTOSA

Tables II and III show results of analysing sera from different epidemiological situations: free regions without vaccination, free regions with vaccination, outbreaks, experimental infection and sera from inoculated cattle with an attenuated strain.

The comparison was done using ELISA 3ABC/EITB system PANAFTOSA; ELISA 3ABC Bommeli; ELISA 3ABC CEDI; ELISA 3B UBI.

The results indicate that significant differences were shown among the NSP assays.

TABLE II. COMPARATIVE RESULTS OF DIFFERENT NSP ASSAYS WITH REFERENCE SERA PANEL OF PANAFTOSA

| Suero | Especificaciones | ELISA 3ABC + EITB - PA | Bommeli | CEDI | UBI |
|---|---|---|---|---|---|
| C.N | Suero Control Negativo CPFA | Neg | - | Neg. | - |
| C1 | Suero Control Positivo 1 ELISA C | **Pos.** | Neg. | **Pos.** | Neg. |
| C2 | Suero Control Positivo 2 ELISA C | **Pos.** | Neg. | **Pos.** | Neg. |
| C3 | Suero Control Positivo 1 EITB CP | **Pos.** | Neg. | **Pos.** | Neg. |
| S.5 | 13 dpi Exper. | **Pos.** | **Pos.** | **Pos.** | **Pos.** |
| S.6 | 11 dpi Exper. | **Pos.** | **Pos.** | **Pos.** | **Pos.** |
| S.7 | 15 dpi Exper. | **Pos.** | Ind. | **Pos.** | **Pos.** |
| S.21 | 34 dpi Exper. | **Pos.** | Neg. | **Pos.** | Neg. |
| S.9 | Suero positivo LMR | **Pos.** | **Pos.** | **Pos.** | Neg. |
| S.10 | Suero débil positivo LMR | **Pos.** | **Pos.** | **Pos.** | **Pos.** |
| S.11 | Suero débil positivo LMR | **Pos.** | Neg. | **Pos.** | Neg. |
| S.13 | 49 dpi - Cepa atenuada | **Pos.** | Neg. | **Pos.** | Neg. |
| S.14 | 56 dpi - Cepa atenuada | **Pos.** | Neg. | **Pos.** | Neg. |
| S.15 | 63 dpi - Cepa atenuada | **Pos.** | Neg. | **Pos.** | Neg. |

The sera are used routinely in the control of the PANAFTOSA ELISA and immunoblot tests. The results for the two PANAFTOSA tests are shown in left column. The CEDI test is in complete agreement for all sera. The Bommeli ELISA failed to detect positivity for 8 of the 12 sera. The UBI test failed to detect 8 /12 sera and the results indicate it has a similar sensitivity to the Bommeli test. The Bommeli and UBI tests have a lower sensitivity as compared to the CEDI.

TABLE III. COMPARATIVE RESULTS OF DIFFERENT NSP ASSAYS WITH REFERENCE SERA PANEL OF PANAFTOSA AND SAMPLES

| Serum | Description | ELISA 3ABC + EITB - PA | Bommeli | CEDI | UBI |
|---|---|---|---|---|---|
| C.N | Negative Control sera PANAFTOSA | Neg | - | Neg. | - |
| C1 | Weak Positive control sera PANAFTOSA | **Pos.** | Neg. | **Pos.** | Neg. |
| C2 | Strong Positive control sera PANAFTOSA | **Pos.** | Neg. | **Pos.** | Neg. |
| C3 | Positive Control sera EITB PANAFTOSA | **Pos.** | Neg. | **Pos.** | Neg. |
| S.5 | 13 dpi Exper. | **Pos.** | **Pos.** | **Pos.** | **Pos.** |
| S.6 | 11 dpi Exper. | **Pos.** | **Pos.** | **Pos.** | **Pos.** |
| S.7 | 15 dpi Exper. | **Pos.** | Ind. | **Pos.** | **Pos.** |
| S.21 | 34 dpi Exper. | **Pos.** | Neg. | **Pos.** | Neg. |
| S.9 | WRL Positive Control sera | **Pos.** | **Pos.** | **Pos.** | Neg. |
| S.10 | WRL Weak positive control sera | **Pos.** | **Pos.** | **Pos.** | **Pos.** |
| S.11 | WRL Weak positive sera | **Pos.** | Neg. | **Pos.** | Neg. |
| S.13 | 49 dpi - atenuated strain | **Pos.** | Neg. | **Pos.** | Neg. |
| S.14 | 56 dpi - atenuated strain | **Pos.** | Neg. | **Pos.** | Neg. |
| S.15 | 63 dpi - atenuated strain | **Pos.** | Neg. | **Pos.** | Neg. |
| S.16 | 77 dpi - atenuated strain | **Pos.** | Neg. | **Pos.** | Neg. |
| S.17 | 70 dpi - atenuated strain | **Pos.** | Neg. | **Pos.** | Neg. |
| S.18 | 532 dpi - OP(+) | **Pos.** | **Pos.** | **Pos.** | Neg. |
| S.19 | 602 dpi - OP(+) | **Pos.** | **Pos.** | **Pos.** | Neg. |
| S.20 | 615 dpi - OP(+) | **Pos.** | **Pos.** | **Pos.** | Neg. |
| S.8 | Outbreak w/vaccination OP(+) 90 d after ou | **Pos.** | Neg. | **Pos.** | Neg. |
| S.22 | Outbreak w/vaccination OP(+) 90 d after ou | **Pos.** | **Pos.** | **Pos.** | **Pos.** |
| S.12 | Outbreak | **Pos.** | **Pos.** | **Pos.** | Neg. |
| S.23 | Outbreak | **Pos.** | Neg. | **Pos.** | Neg. |
| S.24 | Outbreak | **Pos.** | **Pos.** | **Pos.** | **Pos.** |
| S.25 | Outbreak | **Pos.** | **Pos.** | **Pos.** | **Pos.** |
| S.26 | Outbreak | **Pos.** | **Pos.** | **Pos.** | **Pos.** |
| S.27 | Outbreak | **Pos.** | Neg. | **Pos.** | Neg. |
| S.28 | Outbreak | **Pos.** | **Pos.** | Neg. | **Pos.** |
| S.29 | Outbreak | **Pos.** | **Pos.** | Neg. | **Pos.** |
| S.30 | Outbreak | **Pos.** | **Pos.** | **Pos.** | **Pos.** |
| S.31 | Outbreak | **Pos.** | **Pos.** | **Pos.** | **Pos.** |
| S.32 | Outbreak | **Pos.** | **Pos.** | **Pos.** | **Pos.** |
| S.33 | 8 dpi - Bovine 1 | **Pos.** | Neg. | **Pos.** | Neg. |
| S.34 | 9 dpi - Bovino 1 | **Pos.** | Neg. | **Pos.** | Neg. |
| S.35 | 10 dpi - Bovino 1 | **Pos.** | Ind. | **Pos.** | **Pos.** |
| S.36 | 11 dpi - Bovino 1 | **Pos.** | **Pos.** | **Pos.** | **Pos.** |
| S.37 | 8 dpi - Bovino 2 | **Pos.** | Neg. | **Pos.** | Neg. |
| S.38 | 9 dpi - Bovino 2 | **Pos.** | **Pos.** | **Pos.** | Neg. |
| S.39 | 10 dpi - Bovino 2 | **Pos.** | **Pos.** | **Pos.** | **Pos.** |
| S.40 | 11 dpi - Bovino 2 | **Pos.** | **Pos.** | **Pos.** | **Pos.** |

The results indicate the lower sensitivity of the Bommeli and UBI tests as compared to the PANAFTOSA and CEDI, using the cut-off criteria ascribed by suppliers. The Bommeli test misses 18/39. The UBI also misses 22/39. Generally where there is a higher amount of antibody the Bommeli and UBI reflect a positive result. The CEDI test fails to detect only 2/39 sera found to be positive by PANAFTOSA combination of ELISA and immunoblotting.

### 3.5. Control of vaccine purity in terms of NSP contents

All vaccine batches manufactured in Argentina are controlled by SENASA to assure the absence of reactivity against NSP assays used in the sero-surveillance monitoring, according to OIE recommendations.

For licensing of the vaccine, the manufacturer needs to demonstrate that the product do not interfere with the serological NSP tests. The vaccines are released if they do not induce antibodies against NSP in vaccinated and revaccinated cattle. The sera tested for NSP correspond to the sixteen cattle used in the potency test.

An example of a vaccine, from a manufacturer without approved license, is presented in Fig. 5. In this case cattle received three doses of vaccine at 30 d interval. Serum samples were collected at 0, 30, 60 and 90 d of the start of the trial. At 0 d all the animals were not reactive by ELISA 3ABC.

PANAFTOSA. At 30 d post first vaccination one animal was reactive by ELISA. At 30 d post second vaccination, six animals were reactive by ELISA. At 30 d post third vaccination, nine animals were reactive.
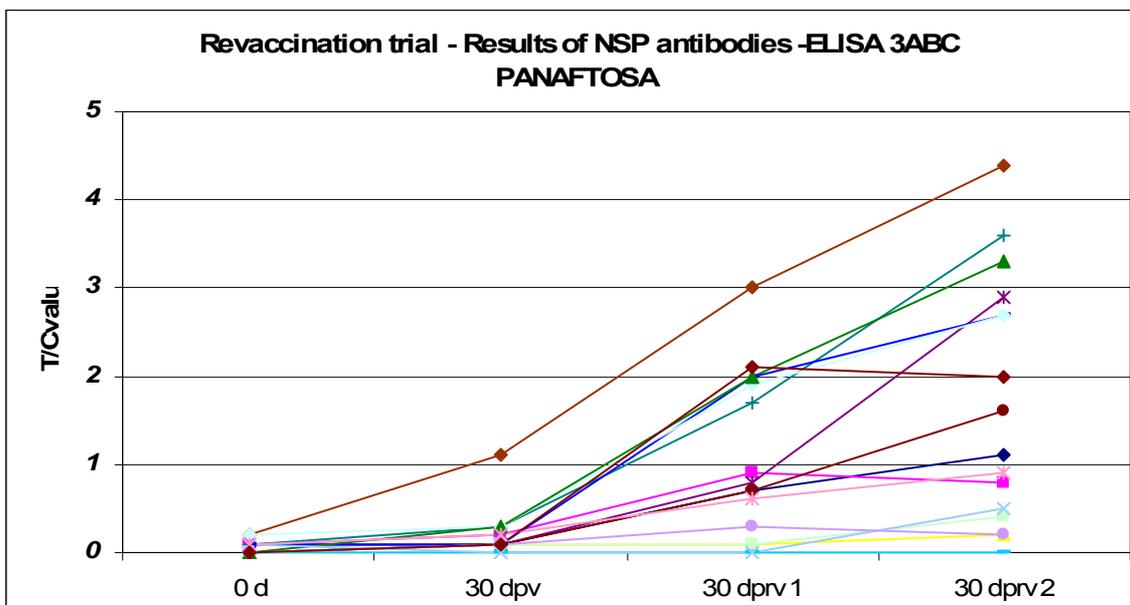


Cut-off value T/C 1.0
The lines indicate the T/C value of each animal serum after the vaccinations.

*FIG. 5. Reactivity to ELISA 3ABC PANAFTOSA in sera from vaccinated and revaccinated cattle purity control of unlicensed vaccine.*

## 4. CONCLUSIONS

During the execution of the Project it was demonstrated that the performance of the complete kits were consistent. In the case of the provision of in-house reagents, great variability in sensitivity and specificity was evident.

In general the NSP assays are useful tools for differentiating vaccinated infected animals in different epidemiological situations, like free areas with vaccination, systematically vaccinated areas, and endemic areas.

The NSP assays showed differences in sensitivity and specificity so it is recommended the selection of kits with high sensitivity and specificity.

It is valuable the availability of confirmatory test with other NSP besides 3ABC.

Due to the importance of the use of vaccines that do not interfere with the NSP serological tests for monitoring surveillance, it is necessary to control the purity of the vaccines regarding the induction of NSP antibodies.

# CONCLUSIONS BY THE TECHNICAL OFFICER

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The CRP reflects the variability in approach for various countries with respect to diagnosis and control of FMD and the variability in resources and the different epidemiological niches where tests are used. The papers from the different countries can be examined in this light.

Ideally the use of tests should be determined solely from the fitness of purpose criteria. This was reviewed in first paper by the technical officer. Often there is confusion when developing tests and then exploiting them, since the purpose of the test and the validation data to fit that purpose have not been examined properly. This was apparent in the CRP, which was also badly affected since developers' kept changing their reagents and performance criteria. This is a lesson we all have to learn along with a proper understanding of the terms diagnostic sensitivity (DSn) and diagnostic specificity (DSp). Often there is a wish for the most sensitive test without appreciating what the needs for the test are. This is associated closely with the prevalence of the disease or analyte, being studied. In the case of NSP test to determine whether herds are infected, then the prevalence should there be an outbreak is likely to be high (a large percentage of animals might expect to be infected). In this case the diagnostic sensitivity of the test needed can be low. Where there is a need to wheedle out single animals in a large herd, for example if we are looking for positive animals a long time after an outbreak situation, then there is a great need to have both a high diagnostic sensitivity and very high specificity. In between the balance of DSn and DSp come the requirements depending on the likely distribution of FMD. Known history of outbreaks and information on animals cannot be excluded and replaced by testing alone.

Full exploitation of the NSP ELISAs and confirmatory tests has not yet been made. One area is the continuous monitoring of animals to see whether there is a low level of FMD circulating, e.g. testing of animals a long time after an outbreak. This monitoring is an essential feature of the OIE pathway to declaring freedom from infection, but sampling regimens and methods based on statistical criteria need to be devised for each country or region, to indicate the best testing strategies. Continuous surveillance also includes the possibilities of using more mobile forms of NSP tests such as the dipsticks. These have been used successfully in Korea in an outbreak situation but are probably highly suitable for supply to local veterinarians who could check animals quickly during transportation. A scheme to use such NSP Dipstick tests is being examined in Bolivia under a Technical Cooperation project.

## Availability of kits

Tests utilizing FMDV NSP for use in detecting antibodies are available from commercial sources. These would be the kits supplied from IDEXX (I-ELISA); Svanova (I-ELISA), UBI (peptide NSP I-ELISA) and CEDI Diagnostics kits (C-ELISA). The prices are roughly in line and negotiations as to purchases can be made through contact with the companies. The PANAFTOSA kit is more in house and not available except in South

America where there is a mandate to provide kits to laboratories. Dipstick NSP ELISAs are also available.

## Performance of kits

The test differed in DSp and DSn, although this conclusion is mainly based on the cut-off criteria of the manufacturers. In turn this is based on their validation data using limited sera both in numbers and geographical spread as well as epidemiological definition. Where direct comparisons have been made between the kits, using the same sera at the same time, it has been shown that adjustment of the cut offs allows a far better fit to be made, in terms of DSn particularly.

## Post infected non vaccinated animals

The tests available are suitable for screening cattle and pig sera to show an outbreak and to follow on after outbreaks. Less data is available for sheep and goats however; there is no reason to suspect that the diagnostic potential is lower for these groups. After infection, the expected and measured prevalence is high so that the variation at the herd level does not drastically affect any of the tests and their DSn. The DSp of the tests for this purpose is also satisfactory.

Differences in performance do confuse results some times (around 4 months) after an outbreak. Here the antibody level in most animals has decreased to near base line (zero antibody levels) so that the variation in testing and analytical sensitivity differences between the tests comes into play which in turn affects the DSn.

The analytical sensitivities of tests were shown to be different in the CRP by using standard sera and titrated in dilution ranges using a constant serum matrix, reflecting the sample concentration of serum. The cut off levels set by manufacturer's indicated that the UBI was set at a low sensitivity (consequently is highly specific). This DSn and DSp relationship for the UBI is also affected by the use of peptide antigens which limit the antigenic target for antibodies.

There were some problems noted from peripheral studies with detecting antibodies against FMDV SAT 2 and 3 using the serotype peptides (O and A) in the test. This reflects the variation noted in sequence data in the NSP region, and this has been addressed more recently. Variation in the ability to detect post-infected cattle SAT sera was also seen using I-ELISA and 3ABC.

## Vaccinated animals

The specificity for detecting anti NSP in the face of vaccination was proven for all the tests; however, this is complicated by the specific vaccine used. The quality issue for purification has to be dealt with. In S. America this has been a major issue and improving the purification process was shown to drastically reduce the number on animals producing anti-NSP responses after vaccination. The DSn is drastically affected where there are animals which do convert after vaccination and all vaccines should be assessed in the light of the NSP test being used to assess post infection antibodies to NSP. The work by Braga shows what is possible using certain vaccines. The overall conclusion is that vaccines produced to high quality levels without contaminating NSP do allow NSP tests to determine antibodies against replicating virus. Here the difference in DSp of the UBI affects greatly the DSp for tests

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where poor quality vaccines are used. Where this is practised, the UBI test can be recommended since it does not recognise the antibodies produced against processed NSP from vaccines as compared to all tests utilizing 3ABC.

## Carrier animals

The risk from carrier animals in spreading disease is probably extremely small. However, the non acceptance of zero risk is behind the alarm and measures taken where FMD infects a none FMD country, making it the leader in feared diseases. The CRP showed that the antibody response to NSP in carriers can be very prolonged (years). This would be a good marker of carrier status; however, some animals do not produce anti-NSP (or much antibody at all) and some produce transient antibodies (in line with antibodies produced against structural antigens). Therefore, the use of anti NSP testing to identify carriers has to bare in mind the data surrounding the system being studied. In a population, for a long time after an outbreak (a year or 18 months) there will be animals, in the absence of clinical disease with antibodies against NSP (carriers) as well as carriers without antibodies to NSP. So it is possible to identify some animals which are carriers, but not all, using NSP tests. Epidemiological assessment of populations in time (suitable random or purposive sampling) through surveillance, can determine the rate of change of populations as to the antibody profile for anti NSP antibodies and antibodies to structural proteins The decay of both sets of antibodies after an outbreak and any increase in either, indicates residual infection. This is most marked where infection may induce hard to see clinical signs, for example in sheep and goats. It is likely that surveillance of sheep and goats using NSP tests will trigger alarm as to the number of animals with antibodies and hence potential threat (as assumed under zero risk mentality). There is no doubt that after outbreaks involving cattle and sheep that sub clinically infected sheep can be identified and traced for an extended time. This poses great problems with declarations of freedom from FMD virus to the OIE. Data on molecular studies (PCR) and tissue culture isolation, confirms the difficulty of analysing probang samples. The isolation rate for RNA or infectious virus is highly variable and does not correlate to the NSP tests. Although carriers are regarded as being capable of spreading virus, it is amazing that the use of the probang has no parallel in nature. The severe nature of scraping the cells from an animal's throat is never repeated in the wild and no one sees an animal scraping its throat on a bush! The likelihood therefore of virus being disseminated in this way is very low indeed.

## Pigs

The CRP indicated that some pigs produce large quantities of antibodies against NSP in the face of very heavy vaccination and where there is no evidence of infection. Pigs are not regarded as carriers. This means that virus is infecting and circulating in herds without clinical manifestation. This needs to be addressed and the management of pig herds in countries where heavy and efficient vaccination keeps clinically observed disease out of sight.

## Animal movement and quarantine control

Where there is an export potential, the screening of animals as they move and in holding stations or strict quarantine, is usefully done using NSP tests. All animals can be assessed reducing the errors of the sampling frame statistic. Ideally the Dipstick test can be used by personnel on the ground and any animals found positive be removed and measures taken to delay others from moving or sending them back to their origin. Because the requirement for vaccination is made, it is widely and erroneously assumed, that animals are immune and non infected. This is not true and animals can become infected as they travel.

Vaccination is never the proof of lack of virus (see Myanmar and Thailand data for MTM area of trade). A complete management package for the use of NSP tests has to be worked out to assure animals as negative for FMD at different times during transportation. This also applies to a constant monitoring of any population and methods to test small groups of animals, for example, in trucks by veterinary staff armed with NSP dipsticks, could greatly help monitor infected animals.

## Standards

Through the CRP, the IAEA contracted a South African laboratory to produce experimentally infected SAT sera and received these for characterisation. The sera will be used to compare tests and provide working standards. This has also been extended to type O, A, C and Asia 1 sera from cattle.

## Quality control

All the kits can be improved by addition of a better set of controls. It is imperative that a medium activity control is given for the assays whereby control charts can be plotted to compare the performance of the tests on each plate, from day to day and between laboratories. Ideally samples in the Indirect ELISA should be measured against the medium control and not the strong positive.

## Final comments

It was a great pleasure to work with all the counterparts, the Research Contract and Agreement holders. All were enthusiastic and friendly, dedicated and conscientious. No papers from the Agreement holders are presented although they were also enthusiastic and very learned, most of the information they gave is published in peer reviewed journals. Final thanks to the various representatives of the commercial companies of UBI, IDEXX (then Bommeli Diagnostics), CEDI and SVANOVA, who greatly supported the CRP through supply of kits and attendance at the RCMs. Involvement of commercial companies also accelerated the kit certification process by OIE.

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