"Observations and developments, under the Co-ordinated Research Program D3.20.20. “The use of non-structural (NS) antigens of FMD virus to assess antibodies in vaccinated and infected livestock"

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Observations from CRP D3.20.20.

1. Background

The development of assays to differentiate between FMD infected and vaccinated livestock, has been going on Internationally for at least twelve years. The target system has been the Enzyme Linked Immunosorbent assay (ELISA) using various antigens from of the non-structural (NS) proteins of FMD. All initial studies were made at Institutions, where FMD could be studied. Europe and S. America were the leaders in this. Laboratories within the EU got together under a Concerted Action initiative by the EU and exchanged results and methods. In S. America the PANAFTOSA created assays. Some cross-fertilisation of results was eventually made between S. American and European laboratories. The USA and Spain also have developed assays. At this moment there are three kits, which can be regarded as sustainable in supply. The performance of the kits should always be examined in the light of the perceived needs in the context of real science and the public demand generating the pressure to try and limit culling of non-infected or in-contact animals in outbreaks. The “fit for use” aspect should be a foremost consideration.

1.1. Coordinated Research Programme

A coordinated research programme (CRP) consists of supporting individual researchers for up to 5 years, as well as obtaining the services of Agreement Holders from leading Institutions, to help run the research. Technical contracts can be awarded to prepare reagents etc. Research Coordination Meetings (RCM) are held every 15-18 months to allow data presentation and discussion as well as to prepare workplans.

The CRP dealing with NS proteins, began in 1999 and the first RCM was held in Rio de Janeiro, March 20th -24th, 2000. The meeting was attended by 14 of the fifteen Research Contract Holders (RCH) and all six of the Agreement Holders (AH’s) as well as representatives of United Biomedical Incorporated.

Three main groups exist in the CRP, reflecting various national developmental states with regard to control of FMD. These were from South America, Argentina (2 Institutions), Colombia, Uruguay, Paraguay, Brazil, Peru; S.E. Asia, Thailand, Philippines, Laos, Malaysia,
Observations from CRP D3.20.20.

Myanmar, Hong Kong, China and Africa (S. Africa). The various needs of each group varied according to the phase of disease control in different countries and this theme emerged through the meeting. The supply of three sets of reagents was made in the first year for examination. These were from:

1. **Institute for Animal Health (IAH) Pirbright, England/Istituto Zooprofilatico Sperimentale, Brescia, Italy.** An *Indirect ELISA based on 3ABC antigen expressed in E.coli*. Here the 3ABC was either captured via a monoclonal antibody attached to the plastic plate matrix or used directly on plate.

2. **United Biomedical Inc., USA. (Commercial).** an *Indirect ELISA based on peptides (synthetic) 3A/3B directly attached to plastic matrix.*


Reagents from (1) and (3) were facilitated through technical contracts, whereas (2) was made available free of charges including their shipment.

Data was obtained by examination of one or more of the assays supplied, from testing an assortment of sera selected from existing serum banks or using sera collected specifically. Other test data, particularly from the Liquid Phase Blocking ELISA (LPBE) were also presented. This was reviewed and summarized in the 2nd RCM in Geelong, March 2002. A complete report of all data has been prepared and can be made available in hard copy. Basic conclusions reached about data using the reagent sets studied were summarized at the 2nd RCM, held in Geelong, Australia in March 2002.

- There were differences in diagnostic sensitivities of the assays detecting pig and cattle antibodies, as illustrated by differing results with serum from animals early after infection or late after infection.

- The diagnostic specificity of each of the tests was similar.
Observations from CRP D3.20.20.

- There were some difficulties with the Indirect ELISAs not being efficient at picking up certain species of sera e.g. buffalo sera in Philippines, and giraffe sera in S. Africa.

- The test from Denmark required a truly competitive format to achieve higher diagnostic sensitivity. A revised protocol was used successfully.

- Differences in vaccine formulation (presence of contaminating NS) affects results, as to whether antibodies against non-structural proteins are produced. This could be a problem in assessing results where some unlicensed vaccines are used.

- The ELISA devised in PANAFTOSA should be included for parallel testing.

- An estimation of the relative analytical sensitivity of each assay should be made.

- Immuno-blotting techniques have a role in surveillance in certain situations.

- The costs of “kits” should be addressed. It is vital that the cost element be identified early so that users can assess needs and cost these for future use in control programmes.

- The exact contents of a kit should be worked out and stated with protocols.

- Reference sera should be identified and volumes assessed for possible future use in reference panels for test comparison.

At the time of the 2nd RCM, in 2002, and to date, three commercial kits are available. It was decided that these would be focused on as the main sustainable kits for antibody measurement and that other earlier developments were regarded as interesting, but not immediately applicable to wide scale testing needs. These are shown in Table I and some features of the assays are mentioned.
2. Analytical and diagnostic sensitivities and specificities

These are required actors to allow a confidence statistic to be associated with any results. The complications of having several tests being developed using a variety of antigens and formats, illustrates a common difficulty in arriving at conclusions concerning the effective use of any tests supplied from various sources.

The various assays have been validated by the suppliers, mainly through the use of a limited number of sera available. The availability varied with the species. The sera selected were used to determine specificity with regard to measuring the population statistic of a number of negative sera, either as naïve animal population or by examination of post-vaccinated sera, with sensitivity being assessed with regard to sera measuring antibodies after experimental infection. All the assays were thus validated to a level within the frame of the sera examined. This validation sets the guidelines as to likely performance in the field conditions, but has to be regarded as a limited exercise, in that all conditions met in the field cannot be reproduced using mainly experimental sera.

An advantage of the CRP is that 15 laboratories can provide a wide range of sera to allow validation to be updated, provided that there is good knowledge on the sera collected with regard to vaccination and infection history. There will be variations in diagnostic performance since the antigens used are different. Some have more limited antigenic spectrum and thus may be affected were a population of antibodies is limited against that antigen. Conversely, limiting antigenic spectrum may reduce ‘background’ and effectively increase sensitivity. This balance is a compromise between ability to detecting antibodies after infection (diagnostic sensitivity) versus the reduction in false positivity (specificity). This was reflected in earlier results comparing the UBI kits with the progenitor kits of Bommeli Diagnostics from Brescia and Pirbright. Direct comparisons of overall performance were made using the Brescia and Pirbright reagents against those from PANAFOTSA, where very similar results were obtained. No direct comparison of the tests using the same sera have since been made. It is hoped that the kits will be compared soon in an exercise involving all
the contract holders in the CRP. The scheme for this was agreed and kits to allow this will be supplied as soon as possible. The sera will be chosen by the counterparts and results assembled at a single source to allow data processing. The commercial companies will have full access to the results and the data should be published through the OIE. This is an attempt at increasing validation criteria and harmonization of testing. It may prove easier to assemble sera at a single laboratory and perform testing with scientists representing all parties involved.

3. Assay needs

The main thrust for the development of tests in areas where there is incursion of disease with devastating consequences, appears to come from the public, who want to know why apparently healthy animals are culled, with the consequent problems of disposal and ethical considerations. They ask for a test that can tell whether a single animal has been infected or not. Thus, we are asking for a test with 100% sensitivity and specificity, which is never achievable. The assessment of the true diagnostic sensitivity and specificity of the tests for all species remains to be extended, but probably will achieve levels of 99% on both counts. All agree, due to the normal nature of FMD infection, that so far, the tests available can differentiate infected and non-infected animals at the herd level (several animals at least). However, there are factors that involve risk, when decisions when considering leaving apparently healthy and NS negative animals alive to mix with naïve animals at some later date. Table II attempts to summarise some of the various scenarios on infection of livestock with FMD. It is intended to highlight some situations where the test result does not give an equivocal answer. All the scenarios have been observed in some animals studied in the CRP. The numbers of animals in the various groups though cannot be considered as high enough to make categorical statements about the surety of the kits performance.
4. Epidemiological considerations, “Fitness for use” of assays

Certain epidemiological situations may require that a test is increased with regard to its sensitivity or specificity properties and assays can be adjusted to be more or less sensitive and more or less specific. Thus, where it is vital that all positive antibodies are needed e.g. where we are screening for every possible animal with that antibody, the test can be made very sensitive with a consequent increase in the rate of false positive samples. Samples with a near positive or weak positive result can then be studied further, whether by repeat testing with more replicates, or by a different test. This is illustrated with regard to the PANAFTOSA test designed to achieve maximal sensitivity with confirmatory testing of doubtful sera using western blotting techniques. Tests can be reduced in sensitivity so that they are specific. Thus, where there is expected infection, a herd screening to assess prevalence can be used where it is less important that a few percent of positive samples are ‘missed’.

UBI have taken up the position of being able to tailor the antigens used in their test and alter the specificity and sensitivity criteria as validation increase and needs arise. In this way, the specific requirements for a test e.g. following an outbreak in pigs on a large scale, where sensitivity is not the issue; or in testing animals some time after an outbreak to declare a region “free” of replication events in a wide ranging serological survey (where extreme sensitivity is required) can be addressed. This is of relevance when choosing a system for different applications in different countries at different situations with regard to FMD control.

5. Kits

The subject was discussed in some detail in Geelong in March with users and producers. This is a difficult area since there are differing opinions from commercial, Institutional and users on the concept. A perfect kit is defined below. The list highlights areas where the present kits fall down with regard to their use and operator responsibilities.
A good (perfect kit) should be:

1) Available in bulk.

2) Available on demand.

3) Costed, so that users understand the cost per sample.

4) Quality controlled in terms of day to day running.

5) Robust (stable reagents defined performances).

6) Contains all materials and reagents to perform the test.

7) Validated in terms of diagnostic sensitivity.

8) Validated in terms of diagnostic specificity.

9) Contain advise on how to read and interpret the results in the context of fitness for use criteria.

Points 1 and 2 are important and fulfilled by commercial companies where good standards of product are assured. Getting a cost per sample (point 3) from companies has proved difficult. Quality control (point 4) is paramount to assessing whether kits work on arrival and how they are performing plate to plate, day to day, week to week, and operator to operator. Without adequate IQC and descriptions of methods to perform IQC, there is little point in reporting data. This has been a constant factor found by the Joint FAO/IAEA division in its dealings with counterparts and various kits over 15 years and through EQA exercises to assess proficiency of laboratories. Methods to allow users to constantly monitor the tests have been devised and are being used. Without such a system then there is little point in buying kits where only processed data is reported. This is very important when considering the wide-scale use of assays and linked strongly to the acceptance of standards. Methods devised have been offered to the respective kit producers.

Point 5 is not easy to assess since very few kits have been sent on a routine basis. There are problems of delivery in developing countries so that for wide-scale use in less well-developed countries requires attention to robustness and materials that can be sent without the need for refrigeration.
Observations from CRP D3.20.20.

Point 6 produced the most conflict where developing country scientists asked for completeness to allow testing. In particular the tips used in assays are difficult to obtain. Points 7 and 8 have been examined and increased validation (a continuous process) is needed, particularly again where the tests are to be used in developing countries. Point 9 again tries to indicate that there is a joint responsibility for the successful performance of a kit which is often lacking though poor information from the commercial sector.

6. Standard sera

   The establishment of serum banks is important in standardizing assays. This is a most contentious area and one where there is little real progress. Steps taken by the CRP to produce and characterize sera for the NS area, include

1). Contracting OVI, Onderstepoort, to produce post infected cattle sera against FMD types SAT1, 2 and 3. This is thought necessary since certain of the kits do not function as well with the SAT serotypes. This serum will be available hopefully by December, 2002.

2). Identification or preparation of post infected pig serum in Laos (available) and Thailand, (available).

3). The provision of a high titre reference standard for activity from multiple sero type infected cattle from Lelystad. This is available.

   Sera will be sent to the IAEA laboratoires in Seibersdorf, Vienna as soon as possible and after irradiation, form a reference bank established. The sera should also be available to the WRL, AHHL Geelong and any interested commercial concerns involved with the CRP. This will allow characterization. The conditions for storage, methods of dissemination, etc., all have to be worked out in detail. It is imperative that such initiatives are considered important enough to elicit continuous funding and support. Standard sera are needed for two major purposes. One, to act as definitive analytical reference points and two, to act as more extensive panels for validation purposes. Sera from cattle, sheep, goats and pigs from all the various epidemiological situations should be stored. Large volumes are needed and this means a concerted action is needed to produce or identify sera.
7. Risk estimation of carrier animals

Carriers are deemed to be very important in assessing the risks of FMD following an outbreak, with or without vaccination. In fact they are the major concern, which sets the rules about culling all in contact or emergency vaccinated animals. It is generally agreed that carriers pose only a small risk to non-immune animals, in fact the situation where large numbers of carriers existed has been seen frequently but not studied. It is agreed that the risk has to be there, proof of the risk is based on rather tentative almost circumstantial data. For this reason, debates on risk rarely conclude with any figures. There are regions of the world where carriers are common. Mongolia vaccinated and left recovered animals recently following an outbreak. They have many animals that are potential carriers. Such areas should be targeted for study to assess antibodies using NS tests; infectious virus and PCR status and the examination of the mixing of such animals with non-immune populations. This is feasible and provides the non-laboratory field experimental situation to researchers. The figures on how many animals recovering are carriers and whether they produce long-term antibodies against NS are emerging and will add to the confidence that certain populations can be identified.

8. Conclusions

1. Tests involving ELISA technology using 3ABC or peptides from the 3BC region are sufficiently characterized to allow discrimination of vaccinated and infected livestock, at the herd level. Great care must be taken to establish the exact requirements of the assay for different epidemiological situations. Use of the assays in the context of other control measure may establish that there is very low risk from FMD transmission, in leaving certain populations of animals alive, following outbreaks.

2. The present indirect ELISA formats have some problems with dealing with all species of animal. Competition assays developed in Lindhold, Denmark (reported
in this session) using monoclonal antibodies against baculo virus expressed 3ABC have shown great promise and offer a better chance that all species can be examined. This system needs to be exploited at the commercial level. The IAEA in association with Geelong and Vienna veterinary University, is developing a competitive ELISA based on baculo expressed 3ABC using chicken antibodies, where it is hoped supply can be made through developing sustainable kit production in S. E. Asia.

3. Kit supply is not proving as straightforward as it might be due to costing of kits, their full content and licensing considerations when sending to certain countries. The PANAFTOSA reagents, designed for maximum sensitivity for detection of antibodies, have been strongly validated and excellent systems to look at the epidemiology of diseases in S. America well documented. However, there is a reluctance to supply the kit to other countries outside S. America until some preliminary work on sera outside S. America is done. This is affecting the exercise planned for the CRP to compare the UBI, Bommeli and PANAFTOSA kits. Therefore, at present only 2 kits can be considered available off the shelf. UBI and Bommeli.

4. Reference sera are needed badly both for analytical sensitivity comparisons and validation panels.

5. The best way forward in increasing validation data might be to gather sera at a central laboratory and test with all kits in a short time. The exercise to supply UBI, Bommeli and PANAFOTSA kits to 15 laboratories in the CRP is proving logistically very difficult.
### Table I

**Outline of kits available.**

<table>
<thead>
<tr>
<th>System</th>
<th>Antigen used</th>
<th>Properties</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. United Biomedical Inc. (UBI)</td>
<td>Indirect ELISA Peptides (synthetic 3A/ or 3B)</td>
<td>Systems bovine (sheep and goats) and pigs.</td>
<td>Used extensively in pigs in Taiwan. Large scale testing already. Full dossier for licensing. Publications. Contact: Scott Liu [<a href="mailto:SLiu@UnitedBiomedical.com">SLiu@UnitedBiomedical.com</a>]</td>
</tr>
<tr>
<td>3. PANAFTOSA/EMBRABIO</td>
<td>Indirect ELISA 3ABC, E.coli expressed.</td>
<td>Systems bovine (sheep and goats) Pigs not extensive though available.</td>
<td>Heavily used (500,000 sera?) in control programmes. Large experience in evaluating endemic areas using WB and ELISA. Pig assay not exploited as much. Commercial arm is EMBRABIO Brazil for distribution. Publications. Contact: Dra. Ingrid Bergmann <a href="mailto:iberman@panaftosa.ops-oms.org">iberman@panaftosa.ops-oms.org</a></td>
</tr>
<tr>
<td>1. BOMMELI Diagnostics (INTERVET association)</td>
<td>Indirect ELISA 3ABC, E.coli expressed purified.</td>
<td>Systems bovine (sheep and goats) and pigs.</td>
<td>Linked to developments by Pirbright, UK and Brescia (Italy). Original assays were compared to PANAFTOSA and were very similar. Commercialised recently. Full dossier for licensing. Contact: Dierk.rebeski <a href="mailto:dierk.rebeski@intervet.com">dierk.rebeski@intervet.com</a></td>
</tr>
</tbody>
</table>
# Table II

## Relationship of disease status of animals to test results.

<table>
<thead>
<tr>
<th>Level initial antibody produced</th>
<th>Result NS ELISA</th>
<th>Likely Status of animal</th>
</tr>
</thead>
<tbody>
<tr>
<td>1, Negative animal not infected</td>
<td>NON</td>
<td>N</td>
</tr>
<tr>
<td>2a) Infected 0-28d CS</td>
<td>HIGH</td>
<td>P</td>
</tr>
<tr>
<td>2b) Infected 0-28d CS</td>
<td>LOW</td>
<td>P/N</td>
</tr>
<tr>
<td>2c) Infected 45d CS</td>
<td>HIGH</td>
<td>P</td>
</tr>
<tr>
<td>3a) Infected 45d</td>
<td>LOW</td>
<td>P/N</td>
</tr>
<tr>
<td>3b) Infected 45d</td>
<td>NON</td>
<td>P</td>
</tr>
<tr>
<td>4a) Infected 160d</td>
<td>HIGH</td>
<td>N/P</td>
</tr>
<tr>
<td>4b) Infected 160d</td>
<td>LOW</td>
<td>N</td>
</tr>
<tr>
<td>5) Vaccinated not infected</td>
<td>NON</td>
<td>N</td>
</tr>
<tr>
<td>6a) Vaccinated infected 28d PI</td>
<td>HIGH</td>
<td>P</td>
</tr>
<tr>
<td>6b) Vaccinated infected 28d PI</td>
<td>LOW</td>
<td>P/N</td>
</tr>
<tr>
<td>7a) Vaccinated 45d PI</td>
<td>HIGH</td>
<td>P/N</td>
</tr>
<tr>
<td>7b) Vaccinated 45d PI</td>
<td>LOW</td>
<td>P/N</td>
</tr>
<tr>
<td>8a) Vaccinated 160d PI</td>
<td>HIGH</td>
<td>P/N</td>
</tr>
<tr>
<td>8b) Vaccinated animal 160d PI</td>
<td>LOW</td>
<td>N/P</td>
</tr>
<tr>
<td>8c) Vaccinated animal 160d PI#</td>
<td>NON</td>
<td>P</td>
</tr>
<tr>
<td>9) Multiply vaccinated animals</td>
<td>HIGH/LOW</td>
<td>N</td>
</tr>
<tr>
<td>10) In contact animal 0-28d CS</td>
<td>HIGH</td>
<td>P</td>
</tr>
<tr>
<td>11) In contact animal 28d NCS</td>
<td>LOW (Non)</td>
<td>P</td>
</tr>
<tr>
<td>12) In contact animal 45d onwards</td>
<td></td>
<td>P</td>
</tr>
<tr>
<td>13) Infected no antibodies</td>
<td>NON</td>
<td>N</td>
</tr>
<tr>
<td>14) NS-contaminated vaccine</td>
<td>HIGH</td>
<td>P</td>
</tr>
<tr>
<td>15) Late development of antibodies against virus e.g. in pigs vaccinated then challenged 0-21d #</td>
<td>NON</td>
<td>N</td>
</tr>
<tr>
<td>16) Late development of antibodies against virus e.g. in pigs vaccinated then challenged 21d-35d NO clinical signs#</td>
<td>HIGH</td>
<td>P</td>
</tr>
</tbody>
</table>

# = (Observed under CRP); NCS = No clinical signs; CS = Clinical signs; N = NEGATIVE (not infected); P= POSITIVE (infected); HIGH means production of high titre of antibodies; LOW means production of low titre of antibodies; NON means that no antibody is produced.