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Management of classical swine fever and foot-and- mouth disease in Lao PDR

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**Proceedings of an international workshop held in Vientiane,
Lao PDR, 20–21 November 2006**

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2008

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Published by the Australian Centre for International Agricultural Research (ACIAR)
GPO Box 1571, Canberra ACT 2601, Australia
Telephone: 61 2 6217 0500; email: <aciarc@aciarc.gov.au>

Conlan J.V., Blacksell S.D., Morrissy C.J. and Colling A. (eds) 2008. Management of classical swine fever and foot-and-mouth disease in Lao PDR. Proceedings of an international workshop held in Vientiane, Lao PDR, 20–21 November 2006. ACIAR Proceedings No. 128, 98 pp.

ISBN 978 1 921434 98 3 (print)

ISBN 978 1 921434 99 0 (online)

Technical editing by Jo Mason, Mason Edit, Adelaide, Australia

Design by Clarus Design Pty Ltd, Canberra, Australia

Printing by Goanna Print, Canberra, Australia

Diagnostic tests for the control of classical swine fever and foot-and-mouth disease in South-East Asia: An overview

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Abstract

Classical swine fever (CSF) and foot-and-mouth disease (FMD) are two major trans-boundary animal diseases (TADs) having economic impact on the South-East Asian region. This paper describes the various diagnostic tests available for CSF and FMD, the limitations of each and their potential application in a low-technology setting. The need to have complementary field and laboratory operations including suitable samples and transport methods are discussed, and examples are given. The importance of a quality assurance system to assess the accuracy and precision of diagnostic results is highlighted.

Introduction

Livestock are highly important in the agriculturally based economic and social structures of Asia. Endemic and periodically epidemic foot-and-mouth disease (FMD) has a serious impact on food security (including crop production through its effect on draught animals), rural income generation, and national economies by impairing livestock trade. Consequently, the poorest sectors of the community

are the most seriously affected. The progressive control of FMD is both a national and regional priority (Khounsy et al. 2008, in press). FMD is the most contagious disease of mammals and can cause severe economic loss in susceptible cloven-hoofed animals. While the disease usually does not cause high levels of mortality, it results in productivity losses and the lameness it induces severely limits the uses of cattle and buffalo for traction, which is of major importance to the livelihoods of poor farmers.

Classical swine fever (CSF) is known internationally as one of the most serious diseases of pigs. Infection may result in mortalities of up to 100% in the acute form or reproductive failure and increased susceptibility to other infections. CSF causes large financial losses to both commercial and smallholder pig farmers, contributing to rural poverty. Control of the disease is attempted by vaccination. The economic burden of CSF to the region is difficult to quantify without an accurate diagnostic capability, but there is consensus that it is the most serious disease faced by the pig industry.

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All farmers and governments in the region spend large amounts of money on FMD and CSF vaccines but outbreaks still occur, causing the farmers to vaccinate more frequently. Not all existing laboratories in the region have the necessary capability to confirm the efficacy of FMD and CSF vaccines and to provide an accurate diagnosis. This paper will discuss the different options available for the detection of FMD and CSF antigen and antibody that can be applied to control the diseases. The control of trans-boundary animal diseases (TADs) such as FMD and CSF can only be achieved by taking a regional approach with countries working together. The application of appropriate diagnostic tests under a quality assurance system to ensure accurate and precise results, combined with surveillance and a disease investigation program under a well-resourced animal health network, is vital for disease control.

Animal health network

The diagnosis and control of infectious livestock disease is an important role of the animal health network, comprising both field and laboratory personnel and requiring complementary field and laboratory operations. The animal health network is active during periods of disease surveillance and outbreaks.

During surveillance, laboratory staff conduct post-vaccination testing to both confirm vaccination success and to determine and monitor disease prevalence in target populations. The field veterinarians collect information on vaccination history, which includes vaccines used, disease outbreaks and health status of animals. It is important to involve laboratory diagnosticians, field veterinarians and epidemiologists in the planning of surveillance studies. This will ensure that critical parameters, such as suitable samples, test performance characteristics and accurate prevalence estimates, are available for the design of sampling frames for surveillance studies.

During a disease outbreak situation, field veterinarians are charged with the responsibility of collecting outbreak information, making a clinical diagnosis, collecting samples from suspected cases, and implementing control measures such as disinfection and quarantine and movement of animals. The laboratory staff support and complement the field investigation by conducting tests to confirm the clinical diagnosis and isolate the causative agent for

further characterisation, and performing molecular epidemiology studies to establish potential links with other outbreaks. In the case of CSF, a laboratory confirmation is required due to the difficulty of correctly identifying cases based solely on clinical signs. For FMD the laboratory confirmation is important to establish the serotype responsible for the outbreak.

Classical swine fever

Clinical diagnosis

It is difficult to accurately and confidently predict the CSF infection status of a herd based on clinical findings alone. The clinical signs and lesions associated with CSF can vary depending on the virulence of the virus and, importantly, individual pigs may show different signs when infected with the same virus strain. Clinical diagnosis is further complicated by inter-clinician variation. The experience of the clinician is very important but, in all cases, samples should be collected and tested in the laboratory to confirm or deny a suspicion based on clinical findings.

Laboratory diagnosis

The quality of laboratory testing is only as good as the samples collected and submitted. Suitable samples, which for CSF include spleen, tonsil, lymph node, whole blood and kidney tissue, will maximise the chances of making a correct diagnosis. Samples should be collected from no fewer than four animals showing clinical signs, with samples of approximately 2 g of tissue and 10 mL of blood from each animal transported on ice and reaching the laboratory as soon as possible after collection. A good history of the animals from which the samples were collected is required, as are details of the outbreak investigation or surveillance. This information links the diagnostic results with outbreak and control efforts.

Isolation and specific detection of virus in tissue culture

In-vitro isolation and subsequent detection of CSF virus is achieved on porcine kidney (PK15) cells or other suitable cells such as primary pig kidney (PK), swine kidney (SK6) or swine testis (ST), and is considered one of the most sensitive diagnostic tests. However, virus isolation (VI)

requires specialised facilities for cell culture and handling of virus, and is expensive to maintain. Reference laboratories require this test for characterisation of virus isolates. CSF virus grows in cell culture approximately 18–24 hours after inoculation but samples must be passaged on cells for three 4-day periods before being declared negative. The identification of virus isolates is carried out using specific antiserum- and immuno-techniques on fixed cell cultures, antigen capture (AC)-ELISA or polymerase chain reaction (PCR) tests, usually 48 hours post infection.

Antigen detection

There are a number of techniques for detection of antigen, allowing for rapid and cheap detection of CSF from field samples. In the case of ELISA, testing can be scaled up to process a large number of samples in a relatively short period of time. In-house antigen detection ELISAs (eg AAHL ELISA; Shannon et al. 1993) are commonly used and commercial antigen detection ELISA kits are available from many companies, most commonly CEDI Diagnostics (the Netherlands), IDEXX (USA) and Symbiotics (France). The antigen detection ELISA for CSF gives a result in 3–5 hours depending on the test used, with some tests having an overnight incubation step. New research in Lao PDR, as part of the ACIAR project, has led to the development of a rapid antigen detection ELISA test in a tube using immunomagnetic beads (IMB) as the solid phase (Conlan 2006; Conlan et al. 2008a). The IMB test can be used in the field and read by eye with a result in 60–90 minutes. Immunocytochemistry-based tests such as the fluorescent antibody test (FAT) and immunoperoxidase (IPX) staining are also used to detect CSF virus antigen in cell culture and tissue sections, and results can be achieved within 2 hours.

Molecular technologies

With improvements in PCR technology and advances in methodologies, the detection of viral RNA as a diagnostic tool has now largely surpassed the more traditional procedures such as virus isolation and FAT. There are a number of conventional and real-time PCR methods available for the detection of CSF genome. The real-time PCR (Ophuis et al. 2006) methods currently available are rapid and have high diagnostic sensitivity and specificity. Because the analytical sensitivity of PCR is also greater than other tests, viral genome can be

detected in smaller amounts and therefore sooner after infection, which has important implications for control efforts. Molecular technologies also allow the investigator to perform genetic characterisation of virus isolates and undertake molecular epidemiological studies to identify infection sources and virus evolution. Molecular technologies are, however, expensive and require high-quality samples with intact RNA. When samples are transported at ambient tropical temperatures, as is the case in Lao PDR, sample degradation has been shown to be detrimental to diagnostic performance (Blacksell et al. 2004).

Serological detection

Detection of antibodies to CSF virus has limited scope in diagnosis, particularly if the focus is on the early detection of virus in a herd or if vaccination is undertaken. Serum antibodies to CSF virus typically appear approximately 10–21 days after infection. Serological testing is, however, an important component of a disease control program to monitor the success of vaccination. Antibody detection is best achieved by the ‘gold standard’ neutralising peroxidase linked assay (NPLA). However, because this test requires tissue culture, it is time consuming, expensive and not suitable for the rapid screening of large numbers of samples. Other methods include in-house ELISAs (Colijn et al. 1999) such as the complex trapping blocking (CTB)-ELISA from the Australian Animal Health Laboratory (AAHL) and ELISA kits that can be purchased from commercial suppliers such as IDEXX and CEDI Diagnostics. Not all diagnostic tests are equally suitable to monitor sero-conversion after vaccination. An example is the AAHL CTB-ELISA that is of limited value to detect post-vaccination antibodies in pig sera because its MAb is specific for the NS3 protein of the crude antigen extract. These antigens are normally exposed after infection but only in limited quantities after vaccination. On the other hand, the NPLA and commercial ELISAs, such as the CEDI ELISA, are more sensitive for the detection of post-vaccinal antibodies because the CEDI ELISA uses a baculovirus expressed E2 protein subunit and an E2-specific MAb. Under experimental conditions with 20 vaccinated pigs, the CEDI ELISA showed a similar sensitivity to detect post-vaccinal antibodies as the NPLA, which is considered the gold standard (Conlan et al. in press; Conlan et al. 2008b).

Foot-and-mouth disease

Clinical diagnosis

Clinical signs of FMD vary between species. In cattle, onset of FMD is initially characterised by pyrexia, anorexia and shivering, followed by smacking of the lips, grinding of the teeth, drooling, lameness, and stamping or kicking of the feet. These symptoms are caused by vesicles on buccal and nasal mucous membranes and/or between the claws and coronary band that will rupture, leaving erosions. Recovery generally occurs within 8–15 days although complications can include superinfection of lesions with bacteria or screwworm infestation, hoof deformation, myocarditis, abortion, death of young animals and permanent loss of weight. Post-mortem lesions on rumen pillars and in the myocardium, particularly of young animals (tiger heart), may be evident. In sheep and goats the lesions are less pronounced and foot lesions may go unrecognised. Pigs may develop severe foot lesions, particularly when housed on concrete, and there may be high mortality in piglets. The differential diagnosis is species dependent and includes vesicular stomatitis, swine vesicular disease and vesicular exanthema of swine, which are all clinically indistinguishable from FMD.

Laboratory diagnosis

Virus isolation

As with CSF, virus isolation is expensive to maintain and requires specialised facilities for cell culture and virus handling. Virus isolation and characterisation is important to compare circulating viruses with vaccine strains (*r*-value) to maximise vaccine effectiveness. FMD virus (FMDV) will grow in a wide range of primary and continuous *in-vitro* cell cultures. The most sensitive cell culture for the isolation of FMDV is primary bovine thyroid (BTY) cells (House and House 1989). Continuous cell lines such as baby hamster kidney (BHK), lamb kidney (LK) and the pig kidney cell lines IB-RS-2 and MVPK-1 are also susceptible to FMDV infection. The sensitivity of virus isolation will depend on the quality and type of cells used as well as the quality of the sample.

Antigen capture ELISA

The antigen capture (AC)-ELISA or serotyping ELISA is the test of choice for countries endemic with FMD and is the recommended test for the detec-

tion of FMD antigen (Office International des Epizooties 2004). The FMD AC-ELISA provides detection of FMD antigen and identification of serotype in the case of an FMD-positive sample, and was developed in its current form by Roeder and Le Blanc Smith (1987) and Ferris and Dawson (1988). The FMD AC-ELISA replaced the complement fixation test for primary FMD diagnosis and serotype identification because of its increased specificity and sensitivity and because it is not affected by pro- or anti-complementary factors in the test sample. Standard reagents for the FMD AC-ELISA are produced at the World Reference Laboratory (WRL) for FMD, Pirbright, United Kingdom. At the Regional Reference Laboratory (RRL), Pak Chong, Thailand, reagents for the detection of serotypes A, Asia 1 and O are routinely produced for use in Asia. Sample quality is important as lesions older than 4–5 days have less antigen; however, samples unsuitable for virus isolation can be tested by ELISA. The ELISA allows high throughput testing of samples and is well suited to low-technology settings. Higher throughput can be achieved with robotics and other equipment and is mainly used in large laboratories which can afford to purchase and maintain this capability.

Molecular technologies

In the years since the advent of genetic diagnostic techniques nearly 2 decades ago, more than 50 different nucleic acid hybridisation and various PCR methodologies have been reported for the diagnosis of FMD. Recently, real-time PCR methods (TaqMan, molecular beacons, Primer-Probe Energy Transfer system) have been developed for FMD diagnosis and are now the mainstay for FMD genetic diagnosis (Reid et al. 2002; Oem et al. 2005). Evaluation of real-time PCR methods with conventional diagnostics (Shaw et al. 2004; Ferris et al. 2006) concluded that PCR was generally more sensitive and rapid, and is ideal for samples which contain low concentrations of virus. By introducing nucleic acid extraction and pipetting robotics, together with multichannel real-time PCR machines, diagnostic procedures have become rapid, robust and automated but may not be best suited to low-technology settings. Another promising development for developing country laboratories is the one-step, reverse transcription loop-mediated amplification (RT-LAMP) assay, which enables FMD virus to be detected in under 1 hour in a single tube without thermal cycling (Dukes et al. 2006).

Serological methods

The FMD liquid phase blocking (LP)-ELISA was developed for the detection of FMD antibodies because of the drawbacks of the conventional virus neutralisation tests (VNTs), which included slowness of the test (up to 3 days), the use of live virus and cell cultures, and the difficulty in reproducing results, all which could be countered by the use of ELISA. The FMD LP-ELISA can detect antibodies against all seven FMD serotypes using polyclonal rabbit and guinea pig IgG antibodies to detect residual FMD antigen following an in-vitro incubation of test serum and FMD antigen (the 'liquid phase'). Results from the FMD LP-ELISA indicated a high degree of correlation with VNT results for post-infection and vaccinated animals, and it was suggested to be a suitable alternative to the VNT (Hamblin et al. 1986a, 1986b, 1987). It was also suggested that the FMD LP-ELISA could be used to estimate in-vivo protection to FMD challenge (Hamblin et al. 1986a, 1986b, 1987).

The FMD LP-ELISA is one of the recommended ELISA methods for the detection of FMD antibodies (Office International des Epizooties 2004) and is the primary test for determining vaccine titres, being used throughout Asia (Blacksell et al., in press). Recently, the FMD competitive (C)-ELISA has been developed for all seven serotypes of FMD in response to the FMD LP-ELISA being less conducive to large-scale testing and automation. The FMD C-ELISA was developed using the same reagents as the FMD LP-ELISA but without the 'liquid-phase' step, allowing a result in the same day (4–5 hours). The FMD C-ELISA was found to be more robust, sensitive and specific than the FMD LP-ELISA, and was used in the recent UK FMD outbreak to allow rapid screening of serum samples for FMD antibodies.

FMD non-structural protein assays

Viral replication in FMD-infected animals induces an immune response against the non-structural (NS) protein of the FMD. The response against NS proteins is not serotype specific and indicates infection with any of the seven serotypes. Animals which are not infected with FMD but vaccinated normally don't develop a detectable antibody response against NS protein in the ELISA. Nevertheless, repeatedly applied, low-quality vaccines, (e.g. lack of viral inactivation and purification) may induce a false positive result in this test. In these

cases the history from the field, e.g. about potential outbreaks/infection, identification of vaccine and number of vaccinations received, is important for correct interpretation of the result.

The use of vaccine for control of FMD has led to the development of a number of assays for the detection of NS antibodies to discriminate between vaccinated animals and those that have been infected. AAHL, with the support of IAEA, has developed an in-house FMD NS 3ABC C-ELISA (Morrissey et al. 2007). It uses baculovirus expressed 3ABC antigen and a competing antibody, which is produced in chicken. This ELISA has been used and validated in the region (IAEA TECDOC 2007). There are a number of commercial ELISA kits (de Bronsvort et al. 2004) available from CEDI Diagnostics (baculovirus 3ABC expressed antigen), Bommeli (E-coli 3ABC expressed antigen) and UBI (synthetic 3B antigen), which are the most common NS-ELISAs in use in the region. The CEDI Diagnostics kit and the AAHL kit are both competitive ELISAs that can be used for all species, whereas the other kits are indirect ELISAs with separate kits for ruminants and pigs. The CEDI Diagnostics kit has been found to be the most sensitive and specific kit of those used in the region (Brocchi et al. 2006). Comparisons between the kits from CEDI and AAHL have shown that both ELISAs have similar performance characteristics when applied in the region.

Quality assurance and quality control (QA/QC)

'Quality is fitness for the intended purpose'. Quality assurance (QA) is a system designed to assure test facility management of compliance with a quality standard, e.g. AS ISO 17025-2005 'General requirements for the competence of testing and calibration laboratories'. Quality control (QC) is the technical realisation of the QA concept, e.g. calibration, assay validation, precision and accuracy of test results. QA and QC principles are crucial requirements to comply with quality standards such as ISO 17025-2005 or the OIE's 'Quality standard and guidelines for veterinary laboratories: Infectious diseases'.

The key components of QA are:

- paperwork/documentation of all tests into standard protocols
- validation data for diagnostic tests being used in the laboratory

- staff training and accreditation
- internal quality control (IQC)—positive and negative controls included in each test run
- analysis and charting to document results from IQC controls used in each test
- external quality assurance—successful participation in proficiency test rounds
- documentation on all sample collection, storage and transport from the field, and storage and handling in the laboratory
- calibration of equipment and calibration records
- laboratory accreditation to a standard, e.g. ISO 17025-2005.

Quality control of diagnostic tests is achieved through a combination of IQC and external quality assurance (EQA). Repeatability and reproducibility are measurements of precision and results are of particular value to monitor the validity of test results (De Clercq et al. 2008).

IQC is useful to measure the **repeatability** of test results in a laboratory. Ideally, internal controls should be included as replicates in each test run and should cover at least the critical range of test results to be expected, e.g. strong positive control (C++); weak positive control, which is slightly above the cut-off (C+); and negative control. Analysis of IQCs will give information about intra- and inter-assay variation, intra- and inter-operator variation, day-to-day variation etc. Critical parameters are basic statistics such as mean values, standard deviation, coefficient of variation, range, and upper and lower control limits. Results can be charted and recorded as Levey-Jennings charts. This approach helps to identify trends in assay performance and is useful to prompt preventive corrective actions or troubleshooting. IQC data can also be useful to assess measurement of uncertainty, e.g. continued measurement of replicates of an internal positive control close to the cut-off (see <http://www.scahls.org.au/policyguidelines/Worked_MU_examples.doc>).

EQA or proficiency testing (PT) measures the **reproducibility** of a test and its performance in different laboratories. It helps to standardise test results for the same test in different laboratories (inter-laboratory comparison, ring test or external quality assurance) or to harmonise test results from different tests in different laboratories (proficiency test round). Successful and regular participation approximately twice a year in EQA programs is an essential component of ISO 17025-2005 or OIE quality standard requirements, and therefore a pre-condition for accreditation.

Equipment calibration and maintenance is another important part of QA because it helps to ensure that tests are giving correct results. It is important that laboratories have a budget to allow them to maintain and calibrate their equipment. In summary, QA and QC are crucial elements in a laboratory's quality system and need to be well established to achieve accreditation to internationally accepted standards.

Discussion

Effective diagnosis and control of livestock diseases requires a strong animal health network where laboratory staff, field veterinarians and epidemiologists work together. Laboratories contributing to the diagnostic network must be able to carry out diagnosis with OIE recommended or alternative tests within a recognised QA system. OIE reference laboratories play an important role in monitoring the disease situation in a country and ensuring that continued, updated and accurate information is forwarded to OIE. This is especially important with TADs, zoonotic, and new and emerging diseases because of their global threat.

The laboratory network in a country is made up of laboratories at different levels of standard and capability, from the national laboratory down to the province and district levels. The diagnostic tests used in these laboratories will differ according to their respective capabilities (Tables 1 and 2).

The national laboratory may have the full range of diagnostic tests, which includes virus isolation and a molecular capability for PCR and sequencing, whereas a provincial or district laboratory will only have low-cost technology. Tests such as ELISAs are the most routinely used for CSF and FMD antibody and antigen detection. ELISAs are cheaper to run than virus isolation and PCR but reagents and equipment are still expensive for laboratories in poorer countries or at the district level. The development of cheaper or low-technology diagnostic tests such as the IMB-ELISA for CSF is important to allow rapid diagnosis close to the disease outbreak, e.g. in a district laboratory. The IMB-ELISA does not require any expensive equipment and can be easily quality assured.

For CSF serology the ELISA is the test of choice for sero-surveillance and post-vaccination testing. The VNT gives greater sensitivity and is used to support or confirm ELISA results. Normally it is available either at the national laboratory or a reference laboratory. The VNT test is still the test of

Table 1. Comparisons among classical swine fever diagnostic tests

Type of test	DSn	DSp	Speed	Cost	Quality of sample required	Degree of proficiency required	High sample throughput	Applicability to reference laboratory	Applicability in a low-technology setting
Virus isolation	*****	*****	.	*****	*****	***	.	*****	.
Molecular technologies	*****	*****	***	*****	*****	*****	***	*****	***
AC-ELISA	***	***	***	.	***	**	*****	***	*****
IMB-ELISA	***	***	*****	.	***	.	***	***	*****
Immuno-cytochemistry	***	***	***	***	***	***	***	***	**
Serology	**	**	***	**	***	***	*****	***** (NPLA and ELISA)	***** (ELISA)

DSn = diagnostic sensitivity; DSp = diagnostic specificity

Table 2. Comparisons among foot-and-mouth disease diagnostic tests

Type of test	DSn	DSp	Speed	Cost	Quality of sample required	Degree of proficiency required	High sample throughput	Applicability to reference laboratory	Applicability in a low-technology setting
Virus isolation	*** (BTY)	***** (BTY)	**	*****	*****	****	**	*****	.
Molecular technologies	*****	*****	***	*****	*****	*****	*****	*****	**
AC-ELISA	***	*****	*****	***	***	***	*****	***	*****
LPB-ELISA	*****	*****	***	***	***	***	*****	***	*****

BTY = bovine thyroid cells (BTY is most sensitive cell line; other cell lines are less sensitive)

DSn = diagnostic sensitivity; DSp = diagnostic specificity

choice when studying maternal antibody levels in piglets to determine the best time for vaccination and vaccine protocols. For detection of CSF, an ELISA is recommended, especially where large numbers of samples are being tested. The CSF PCR for detection of genome is recommended where the laboratory has the capability in place and is testing small numbers of samples. Virus isolation is important for further characterisation and is best carried out in the national laboratory or a reference laboratory.

For FMD serology the LP-ELISA is the test of choice in a country where FMD is endemic, as it is still the only test validated for post-vaccination testing. The C-ELISA is used in FMD-free countries, and can be used to screen sera first as it has greater sensitivity and specificity and allows greater throughput. The NS-ELISA is used with the structural LP-ELISA or the C-ELISA to distinguish infected animals from vaccinated. The NS-ELISA does not identify carrier animals, but rather animals that have been infected with FMDV in the past. The NS-ELISA can be used to indicate disease prevalence, or when a country is declaring freedom from FMD, or in animal trading to indicate that animals have not been exposed to FMDV. The AC-ELISA is used for detection of FMD antigen and is the only test able to rapidly determine the serotype of an FMD outbreak. PCR is important as a confirmation for FMD genome and in further characterisation of FMDV by sequencing. Virus isolation is important in producing high-titred stocks of FMDV for characterisation or for growth of samples with low virus titre. Virus isolation is used in national or reference laboratories due to the high cost of maintaining tissue culture.

The quality of samples submitted to the laboratory is important in achieving precise and accurate results and involves:

- maintaining a cold chain
- collection of appropriate samples for diagnosis
- collection in the appropriate sample collection buffer (i.e. phosphate/glycerol for virus isolation and ELISA).

Training of laboratory staff in the different diagnostic tests for FMD and CSF is an important part of AAHL's overseas projects. Training includes aspects of test validation and application of internal and external quality control and assurance principles to monitor assay reliability. Quality results enable epidemiologists and policymakers to make informed decisions about animal health policies.

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