

Section 3

Diagnosis and vaccination

The molecular epidemiology of classical swine fever viruses from Lao PDR and Asia: A brief review

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Introduction

Classical swine fever (CSF) is a highly contagious virus infection of swine caused by classical swine fever virus (CSFV), a member of the genus *Pestivirus*, family Flaviviridae. The CSFV genome is a positive-sense single-stranded RNA molecule of approximately 12.3 kb. The open reading frame (ORF) encodes a single polyprotein that is post-translationally processed into structural and non-structural proteins. Flanking the ORF are non-coding regions at the 5' (5'NCR) and 3' (3'NCR) ends of approximately 360–385 and 228 bases, respectively. Molecular epidemiology techniques (also known as phylogenetic techniques) have been successfully applied to the investigation of CSF viruses to explain geographical and/or temporal relationships.

Phylogenetic taxonomy

The first major study into the nature of CSF virus phylogeny examined the 5'NCR, E2 and NS5B genomic regions of a large number of CSF viruses that were divided, on the basis of genetic similarity, into two major genogroups and further subgroups (Lowings et al. 1996). These groupings have subsequently been adopted as the standard nomenclature for CSFV genogroup assignment. Minor refinements and expansion of the taxonomic groupings

have occurred with the introduction of a third genogroup and designation of additional subgroups (Paton et al. 2000).

Methodologies

Reference CSFV genetic data is stored in the form of nucleotide sequence in genetic databases such as GenBank. The University of Hannover's School of Veterinary Medicine in Germany has established a dedicated internet-based database that holds nucleotide sequences for 5'NCR, E2 and NS5B genomic regions (Greiser-Wilke et al. 2000), enabling researchers worldwide to access the sequences for local analysis.

Four regions of the CSFV genome—the 5'NCR (Hofmann et al. 1994), E2 gene (Lowings et al. 1996), NS5B gene (Paton et al. 2000) and 3'NCR (Björklund et al. 1998)—have been successfully employed for phylogenetic analysis of CSF virus isolates. The 5'NCR has proved to be a popular and reliable genomic region for the study of pestivirus phylogenetics. The first report of the successful analysis of the 5'NCR was by Vilcek et al. (1994), in which the 324/326 primer set (also employed in the 5'NCR analysis presented in this chapter) was employed to discriminate pestiviruses using phylogenetic and RFLP analysis. Further reports have employed the 324/326 primer set for amplification of the 5'NCR (Sakoda et al. 1999; Stadejek et al. 1996, 1997) for successful restriction enzyme (RE) or phylogenetic analysis of CSFV isolates. The E2 gene has also been used extensively for CSFV phylogenetic analysis. Investigation of a 190-nucleotide (nt) region at the 5' end of the E2 gene has formed

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the basis for the assignment of genogroups and is preferred over the 5'NCR because of the higher bootstrap confidence levels associated with E2 analysis (Paton et al. 2000).

Molecular epidemiology of Asian CSF viruses

South-East Asia

The distribution of CSFV genotypes in Asia is presented in Table 1. Studies of the distribution of CSFV genotypes in South-East Asia have been conducted in Lao PDR, Vietnam, Thailand and Malaysia.

In Lao PDR two studies have examined the phylogenetic relationships of the 5'NCR (Blacksell et al. 2005) and the E2 gene (Blacksell et al. 2004) of Lao PDR CSF virus samples. All Lao CSFV isolates examined belong to subgroups 2.1 and 2.2. There was a strong geographical relationship between CSFV isolates from the northern (subgroup 2.1) and southern (subgroup 2.2) regions of Lao PDR. As the viruses assessed in the studies were only collected over a period of 2.5 years, it was not possible to assess the level of temporal variation in Lao PDR CSFV isolates. The majority of Lao CSF viruses belonged to subgroup 2.2. Clear phylogeographic clusterings were evident for subgroup 2.2 viruses originating from provinces in the southern region, most notably in Champassak province. An important finding was that no subgroup 2.2 virus isolates were detected in the northern region of Lao PDR. Of the isolates belonging to subgroup 2.1, all originated from the northern and central regions of Lao PDR, with none being detected in the southern region. The northern region of Lao PDR is very mountainous with poor transport infrastructure. This isolation

forms a natural barrier from other regions of Lao PDR. It is therefore not unexpected that CSFV isolates from these areas may be distinct, as verified by the absence of subgroup 2.2 viruses in the northern region. The presence of subgroup 2.1 viruses in the central region is most probably due to the movement of infected pigs or pork products to Vientiane City for sale. A recent study of the viral diseases of pigs in Vietnam during 1999 to 2003 found subgroup 2.1 and 2.2 CSF viruses (Kamakawa et al. 2006) that are the same as those found in Lao PDR.

Other countries in South-East Asia demonstrate a large number of CSFV genotypes. In Thailand subgroup 1.1, 2.1, 2.2 and 3.4 CSFV strains have been identified from historical and contemporary virus isolates (Parchariyanon et al. 2000a, 2000b). Three viruses of Thai origin were shown to belong to genogroups 1 and 3 (Sakoda et al. 1999). Malaysian CSF viruses isolated in the 1980s were determined to belong to genogroups 1.2 and 2.1 by analysis of the 5'NCR, E2 and NS5B genomic regions (Lowings et al. 1996).

Northern/Eastern Asia

In northern and eastern parts of Asia, studies have been conducted in China, Japan, Taiwan and Korea.

The first report of the genetic characterisation of Chinese CSFV isolates was during a comparison of the NS5B genetic region of worldwide CSF viruses (Björklund et al. 1999). The Chinese 'Wuhan' field isolate and C-strain vaccine were both determined to be members of genogroup 1 (Björklund et al. 1999). More recent phylogenetic studies compared the E2 region and have identified CSFV isolates belonging to subgroups 1.1, 2.1, 2.2 and 2.3 (Tu et al. 2001). The majority of the CSFV isolates belonged to genogroup 2 (89.3%) with the viruses being almost

Table 1. Summary of CSF virus genogroups found in Asia

Region	Country	Sub-genotype	Reference
Central/Northern Asia	China	1.1, 2.1, 2.2, 2.3	Tu et al. 2001
Eastern Asia	Japan	1.1, 1.2, 2.2, 3.4	Sakoda et al. 1999
Eastern Asia	Korea	3.2	Paton et al. 2000
Eastern Asia	Taiwan	2.1, 2.2, 3.4	Pan et al. 2005; Deng et al. 2005
South-East Asia	Lao PDR	2.1, 2.2	Blacksell et al. 2004, 2005
South-East Asia	Thailand	1.1, 2.1, 2.2, 3.4 1.2, 3.3	Parchariyanon et al. 2000a, 2000b; Sakoda et al. 1999
South-East Asia	Malaysia	1.2, 2.1	Lowings et al. 1996; Vilcek et al. 1996
South-East Asia	Vietnam	2.1, 2.2	Kamakawa et al. 2006

equally divided between sub-genogroups 2.1 (48.1%) and 2.2 (41.2%) (Tu et al. 2001). Of most interest is the genetic composition of CSF viruses originating in Yunnan and Guangxi provinces of China that share common borders with Lao PDR and Vietnam, respectively. All virus isolates originating from Yunnan province belonged to sub-genogroup 2.1 (Tu et al. 2001). Somewhat surprisingly, CSF viruses belonging to a range of subgroups, 1.1, 2.1, 2.2 and 2.3, were detected in adjacent Guangxi province (Tu et al. 2001).

Analysis of the 5'NCR of a large number of Japanese CSFV isolates collected during outbreaks from 1951 to 1986 revealed that these viruses belonged to genogroups 1, 2 and 3 (Sakoda et al. 1999). In Taiwan subgroups 2.1, 2.2, 3.4 have been detected (Deng et al. 2005) and in Korea subgroup 3.2 (Paton et al. 2000).

Europe

The most recent CSF outbreaks in Western Europe, such as the Netherlands outbreak of 1997–98 and the United Kingdom and German outbreaks of August–September 2000, have been associated with sub-genogroup 2.1 CSF viruses (Widjoatmodjo et al. 1999).

Phylogeographic relationships between CSFV genotypes: Opportunities and limitations for disease control

There is considerable diversity in the distribution of CSFV genogroups throughout Asia and the rest of the world. Genogroup 2 viruses have the widest distribution in Asia and are the exclusive genogroup in Lao PDR and Vietnam. It is interesting to note the diversity of CSFV genogroups in Thailand and China. While it is not entirely clear why this is the case, trans-boundary movement of infected animals may be the cause. Given the diversity of genogroup distribution in Asia, the monitoring of CSFV genogroups following an outbreak is an excellent tool for tracking trans-boundary disease. A good example is the speculation on the possible origins of subgroup 2.1 viruses responsible for the CSF outbreaks in Western Europe during the 1990s that were thought possibly to be of Asian origin (Hofmann & Bossy 1998; Paton et al. 2000). Molecular epidemi-

ology will only be an effective tool if continued assessment of virus isolates from the region is performed. Mutations occur naturally within the RNA genome and there are potential incursions of new virus strains by the uncontrolled trans-boundary movement of animals within the region. Low-cost methods for genetic typing of CSF virus such as the use of restriction fragment length polymorphism (RFLP) (Parchariyanon et al. 2000b) may provide more appropriate and rapid methodologies for the assessment of field virus isolates in low-technology settings. Further investigations are, however, required to confirm the long-term usefulness of the proposed techniques.

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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 (Morrissy 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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Application of immunomagnetic bead technology for improved diagnosis of classical swine fever virus

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Abstract

Classical swine fever (CSF) is a highly contagious viral disease of swine that causes major losses in all pig production systems in many regions of the world. In Lao PDR, CSF is endemic and outbreaks have adverse effects on the predominantly smallholder farming sector. Laboratory testing is required to accurately identify CSF outbreaks because of the difficulty of making a diagnosis based solely on clinical signs. The National Animal Health Centre, located in the national capital, Vientiane, has the capacity to reliably detect CSF antigen in tissue samples using an antigen capture (AC)-ELISA, and antibodies to CSF virus from serum samples using the complex trapping blocking ELISA. This paper describes the use of immunomagnetic beads (IMB) as the solid phase for the portable detection of CSF antigen in spleen samples and for the reliable detection of antibodies to CSF in animals vaccinated with a lapinised C-strain vaccine. The portable IMB-ELISA for antigen detection was shown to be 100% sensitive and 91% specific in comparison to the AC-ELISA. The IMB-Antibody-ELISA was shown to be 97% sensitive and 95% specific in comparison to the gold standard—neutralising peroxidase linked assay. These new diagnostic tests have the potential to improve CSF management through portable and rapid identification of outbreaks and the reliable and inexpensive monitoring of vaccination programs.

Introduction

Laboratory testing of clinical samples is of paramount importance if classical swine fever (CSF) outbreaks are to be correctly identified (Elbers et al. 2004; Paton and Greiser-Wilke 2003; van Oirschot

1999). Likewise, a rapid turnaround from sample collection to reporting results is necessary to ensure control measures are enacted in a timely manner. In Lao PDR sample submission can be delayed after collection, and delays also often occur once samples have been received at the laboratory. To counter this

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problem, new diagnostic tests have been developed to simplify the process, decrease reporting times and provide reproducible results in a portable format without compromising results (Conlan 2006; Conlan et al., in press). The use of immunomagnetic beads (IMB) for the detection of CSF virus has been described previously (Conlan 2006; Conlan et al., in press), where the results are read by eye without compromising test integrity.

Vaccination is a very important control measure for CSF; however, to maximise the potential benefit of a vaccination program, farmers and veterinary authorities need to be able to monitor herd immunity post vaccination. The neutralising peroxidase linked assay (NPLA) is the gold standard for the detection of antibodies to CSF virus (OIE 2004); however, this test is slow, laborious and expensive, and requires tissue culture facilities. In addition, the complex trapping blocking (CTB)-ELISA currently being used at the National Animal Health Centre (NAHC) is not able to be applied in monitoring vaccination programs. Therefore, rapid inexpensive alternatives to these two tests are required.

The results presented in this paper describe the application of the IMB-ELISA (Conlan 2006; Conlan et al., in press) in a near-to-field format for antigen detection, and its adaptation to a format for antibody detection, the IMB-Antibody (Ab)-ELISA.

IMB-ELISA for antigen detection

Control antigens

Negative and positive control antigen extracts were prepared as pooled 5% w/v homogenates in buffered detergent (1% Nonidet P-40 in phosphate buffered saline (PBS) with 5% normal goat serum (NGS) and 0.07% Proclin 300) (Conlan et al., in press).

IMB-ELISA method

Immunomagnetic beads (Spherotech Inc., USA) were coated with anti-pestivirus goat polyclonal antibody according to methods previously described (Conlan 2006; Conlan et al., in press), with some modification. The IMBs were coated at room temperature overnight instead of for 2 hours. Following coating, the IMBs were blocked, washed and resuspended to 0.20% w/v in storage buffer as previously described (Conlan 2006; Conlan et al., in press).

IMB-ELISA test kits were prepared in dropper bottles (Nalgene, USA) and reagents were prepared at working concentrations. Monoclonal antibody (MAb) 24/10 (Kosmidou et al. 1995), specific for CSF virus E2 protein, was incubated with an equal volume of filter-sterilised NGS for 20 minutes prior to dilution in buffer containing 5% v/v glycerol, 0.5% w/v fish skin gelatine, 0.05% v/v Tween 20, 0.07% v/v Proclin 300 in PBS. Goat anti-mouse horseradish-peroxidase (HRP) conjugate (DakoCytomation, Denmark) was incubated with five volumes equivalent of NGS for 20 minutes prior to dilution in Guardian Peroxidase Stabiliser (Pierce, USA). The chromogen-substrate used in the test kit was TMB Liquid Substrate System (Sigma, USA) and was purchased as ready-to-use.

One drop (~35 μ L) of IMBs was added to 100 μ L of sample and controls in a 1.5 mL tube, mixed and incubated at room temperature (~25 °C) for 30 minutes. The tubes were placed on a magnet (Dexter Magnetic Technologies, USA) for 20 seconds and the supernatant discarded. Three drops (~100 μ L) of MAb were added, mixed and incubated at room temperature for 15 minutes. The tubes were again placed on the magnet, the MAb was discarded and three drops of conjugate were added, mixed and incubated at room temperature for 15 minutes. The tubes were placed on the magnet, the conjugate discarded and the IMBs were washed three times prior to transfer to a new tube. The final wash was removed and two drops (~65 μ L) of chromogen-substrate was added, mixed and incubated for 5 minutes at room temperature (Figure 1). Samples were considered positive if an obvious green/blue colour was visible (scored as 3+, 2+ or 1+ depending on intensity) and negative if the colour remained brown/red (scored as 0) (Figure 2).

Relative diagnostic performance

The relative diagnostic performance of the IMB-ELISA test kit was determined using an AC-ELISA (Fuqing et al. 2000; Shannon et al. 1993) as the reference comparator. During 2004–06, 110 spleen samples from CSF-suspected pigs were submitted to the NAHC, Vientiane. Specimens were transported in buffered glycerol (50% v/v glycerol in PBS), prepared as 5% w/v spleen homogenates in buffered detergent and stored at –85 °C prior to testing. The spleen samples were given a randomised number and tested in the IMB-ELISA test kit.

Kit stability

Four replicates of mid-positive and negative control antigens were tested in the IMB-ELISA at weeks 2, 4, 8 and 12 after preparing a kit. The kits were stored at 4 °C throughout. At the completion of the 5-minute chromogen-substrate incubation, 1N H₂SO₄ was added to stop the reaction, and the optical density was measured at a wavelength of 450 nm (OD₄₅₀).

Results

Of the 110 samples, 34 (31%) were positive by AC-ELISA and 41 (37%) were positive by IMB-ELISA. All samples positive by AC-ELISA were also positive by IMB-ELISA; that is, no false negatives were observed. The relative diagnostic per-

formance of the IMB-ELISA test kit was 100% (95%CI: 87–100) sensitive and 91% (95%CI: 81–96) specific in comparison to the AC-ELISA.

The kit was found to be stable for less than 3 months. After 12 weeks the detection efficiency as indicated by the optical density decreased substantially from an OD₄₅₀ of 0.80 to 0.36, a decrease of greater than 50%.

IMB-Antibody-ELISA for detection of antibodies to CSF virus

IMB-Antibody-ELISA method

The IMBs were coated, blocked and resuspended as described above to a final working concentration of 0.10% w/v. The MAb, conjugate and chromogen-

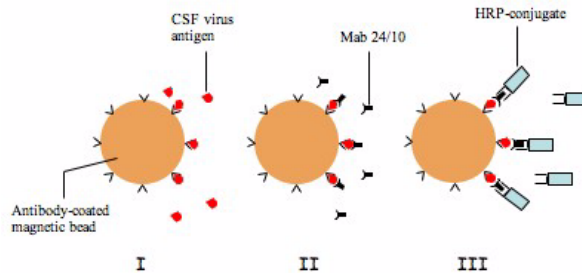


Figure 1. Principle of the IMB-ELISA for antigen detection. **I:** CSF virus antigen capture, **II:** specific detection by monoclonal antibody and **III:** chromogen-substrate colour development

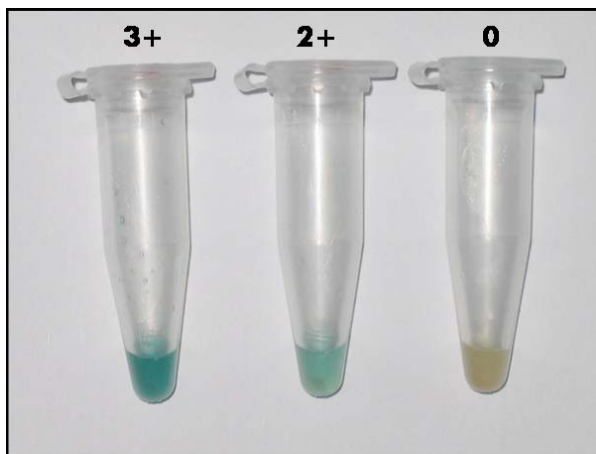


Figure 2. Colour range for test results; positive (bluest, score 3+) to negative (brownest, score 0)

substrate used were the same as those described above. The CSF virus antigen used in the blocking ELISA was the same as that described above diluted 1:2 to 2.5% w/v in buffered detergent.

Twenty-five μL of control or test serum was added to 25 μL of buffered detergent and incubated at room temperature for 5 minutes before the addition of 50 μL of CSF virus control antigen. The serum and antigen were mixed and incubated at 37 °C for 30 minutes followed by the addition of 50 μL of IMBs and shaking for 30 minutes at 37 °C. A no-serum control was included as OD_{max} . The tubes were placed on the magnet for 20 seconds, the supernatant was discarded and 100 μL of MAb added and shaken at 37 °C for 15 minutes. The MAb was removed and 100 μL of conjugate was added and shaken at 37 °C for 15 minutes. The IMBs were washed three times and transferred to a new tube, the final wash was removed and 50 μL of chromogen-substrate added and incubated at room temperature for 5 minutes. The reaction was stopped with 1N H_2SO_4 and the $\text{OD}_{450\text{nm}}$ measured; the percentage inhibition was calculated according to equation (1).

$$\text{PI} = 100 - \frac{\text{OD}_{\text{sample}}}{\text{OD}_{\text{max}}} \times 100 \quad \text{equation (1)}$$

Test samples

Twenty pigs were vaccinated with a lapinised C-strain CSF vaccine in two villages of Bolikhamxay province in central Lao PDR and bled at 0, 35 and 70 days post vaccination. In total, 57 serum samples were tested by NPLA (OIE 2004), Ceditest (CEDI Diagnostics, the Netherlands), CTB-ELISA (Blacksell 2001) and IMB-Antibody (Ab)-ELISA.

Data analysis

The diagnostic cut-off for the IMB-Ab-ELISA was visually assigned after graphically plotting the frequency against intervals of per cent inhibition for the 57 samples assessed, with the NPLA used as the reference test. The diagnostic performances of the IMB-Ab-ELISA and the CTB-ELISA were assessed by calculating relative diagnostic sensitivity and specificity using EpiCalc software (CDC, USA), with the NPLA and CEDI ELISA used as the reference comparators. The level of agreement of the tests was calculated using kappa statistic analysis (Smith 2006), where kappa scores of 0.41–0.60,

0.61–0.80, 0.81–0.99 and 1.00 correspond to levels of agreement of moderate, substantial, almost perfect and perfect, respectively.

Results

The diagnostic cut-off for the IMB-Ab-ELISA was set at greater than or equal to 50% inhibition (Figure 3). At this cut-off there were two false negatives and one false positive when compared to the NPLA. The relative diagnostic sensitivities and specificities of the IMB-Ab-ELISA and the CTB-ELISA are summarised in Table 1.

The levels of agreement between the CTB-ELISA and the CEDI ELISA, and between the CTB-ELISA and NPLA, were less than moderate. The IMB-Ab-ELISA showed almost perfect agreement with both the CEDI ELISA and NPLA.

General discussion

Antigen detection

Appropriate diagnostics are a critical component of CSF management and the speed and efficiency of application will, to a large degree, determine the outcome of a disease-control initiative. The research presented in this paper describes the application of IMB technology to CSF diagnosis in a portable and sensitive format suitable for use in the field. The detection of CSF viral antigen by IMB-ELISA was first described by Conlan et al. (in press), and was found to be a rapid, sensitive, specific and highly repeatable test format with demonstrated high levels of agreement between operators. Minimal training was required to implement the test in a laboratory and the test was not expensive. These combined factors make the test ideal for the conditions seen in a low-technology setting such as Lao PDR where sample submission from remote locations can be difficult. This research demonstrates that the test was successfully adapted to a portable format using dropper bottles for dispensing reagents, performing the test at room temperature and reading the result by eye. Diagnostic performance was good in comparison to an AC-ELISA, with 100% and 91% relative diagnostic sensitivity and specificity, respectively. The estimated shelf life was not as good as was expected using stabilised reagents. After 3 months the test performance dropped to unacceptable levels, and this will need to be corrected in the future.

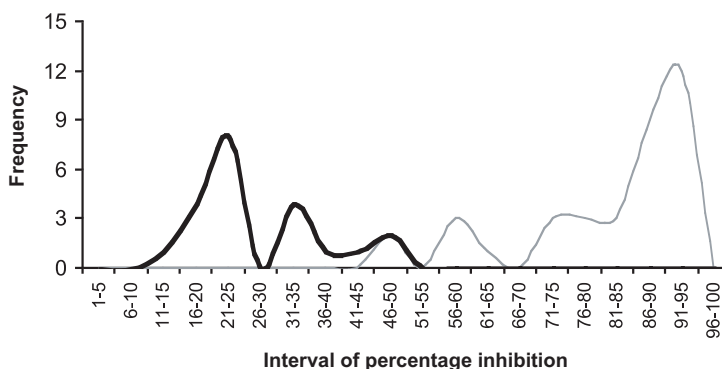


Figure 3. Analysis of the reactivity range of 57 serum samples tested in the IMB-Ab-ELISA. The black line represents samples negative for CSF antibodies (≤ 8 by NPLA) and the grey line represents samples positive for CSF antibodies (≥ 8 by NPLA).

Table 1. The relative diagnostic performance and level of agreement of the IMB-Antibody (Ab)-ELISA and complex trapping blocking (CTB)-ELISA in comparison with two standard antibody tests, the neutralising peroxidase linked assay (NPLA) and the Ceditest

	IMB-Ab-ELISA			CTB-ELISA		
	DSn	DSp	<i>K</i>	DSn	DSp	<i>K</i>
Ceditest	97	97	0.92	40	100	0.34
NPLA	97	95	0.92	39	100	0.32

DSn = diagnostic sensitivity; DSp = diagnostic specificity; *K* = kappa statistic

Monitoring vaccination

Vaccination is the only control and prevention measure undertaken in Lao PDR to minimise the occurrence of CSF in village production systems. A slaughter policy during an outbreak does not exist; however, in some villages, quarantine systems have been set up to decrease the risk of introducing disease into a village. Farmers and animal health officials are, therefore, highly reliant on the success of vaccine delivery and need suitable resources to accurately monitor vaccine uptake. The CTB-ELISA currently used in Lao PDR is not suitable for this purpose; this study demonstrated that its level of agreement with the NPLA test was very poor (0.32) and it showed low test sensitivity (39%) for the detection of vaccinated sero-positive animals. The adaptation of the IMB-ELISA into an antibody detection format has produced promising early results. The level of agreement with the NPLA was almost perfect (0.92) and the sensitivity and specificity were very high (97% and 95%, respectively). At this stage of test development, proof

of principle has been clearly demonstrated but too few samples have been tested to give a clear indication of test performance. Further work is required to adapt the test to a plate format to increase speed and the number of samples that can be tested.

Conclusions

Immunomagnetic bead technology is adaptable and versatile and can provide a platform for appropriate diagnostic test development in a limited-resource setting such as Lao PDR. The IMB-ELISA for CSF is inexpensive, portable, stable and a reliable test that requires minimal training to implement and will improve diagnostic services for pig farmers. The IMB-Ab-ELISA, while in the early stages of development, shows strong agreement with the 'gold standard' NPLA and could be a valuable tool for monitoring vaccine uptake.

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Classical swine fever virus vaccine stability in Lao PDR

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and Laurence Gleeson^{1,2}

Abstract

Classical swine fever (CSF) virus is a highly contagious but vaccine-preventable disease of swine. A locally produced lapinised C-strain vaccine is used to control CSF in Lao PDR; however, vaccine failure has been reported. The CSF vaccine is produced at the National Vaccine Production Centre (NVPC) as a freeze-dried rabbit spleen homogenate in a rubber stoppered glass vial and stored at -20°C with a recommended shelf life of 1 year. This paper describes two studies to (i) determine the stability of the locally produced vaccine when stored at 4°C and -20°C and (ii) determine if the vaccine elicits a protective immune response when delivered to village pigs under good transport conditions. The vaccine was found to be stable for only 4 months when stored at -20°C and for less than 3 months when stored at 4°C . Under field conditions, vaccine stored at -20°C for 2 months and transported at temperatures less than 1°C elicited an immune response in 89% of vaccinated pigs by day 35 and 100% of pigs by day 70 post vaccination.

Introduction

The control and management of classical swine fever (CSF) virus in an endemic country is reliant on rapid disease recognition and the use of an effective vaccine. Commercially available vaccines for CSF are widely available, the most common being live attenuated virus vaccines or subunit vaccines of the immunodominant E2 protein. The most effective live attenuated vaccines are based on wild-type virus strains and include the C-strain vaccine, the Japanese guinea-pig-exaltation-negative (GPE-) strain derived from the virulent ALD strain, the cell culture

adapted Thiverval strain derived from the virulent Alfort strain (de Smit 2000; van Oirschot 2003) and the PAV-250 strain (de Smit 2000). Traditionally, C-strain vaccine was produced from the organs of rabbits inoculated with a working seed (Terpstra et al. 1990). The C-strain virus has subsequently been adapted to cell culture systems for large-scale production of vaccine using the swine kidney cell line SK-6 (Terpstra et al. 1990) or minipig kidney (MPK) cells (Ferrari 1992; Rivero et al. 1988).

In Lao PDR the CSF vaccine is produced from the live attenuated C-strain virus from homogenised rabbit spleen and mesenteric lymph nodes freeze dried in the presence of stabilisers in a rubber stoppered vial (Khounsy et al. 2007). Only a small proportion of pigs are vaccinated in Lao PDR and vaccine failure has previously been documented and attributed to one or more of the following: (i) vaccine not viable at manufacture, (ii) inactivation due to incorrect storage and transport, (iii) incorrect administration of the vaccine or (iv) the presence of

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maternally derived antibodies. The research presented in this paper specifically addresses the second factor, vaccine stability during transport and storage. The 'cold chain' in Lao PDR is perhaps the most important factor limiting the successful delivery of temperature-sensitive live virus vaccine that requires storage at -20°C . Figure 1 represents a descriptive model of the cold-chain limitations under ideal conditions, where several temperature fluctuations occur and storage at provincial and district levels is inadequate according to the manufacturer's recommendations.

To determine if the temperature fluctuations could be eliminated from the transport equation by storing vaccine at 4°C instead of -20°C , the long-term stability of the vaccine was assessed at these two temperatures. Secondly, the ability of the vaccine to elicit a protective immune response in village pigs was assessed.

Vaccine stability

Materials and method

A single batch (05/2004) of the lapinised CSF C-strain vaccine was procured from the National Vaccine Production Centre (NVPC) and transported on ice to the National Animal Health Centre (NAHC), Vientiane, Lao PDR. The vaccine batch was assessed by vaccinating four pigs that had been brought into the laboratory pens, allowed to accli-

mate and treated with ivermectin and antibiotics to eliminate any infection. Two pigs were included as non-vaccinated controls.

One month after procuring the vaccine, an adequate volume of vaccine was stored at 4°C and the remainder kept at -20°C . Temperature was monitored for both lots of vaccine using a temperature logger (Thermocron, OnSolution, Australia) at 20-minute intervals. After 3 months' storage at 4°C , one group of four pigs was vaccinated with vaccine stored at 4°C and a second group with vaccine stored at -20°C for 4 months, and one unvaccinated pig was included as a control. As above, pigs were treated with ivermectin and antibiotics to eliminate infection prior to vaccination. After 4 months' storage at 4°C , the above protocol was repeated. Pigs were bled on days 0, 10, 14, 21 and 28 post vaccination (pv) to monitor neutralising antibody titre by the neutralising peroxidase linked assay (NPLA) (OIE 2004).

Results

Vaccine stored frozen during the experiment was held at an average temperature of -18.28°C with a standard deviation of 1.28. Vaccine stored in the refrigerator was held at an average temperature of 4.34°C with a standard deviation of 0.73.

All pigs vaccinated during the pre-trial assessment of batch number 05/2004 were CSF antibody negative on day 0 and all four vaccinated pigs were

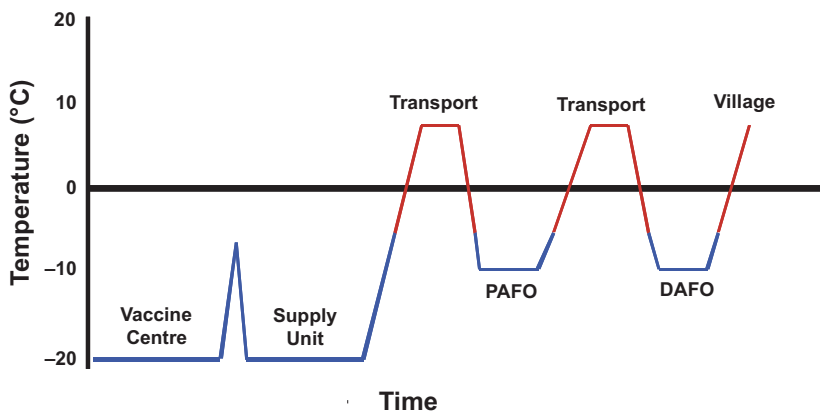


Figure 1. Conceptual model of CSF vaccine delivery in Lao PDR. The blue line represents adequate temperature and the red line represents substandard storage temperatures. PAFO = Provincial Agriculture and Forestry Office; DAFO = District Agriculture and Forestry Office. Storage times at PAFO and DAFO can be variable, depending on demand for vaccine.

positive for the presence of CSF virus neutralising antibodies on day 35 pv, with a median titre of 1:44 (range 1:32–1:44). The median antibody titre on day 28 pv was 1:32 (range 1:22–1:32). Antibodies were first detected in one pig on day 10 pv, three pigs on day 14 pv and all pigs on day 21 pv.

For the pigs vaccinated with vaccine stored at 4 °C for 3 months, the median neutralising antibody titre was 1:4 (n=4; range 1:4–1:32); no significant difference could be demonstrated in comparison to the pre-trial assessment (Fisher Exact Test, $p=0.07$) due to the small sample number. However, storage at 4 °C for 3 months was four times more likely to result in vaccine failure than new vaccine (risk ratio=4, 95%CI: 0.73–21.84). For the pigs vaccinated with vaccine stored at 4 °C for 4 months, the median neutralising antibody titre was 1:4 (n=4; range 0–1:8), a significant difference when compared to new vaccine (Fisher Exact Test, $p=0.01$).

For the pigs vaccinated with vaccine stored for 4 months at –20 °C, the median neutralising antibody titre was 1:36 (n=4; range 0–1:352); no significant difference could be demonstrated in comparison to the pre-trial assessment (Fisher Exact Test, $p=0.5$) and the risk of failure after storage for 4 months at –20 °C was low (risk ratio=1.33; 95%CI: 0.76–2.35). For the pigs vaccinated with vaccine stored at –20 °C for 5 months, the median neutralising antibody titre was 1:4 (n=3; range 0–1:8), a significant difference when compared to new vaccine (Fisher Exact Test, $p=0.01$). One of the four pigs in this final group died during the experiment and no CSF virus antigen was detected in its organs.

Discussion

The cold chain for the delivery of frozen vaccine in Lao PDR is poor; as a result, the delivery of frozen vaccine to village pigs requires several freeze–thaw cycles and substandard storage temperatures. It is well recognised that CSF virus is adversely affected by temperature fluctuations such as repeated freezing and thawing. The principal aim of this research was, therefore, to determine if the locally produced CSF vaccine could be stored at 4 °C for prolonged periods of time and remain immunogenic. An added component of this research was one of quality assurance—determining an estimate of vaccine shelf life when stored at –20 °C.

The results of this study show that, for this batch at least, the vaccine cannot be stored at 4 °C for extended periods of time and remain viable. After

3 months' storage at 4 °C at the NAHC with no temperature fluctuations, the vaccine was not able to elicit a good immune response in test animals. Somewhat surprisingly, this study found that the vaccine is not stable for at least as long as the manufacturer recommended. The vaccine was still viable after 4 months' storage at –20 °C but was unable to elicit a good immune response in test animals when stored for 5 months. This experiment was conducted under ideal conditions of storage and transport; it is anticipated that under field conditions the vaccine stability would be even less. Many provincial and district vaccine storage freezers are unable to maintain temperatures in the range of –15 °C to –20 °C. To navigate through the constraints of delivering a quality CSF vaccine to a village pig, a great deal of planning will be required on the part of the Lao animal health service. More research and investment is required to address the quality of CSF vaccine produced at the NVPC and its subsequent delivery to village farms.

Vaccine delivery at the village level

Material and methods

Two villages in Bolikhan district, Bolikhamxay province, were selected for this study. Thirty CSF vaccine vials (300 doses) were procured from the NVPC (batch number: 03/2006) and stored frozen at the NAHC for approximately 2 months. The vaccine was transported in an ice box to the selected villages and the temperature was monitored throughout with a temperature logger (Thermocron, OnSolution, Australia) at 20-minute intervals.

All pigs in the villages were vaccinated (excluding pregnant sows and piglets <1 week of age). Blood samples for serology were collected from 10 pigs in each village prior to vaccination and again 35 and 70 days post vaccination. Sera were tested in the complex trapping blocking (CTB)-ELISA at the NAHC, and a portion of the samples were also sent to the CSIRO Australian Animal Health Laboratory (AAHL), Geelong, Australia, for testing by the NPLA and Ceditest (CEDI Diagnostics, the Netherlands) for CSF antibody.

Results

The average storage temperature at the NAHC over the 2 months prior to vaccination was –21.2 °C (standard deviation: 1.8). During transport to the village, the temperature within the icebox was main-

tained at or below 0 °C, with logger readings gradually increasing from -14.5 °C to 0 °C.

By NPLA, one pig was weakly positive for antibodies to CSF virus on day 0 pv (titre=1:8) and the remaining 18 pigs were negative for neutralising antibodies. One serum sample could not be tested by NPLA due to cell toxicity. By day 35 pv, only 19 pigs remained in the cohort and 17/19 (89%) were positive for the presence of neutralising antibodies; however, only 6/19 (32%) pigs had antibody titres \geq 1:32. On day 70 pv, 18 pigs remained in the cohort and all were positive for the presence of neutralising antibodies, with 17/18 (94%) having an antibody titre \geq 1:32. The Ceditest had very strong agreement with the NPLA results (κ =0.88) and diagnostic specificity and sensitivity were 90% and 97%, respectively. The CTB-ELISA, on the other hand, had very poor agreement with the NPLA results (κ =0.32) and diagnostic specificity and sensitivity were 100% and 39%, respectively.

Discussion

This study has clearly demonstrated that, under ideal storage and transport conditions, a relatively new batch of vaccine can elicit a protective immunity in village pigs. Seventy days after vaccination, 94% of pigs had an antibody titre \geq 1:32, which, for epidemiological purposes, affords complete protection and prevents virus shedding (Terpstra and Wensvoort 1988). Post-vaccinal antibody titres can continue to increase for up to 12 weeks (Dahle and Liess 1995; Terpstra et al. 1990; Terzic et al. 2003), and this was observed during this study. The NVPC recommends that the vaccine be stored at -20 °C for up to 1 year; however, as demonstrated above, the vaccine loses viability after 4 months' storage under recommended conditions. The capacity of rural agricultural offices to hold vaccine at -20 °C is low regardless of the timeframe it can be stored for; therefore, the critical issues of vaccine stability and delivery remain. Future strategies for the improvement of vaccine delivery at the village level need to be put in place if farmers are expected to embrace this technology.

This study has also highlighted the critical issue of having the capacity to monitor vaccination success. The only antibody detection test routinely available for CSF in Lao PDR is the CTB-ELISA. However, this test was unable to reliably detect vaccinated positive animals, with a sensitivity of just 39% and a

very low level of agreement with the NPLA (κ =0.32). Additional work is required to increase the capacity of the laboratory to enable the detection of vaccine-related serological responses. The Ceditest and NPLA are expensive tests in comparison to the CTB-ELISA, and could not be introduced into mainstream laboratory testing without the continued support of foreign donors. Research is required to develop a simple and inexpensive alternative to the CTB-ELISA that is capable of sensitive and specific detection of vaccinated positive animals.

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Recommended vaccine programs for village-based pig production systems in Lao PDR

Syseng Khounsy¹, Tess Vitesnik^{1,2} and James Conlan¹

Introduction

Classical swine fever (CSF) virus is endemic in Lao PDR, with major outbreaks each year resulting in significant production losses in all farming systems, including smallholder, semi-intensive and intensive farms. CSF is a vaccine-preventable disease and there are many vaccines, both live attenuated and subunit, commercially available. In Lao PDR a variety of livestock vaccines are produced at the National Vaccine Production Centre (NVPC), which is located at Nongteng village 15 km from Vientiane. The NVPC was established in 1980 and produces vaccines for CSF, haemorrhagic septicaemia, Newcastle disease, fowl cholera, infectious bronchitis, fowl pox and duck plague. CSF vaccine is produced from the live attenuated C-strain virus from homogenised rabbit spleen and mesenteric lymph nodes freeze dried in the presence of stabilisers in a rubber stoppered vial and stored at -20°C .

Storage and transport of CSF vaccine

The NVPC recommends that, under correct storage conditions, the shelf life of CSF vaccine is 12 months; however, many provincial and district vaccine storage freezers do not have the capacity of -20°C storage (-10°C is the normal limit in small domestic refrigerator-freezers). The CSF virus is an

enveloped virus and, because rapid and frequent temperature fluctuation results in viral death, there can be a significant decrease in the live virus titre of each dose of vaccine under such conditions. Therefore, temperature fluctuations should be avoided to maximise the amount of live virus in each dose administered to the pig.

Vaccine should be transported on ice in a well-insulated 'cool-box' to prevent the transport temperature exceeding 4°C ; if long transport times are anticipated, sufficient ice or ice-packs need to be included. In Lao PDR vaccine delivery follows a chain from the NVPC through different government offices before delivery to the farm (Figure 1).

Delivery of vaccine at the village level

The CSF vaccine is supplied freeze dried in a 10-dose vial and needs to be reconstituted in 10 mL of sterile distilled water which is provided by the manufacturer. Once reconstituted, the vaccine should be used as quickly as possible and not re-used the next day. The following procedures should be followed during a vaccination program in a village:

- Sterile technique should be used when reconstituting vaccine.
- Unused reconstituted vaccine should be discarded.
- Once reconstituted, vaccine should be used in only one village.
- Pigs should be adequately restrained and 1 mL of vaccine administered intramuscularly.
- A pig snare can be used to restrain larger pigs.
- Sterilised/sterile syringes should be used when administering vaccine.
- Syringes and needles should be sterilised between uses in different villages.

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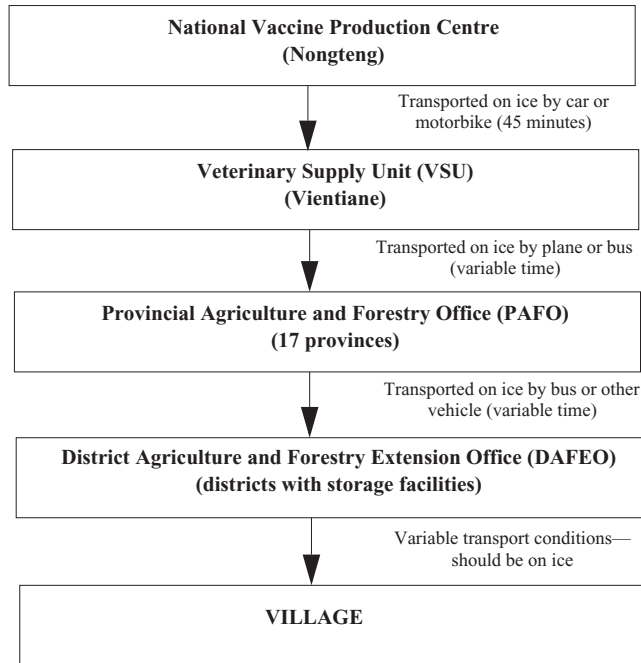


Figure 1. CSF vaccine delivery chain in Lao PDR

Recommended vaccine strategy by animal age class

Sows: Sows should be vaccinated every 6 months or at least once per year. Pregnant sows should not be vaccinated.

Boars: Boars should be vaccinated every 6 months or at least once per year.

Piglets (of vaccinated sow): Every effort should be made to allow newborn piglets access to colostrum within the first 12 hours after birth. Piglets should be vaccinated no earlier than 5–6 weeks of age to avoid virus neutralisation by maternally derived antibodies. A booster dose is recommended 4 weeks after the initial dose.

Piglets (of unvaccinated sow): Piglets should be vaccinated at 1 week of age and a booster dose given 4 weeks later.

Weaners and growers: If vaccinated as a piglet, a booster dose should be given after 12 months.

Purchased pigs: New pigs purchased from a market, middleman trader or another farmer should be vaccinated and kept in quarantine for a minimum of 2–4 weeks and a booster dose given 4 weeks after

the initial dose. Subsequent vaccinations should be given according to information for boars and sows above.

Factors contributing to vaccination success

Animal-related factors include the following:

- no subclinical infection present
- livestock well fed and watered
- low parasite burden
- livestock old enough for maternal antibodies to have declined
- livestock not challenged with CSF virus before vaccine can generate an immune response.

Vaccine-related factors include the following:

- vaccine not expired
- vaccine proved to be effective by the manufacturer
- vaccine stored and transported at correct temperature and fluctuations avoided or minimised
- vaccine not exposed to heat for long periods of time.

Vaccination procedures include the following:

- correct route of administration
- animal restrained to ensure delivery of the correct dose
- use of diluent supplied by the manufacturer
- leftover doses from multidose containers disposed of safely
- sterile equipment used in multidose containers.

Future research directions for classical swine fever and foot-and-mouth disease in Lao PDR: A facilitated session to capture the skills and experience of workshop delegates

Ross Cutler^{1,2} and James Conlan³

Introduction

At the close of the workshop, a facilitated session was held to explore future directions for the control and management of classical swine fever (CSF) and foot-and-mouth disease (FMD) in Lao PDR. The workshop was attended by experts in their fields from the People's Republic of China, Thailand, Myanmar, Cambodia, Vietnam, Lao PDR and Australia, and other representatives from a range of non-government organisations and international development projects. The session was designed in such a way as to best capture the skills and experience of attending delegates to progress ideas in an environment where all participants were able to make a contribution to the discussion.

The workshop participants were divided into five groups in a manner to ensure an even representation across countries. Two leaders were assigned to each group, one to record answers on a whiteboard and the other to facilitate discussion within the group. The leadership personnel comprised Lao and Australian project staff and the session was facilitated by the first author. The session method is described in Figure 1.

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Outcomes

The first question to be addressed by the groups was:

With regard to FMD and CSF projects in Lao PDR, what have been the successful elements?

The key responses are summarised as follows:

Information exchange (regional and local) during outbreaks

- established networks of farmers—district, provincial, national, international
- network of people to report and respond

Knowledge

- improved management skills, training, disease control skills and public awareness
- international cooperation
- improved capacity at all levels
- farmers sensitised about diseases and control

Vaccination

- benefits of vaccination, how to use, information about transport and storage
- improved knowledge of how to produce and deliver a good-quality vaccine
- increased vaccination coverage
- standardised laboratory and vaccine production and training

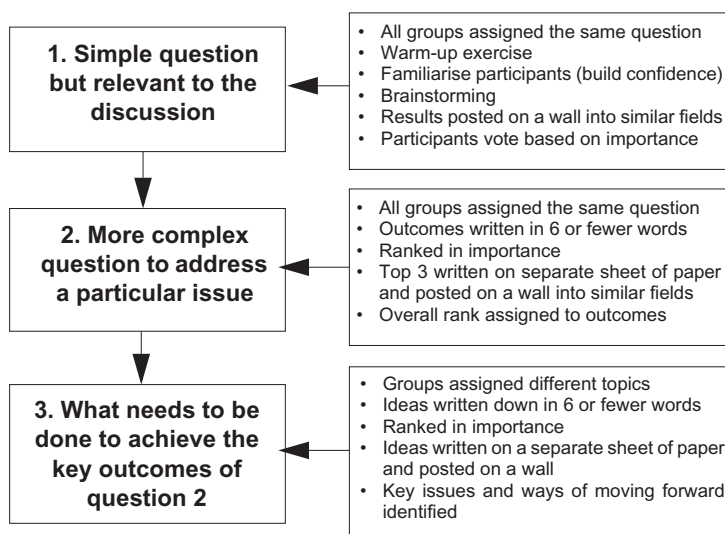


Figure 1. Flow diagram describing facilitated session method

Improved diagnostic capacity: new diagnostic tests

- IMB-ELISA for rapid CSF diagnosis
- quality control improvements
- strengthened sample collection and transport (diagnostic network)

Animal movement control

- local and trans-boundary control.

Each delegate voted for the three most important outcomes of CSF and FMD projects and the results are as follows:

Outcome	Score
Knowledge	15
Vaccination	13
Information exchange	10
Improved diagnostic capacity	7
Animal movement control	2

The session proved successful in introducing the participants to brainstorming and understanding the session format. Delegates were now able to proceed to exploring future possibilities.

The second question was:

What more would you like to see achieved?

The responses and relevant scores were:

Establishment of regional CSF reference laboratory (score 9)

- provision of standard reagents to participating countries
- strain characterisation
- training

Greater disease awareness for farmers and advisers (score 20)

- capacity building for farmers and district agricultural extension staff
- increased capacity of district agricultural extension staff
- organised education material for farmers and provision of better access to education
- increased disease recognition, diagnosis and control capacity
- networking for reporting and response

Training of more veterinary staff (score 20)

- more veterinarians needed in Lao PDR
- training of more veterinary staff

Vaccination (score 36)

- research to develop a heat-stable CSF vaccine
- simple test to check vaccine immunogenicity
- simple test to check post-vaccine immunity
- improved vaccine program (production, delivery and timing)
- encouragement for vaccination (with high-quality vaccine).

Emerging from this question, the most important issues were vaccination, disease awareness for farmers and advisers, and an obvious shortage of veterinary personnel in Lao PDR.

The next question was aimed at addressing these three key points by asking:

What do we do next to:

- a) improve CSF vaccine quality? Group 1
- b) increase the capacity for vaccination? Group 2
- c) increase incentives to vaccinate and promote awareness? Group 3
- d) train more veterinary staff? Group 4
- e) develop greater disease awareness for farmers and advisers? Group 5

The outcomes from this session are summarised below:

a) Improve CSF vaccine quality

- Improvements are needed in quality control at the vaccine production laboratory.
- Testing is needed post vaccination to ensure livestock are protected.
- Improvements need to be made to control the cold chain.

b) Increase the capacity for vaccination

- Ensure enough vaccine is produced to meet demand.
- Ensure enough trained staff are available to supply and administer vaccine.

c) Increase incentives to vaccinate and promote awareness

- Make the vaccine free.
- Raise awareness of the benefits of vaccination among farmers and allied veterinary staff.
- Develop effective advertising to coincide with public awareness.

d) Train more veterinary staff

- Long-term vision is required to correct the human resource deficiency that now exists.
- Short-term specialist training is needed for existing veterinarians and technicians at the district level.
- Encourage and lobby regional governments to provide university places for Lao veterinary students.
- Create university places in regional universities for Lao students.
- Make the options donor friendly—veterinary degrees require a 5–6-year investment, which in many cases is beyond the time frame of a project.
- Develop, in conjunction with universities and international governments, scholarships for Lao students at regional and Australian and New Zealand universities.
- Establish joint funding agreements between Lao and regional governments.
- Encourage donor support.
- Establish postgraduate training for Lao students with a Bachelor of Science degree to upgrade to a Master of Veterinary Science or Master of Science. This is more likely to be a donor friendly option as it will require only a 1–2-year investment; however, there will still be a shortage of trained veterinarians.

e) Develop greater disease awareness for farmers and advisers

- Target farmer groups, exchange information and determine the factors that influence farmers' decision making.
- Assist district and provincial agricultural officers to understand improved techniques of communication.
- Produce effective materials adapted for all people in a range of languages, including all media types (e.g. oral, written, pictorial).
- Improve and extend the university curriculum to include communication skills to upgrade the communication capacity of district and provincial staff.
- Create a suite of education materials and media/ education kits directed specifically at farmers, traders and district agricultural extension officers.
- Use 'mentors' at provincial and district levels to facilitate the dissemination of skills and information.

Conclusions

The facilitated session was able to identify three important areas to focus on in the future for CSF and FMD research. Vaccination was seen as a very important issue for the sustainable prevention of disease, and further research and development will be required to ensure the delivery and use of a quality

vaccine. The human resource deficiency in the veterinary field was highlighted but it was noted that this will be difficult to correct; making options 'donor friendly' will be important. The final key issue highlighted during this session was that of public awareness. For disease control measures to be successful, greater disease awareness at farmer and district levels will be required.

Maximising training outcomes in diagnostic laboratories: A two-way process

Chris Morrissy¹, Lynda Wright¹, Winsome Goff¹, Axel Colling¹, Greer Meehan¹, Michael Johnson¹, Stuart Blacksell², Laurence Gleeson¹ and Peter Daniels¹

Abstract

In this paper we describe the Australian Animal Health Laboratory (AAHL) experiences and approach with training and technology transfer of diagnostic tests for major livestock disease in South-East Asia. Examples are given of successful achievements in Vietnam, Thailand, Indonesia and Lao PDR. In brief, AAHL follows a 3-step approach. The laboratory diagnostician is trained at AAHL in the test methodology (e.g. TaqMan PCR) for a particular disease—for example foot-and-mouth disease (FMD) or highly pathogenic avian influenza (HPAI)—under ideal conditions and in a quality controlled and quality assured environment. The trainee then takes test reagents and protocol to his/her own laboratory to establish and standardise the test locally. Follow-up is provided by a consolidation and troubleshooting visit by AAHL staff to the overseas laboratory and includes a hands-on workshop on diagnostic techniques to remedy in-situ constraints.

Quality control procedures are built into the technology transfer. AAHL organises external quality assurance rounds to monitor the success of the technology transfer and to obtain useful information about potential sources of error. Other important aspects of training are related to biosecurity and biosafety. The close collaboration with South-East Asian counterpart laboratories increases AAHL's awareness of potential new and emerging diseases in the region. Although being directly involved in the development and validation of diagnostic tests for exotic diseases such as FMD, classical swine fever (CSF), HPAI, severe acute respiratory syndrome (SARS) and henipavirus, AAHL scientists are confronted with the limitations of a country (Australia) where these diseases do not exist. The collaborative nature of the projects in South-East Asia allows AAHL to use and validate these tests in an environment where the diseases are present. In summary, these activities enhance Australia's emergency disease preparedness and pre-boundary protection, which are the pillars to maintaining and improving its competitive international trade status. In turn, collaborating South-East Asian (SEA) countries strengthen their own diagnostic and disease surveillance capacity that subsequently leads to improved disease control—a win-win situation for all involved.

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Introduction

The Australian Animal Health Laboratory (AAHL), in its role as an OIE collaborating and reference laboratory, has a strong commitment to the development, validation and transfer of diagnostic technologies in the South-East Asian region. This process also includes training in the use of the technologies, interpretation of results, and aspects of internal and external quality assurance to ensure ongoing precision and accuracy of diagnostic results. An efficient network between clinical information from the field, laboratory diagnostic results and epidemiological data is the basis for informed decisions and adequate livestock health policies.



Figure 1. Major collaborating countries in South-East Asia

Funding agencies

The ability of AAHL to perform training depends on funds from a number of Australian sources including AusAID and AusAid programs (e.g. via the Collaboration for Agriculture and Rural Development (CARD) program), the Australian Centre for International Agricultural Research (ACIAR) and the Department of Agriculture, Fisheries and Forestry (DAFF). International funding agencies that have contributed to programs include OIE, FAO, IAEA and USDA. The majority of countries that AAHL collaborates with are located within the South-East Asian region (Figure 1).

Projects and training courses

AAHL provides support to the region through two types of training programs: workshops/programs lasting 1–2 weeks to 6 months; and projects of 1–3 years' duration. The training is based around the major trans-boundary diseases (TADs) of concern to Australia, the region and the world including foot-and-mouth disease (FMD), classical swine fever (CSF), duck virus enteritis (DVE), avian influenza (AI), and new and emerging diseases including Nipah, Hendra and severe acute respiratory syndrome (SARS).

AAHL has also organised veterinary training courses for Australia and the region. The focus of these courses is on recognising the clinical symptoms of these diseases and the differential diagnoses available, together with the selection of suitable specimens, protocols for their collection and transport, and information needed from the disease investigation. Below is a list of some of the projects in which AAHL has participated as manager or collaborator.

- FMD in Thailand — ACIAR (AS1/1988/35 1985–91; AS1/1992/04 1994–96), AusAID (2005–07)
- CSF and FMD in Vietnam — ACIAR (AS2/1993/875 1995): 'CSF in Vietnam'; AusAID (CSF 2001–03, FMD 2005–08).
- Duck virus enteritis (DVE) in Vietnam — ACIAR (AF2/1991/022 1996–99) in collaboration with University of Queensland: 'Duck plague: improved diagnostic methods and vaccination'.
- FMD and CSF in Lao PDR — ACIAR (AS1/1994/038 1997–2003): 'Improved diagnostic and control methodologies for livestock diseases in Lao PDR and Yunnan Province, PRC'; and ACIAR (AH/2003/001 2003–08): 'Management of CSF and FMD at the village level in Lao PDR'.
- Quality assurance project in Thailand and Indonesia (EQA program in six laboratories) — DAFF (2000–01) in collaboration with Attwood laboratory (ANQAP).

Workshops and training programs are aligned with AAHL's key deliverables in diagnostics and the support of Australia's preparedness for disease emergency. These include diagnostic techniques (i.e. ELISA, haemagglutination assay (HA) and haemagglutination inhibition assay (HI), cell culture, virus isolation, PCR and sequencing); veterinary training (i.e. disease diagnosis, disease investigation and

sample collection); major disease threats; and new and emerging diseases such as FMD, CSF, Nipah virus (Malaysia, Thailand, Indonesia, Taiwan and Japan) and AI (the Philippines, Vietnam, Indonesia, Nepal and Myanmar). There has been an increase in provision of molecular diagnostics training to the region, which gives diagnostic laboratories the ability to perform rapid testing of samples from the field. The PCR test provides increased sensitivity, ability for higher throughput and a rapid result compared to virus isolation. With the aim of determining the need for, and gaps in, improved diagnosis in regional laboratories, AAHL has undertaken an increased number of laboratory assessments.

Major collaborations and outcomes

Vietnam

ACIAR duck plague project

The ACIAR duck plague project in Vietnam, in collaboration with Queensland University and the Cambodian National Veterinary Company (NAVETCO), was a very successful ACIAR project with clear impacts. This project developed new diagnostic tests for diagnosis of DVE that were previously lacking both in Vietnam and Australia. The project's achievements included: the development of diagnostic tests for detection of antigen and antibody including indirect antibody ELISA, antigen capture ELISA, cell culture and virus isolation, and PCR; the development of a new cell culture vaccine that is cheaper and of higher quality; and a leadership role for other projects in Vietnam for CSF and FMD.

AusAID CARD FMD project

The current CARD FMD project in Vietnam has been successful for both Vietnam and Australia. It has given AAHL scientists a chance to enhance AAHL's FMD diagnostic capability as well as better understand FMD at the field level. The aims of the project are to establish an effective laboratory network for the diagnosis and control of FMD by providing resources and training staff in required methods and quality assurance; and to provide accurate data that will help to explain the failure of vaccination to control the FMD virus and develop new effective vaccine application strategies. The CARD project's achievements include:

- The Ho Chi Minh City and Hanoi (National Centre for Veterinary Diagnosis) Department of Animal Health laboratories now have established diagnostic tests for FMD diagnosis including ELISA, cell culture, virus isolation, PCR, nucleotide sequencing and the production of FMD antigen for use in ELISA (reagent production).
- The regional laboratories of Da Nang and Can Tho now have capability for detection of FMD antibody and antigen using ELISA.
- The project has improved the quality control and quality assurance procedures for diagnostic tests.
- Field specimens from FMD-infected animals and other reference populations were received to assist with further validation and sequencing of field isolates.
- The project is being used as a model for Vietnam's National Control Plan for FMD.
- There have been requests for assistance to improve diagnosis and outbreak investigation for AI and goat pox in Vietnam and further collaborations with DAH.

Lao PDR

AAHL has managed ACIAR-funded projects conducting studies on CSF and FMD in Lao PDR since 1997, with a focus on improving the country's animal health network from field to the laboratory. The project has been vital for improving the national diagnostic capacity through development of the laboratories and training of national staff to enable and enhance understanding of the disease situation in Lao PDR. Project outcomes include the implementation of conventional laboratory diagnostics for the diagnosis of CSF and FMD using ELISAs for antibody and antigen detection, as well as studies to determine the most appropriate samples for CSF diagnosis (Khounsy et al. 2007). Furthermore, a novel immunomagnetic bead (IMB)-ELISA was developed for the rapid diagnosis of CSF in low-technology field settings (Conlan et al., in press). Extensive training of local staff in disease investigation and surveillance, and vaccination and quarantine procedures, was conducted. The outcome was the development of a national sample submission network that resulted in a better understanding of the distribution of CSF virus strains from outbreak samples using molecular epidemiology (Blacksell et al. 2004; Blacksell et al. 2005). Detailed studies of FMD epidemiology were per-

formed using serological surveys and antigenic analysis from FMD outbreaks (Blacksell et al., submitted; Khounsy et al., submitted). Studies also demonstrated that local native pigs were less susceptible to CSFV infection when compared to improved breeds (Blacksell et al. 2006). In addition, the project provided opportunities for improved livestock production and understanding of livestock marketing.

Thailand

AAHL has had a long relationship with Thailand, having worked on FMD since 1985. Projects were based at the FMD Regional Reference Laboratory located at Pak Chong in north-eastern Thailand and the Northern Veterinary Research and Diagnostic Center in Lampang in northern Thailand. During the life of these projects, numerous Thai scientists visited AAHL for short- and long-term studies, and Australian scientists were resident in Thailand providing training to local scientists and gaining experience in FMD diagnosis and control measures. The early projects were funded by ACIAR and concentrated on the development, transfer and validation of FMD diagnostic technologies (Blacksell et al. 1994a), examination of local FMD epidemiology (Gleeson et al. 1995; Chamnanpood et al. 1995) and characterisation of Thai FMD strains (Blacksell et al. 1992; Lunt et al. 1994; Doughty et al. 1995a, 1995b). In addition, ACIAR projects developed internal quality assurance programs for the diagnostic assays in use at the time (Blacksell et al. 1994b; Blacksell et al. 1996). The current AusAID FMD CARD project is focusing on quality assurance and the completion of the BSL3 laboratory at Pak Chong. Current project outcomes include:

- BSL3 laboratory completed and in use
- accreditation to ISO 17025 Standard to obtain OIE status as a regional reference laboratory (RRL)
- harmonisation of diagnostic protocols for FMD diagnosis in the region
- training of regional countries to send samples to RRL
- training of regional scientists in FMD diagnostics.

Malaysia

AAHL assisted Malaysia in the control and eradication of Nipah virus from Malaysian pig herds. Nipah virus is a zoonotic disease affecting both animals and humans, and AAHL was able to apply

its experience with Hendra virus, another emerging disease that affected horses and humans in Australia. Providing support in both the laboratory and the field, AAHL staff worked in-country with Malaysian scientists and veterinarians. Major outcomes from the collaboration were:

- the development of diagnostic tests for detection of Nipah virus antigen and antibody including indirect ELISA (Nipah virus ELISA) for antibody detection; virus neutralisation test (VNT) for detection of antibody; and virus isolation, PCR and EM tests for detection of antigen or genome
- AAHL Nipah virus ELISA used for sero-surveillance and eradication of Nipah virus
- support in the laboratory and in the field
- AAHL becoming the OIE collaborating laboratory for new and emerging diseases.

The newly developed indirect Nipah ELISA was vital for the control and eradication of Nipah virus from Malaysia. The test was used to decide on the status of pig farms for the presence of Nipah virus and the culling of pigs in infected premises. The indirect Nipah ELISA had a relative diagnostic sensitivity of 98.99% (94.50–99.97% at 95%CI) and a relative diagnostic specificity of 99.91% (99.78–99.97% at 95%CI) with a kappa value of 0.9697 for ELISA and VNT (Table 1).

Table 1. Relative diagnostic specificity and sensitivity of indirect Nipah ELISA

ELISA	VNT	
	Positive	Negative
Positive	98	5
Negative	1	5333

Technology transfer and training programs: AAHL protocol

AAHL's training program consists of three phases (Figure 2).

During phase 1, trainees receive training at a reference laboratory, such as AAHL, where testing can be performed under ideal and standardised conditions in a quality controlled manner. In phase 2, trainees receive reagents and standard protocols to establish and perform the test at their own laboratories. During phase 3, trainees will be visited by an expert in their own laboratory and given more focused advice and attention based on reported problems including troubleshooting with the test.

During this phase the laboratory will improve its quality system by using and analysing internal quality controls and, ideally, through participation in an external quality assurance program. To make the technology transfer successful and sustainable, the following support systems need to be in place:

- appropriate staff, both trainers and trainees, from collaborating institutions
- appropriate diagnostic methodology to suit the purpose of the new test and the fitness of the test to fulfil this purpose (e.g. whether it is to be used as a screening or as a confirmatory test)
- suitable laboratory infrastructure and budget to implement and maintain the new methodology (e.g. appropriate equipment, suitable working space, supply of critical reagents).

The chances for the training program and technology transfer to be successful increase if a two-way process is followed in which both sides benefit and where participating scientists have ownership and understanding of the outcomes. Monitoring of assay performance is easier and more reliable in a system with established internal and external quality control and quality assurance (OIE Quality Standard 2008). Good and continued communication about the outcome of analysis and interpretation of quality control samples between the two laboratories is an important basis for success. Training in data analysis and interpretation of results is an important step at the end of the diagnostic testing.

There must be a clearly established procedure when a sample needs to be retested, or when a result is inconclusive and a confirmatory test is required. For example, during a surveillance program, a screening test such as ELISA with a high sensitivity, high throughput, short turnaround time and low cost is required. This test may produce some false positive results which will need to be retested with a more specific confirmatory test. Another important consideration is the positive and negative predictive values of a test. For example, during a disease control program, the prevalence of the disease may drop dramatically from 5% to 1% or less. This drop will have a serious impact on the predictive values of a diagnostic test. The lower the prevalence (pre-test probability), the lower the positive predictive value and the higher the percentage of false positive results.

Diagnosis and outbreak investigation

The training program includes disease diagnosis and outbreak investigation for both laboratory and field staff. Recognition of clinical signs and basic understanding of pathology, specimen collection and transport are essential to improve animal health. Also crucial is the use of standardised protocols for diagnostic tests including quality control and quality assurance, and record keeping and data collection for a disease database.

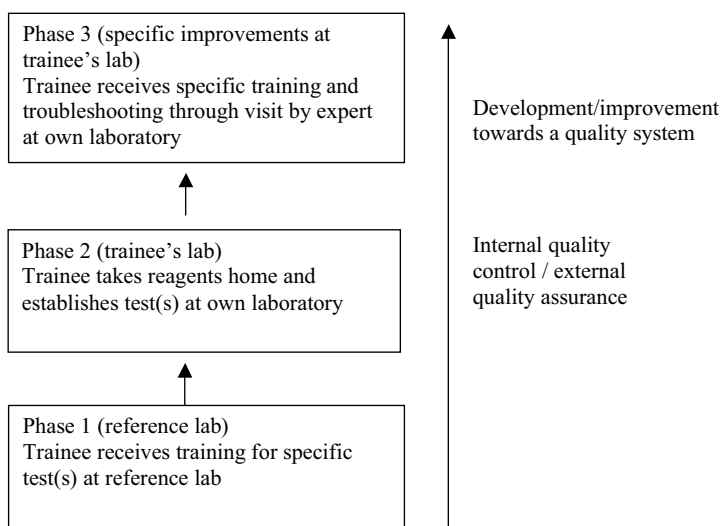


Figure 2. The three phases of AAHL's training program

In our experience a major constraint in the successful realisation of a laboratory test is the suitability of the specimens. Ideally, the technology transfer will therefore involve field veterinarians and village para-veterinary workers who interact with farmers and provide the primary link in the diagnostic chain by selecting and submitting specimens for laboratory examination. Training in submission of appropriate specimens becomes part of the training package, with a focus on building a specimen submission network and improved interaction between field veterinarians and farmers. Involvement of the farmer at the early stage of this process and communication of the benefits of timely and accurate diagnostic results will help to contain the spread of disease and turn the farmer into a major stakeholder in the project.

The collection of specimens should follow a standard protocol. Once the specimens have been collected it is important to transport them under the correct temperature conditions. Serum and tissue samples should be transported at 4 °C and, if possible, tissue samples at -20 °C. The quality of the specimens when received at the laboratory will determine the quality of the diagnostic results. The history of the disease and other important field information about the species (age, sex, vaccination etc.) are important and need to be recorded on the specimen submission sheet. The result of a diagnostic test is only one component of the information required to arrive at a final diagnosis. Clinical and epidemiological findings from the field are also crucial.

Biosafety and biosecurity

Training in biosafety and biosecurity is an important part of a disease investigation, both in the field and in the laboratory. Biosafety is required when collecting specimens or carrying out a post-mortem in the field. Veterinarians need to wear correct personal protective equipment including gloves, gown, mask or respirator, and eye protection. Similarly, laboratory diagnosticians need to follow biosafety rules to be adequately protected against microbiological contamination by using gloves, gown and eye protection and working in class II microbiological safety cabinets (BSCII). Biosafety protocols and standard operating procedures need to be in place to prevent human infection and ensure the safe handling of disease agents. They are an important part of all outbreak investigations, especially with

an increasing number of new and emerging zoonotic diseases.

Biosecurity is related to the physical containment of microbiological contamination and starts in the field when carrying out a post-mortem. Transport of specimens from the field to the laboratory must be done in the correct containers to prevent leakage. Work areas in the laboratory must be designed to prevent release of an agent to the environment. This is achieved through the use of flow hoods and BSCII and HEPA-filtered exhaust air.

Quality assurance and quality control

All training is carried out under a quality system. AAHL is an ISO 17025 accredited laboratory and all its procedures are documented in a quality manual. The overall goal of the training is to enhance the trainee's understanding and application of quality control and quality assurance protocols and the need to have quality controlled and valid test procedures. The use and analysis of internal quality controls such as strong positive, weak positive and negative controls over a critical range of potential test results is an essential technical requirement of ISO 17025-2005 'General requirements for the competence of testing and calibration laboratories'. Results are useful to ensure that a test run is valid and provides important information about test precision. Trends in test performance can be detected early before results get out of control and therefore are useful parameters for preventive troubleshooting (Crowther et al. 2006).

External quality assurance (EQA) or proficiency testing (PT) is carried out by an external laboratory. The PT provider sends out a proficiency panel and samples need to be tested by each participating laboratory. Results are then returned to the PT provider who does the analysis and sends out a brief report to the participants. PT programs are useful to identify sources of bias such as random versus systematic errors and loss of test sensitivity or specificity, and are important tools in the ongoing assay validation process (De Clercq et al. 2008). As part of the technology transfer monitoring process, AAHL has organised a number of PT rounds. Successful participation in PT programs is a crucial requirement for an ISO 17025 accredited laboratory. Australia's National Quality Assurance Program (ANQAP) organiser offers PT rounds for a wide range of tests

on a global level (<<http://www.anqap.com>>). Supranational organisations such as FAO or IAEA have organised a number of external quality assurance programs (e.g. for FMD ELISAs) and participants have used the experience to establish quality systems in their own laboratories (Colling et al. 2008).

Conclusion

The major objective of Australia's disease control projects over the past 25 years has been to provide training to local South-East Asian staff to increase capacity in the diagnostic and clinical aspects of disease control. To maximise training and project outcomes, it is important that training be a two-way process using a collaborative approach (Figure 3).

Such projects also have benefits for AAHL, including opportunities for the validation of current and new diagnostic tests; provision of education and training in support of pre-border quarantine; and training of Australians in disease diagnosis, disease control and surveillance. A collaborative approach improves commitment from the involved parties, communication and understanding, and ownership of project outcomes. OIE has recognised the benefits of this approach and has initiated an era of 'twinning projects' involving OIE collaborative or reference laboratories with other laboratories. Training is more efficient if it is done under a system using internal and external quality control and assurance procedures because results provide useful troubleshooting

information. EQA improves the communication between laboratories about assay and staff performance. Data analysis, interpretation of results and graphical display of mass data are needed to convert pieces of information into knowledge. Field staff must be included in the training to collect suitable and fresh specimens and so improve the quality of information about the disease outbreak. Training in biosafety and biosecurity is very important to minimise human exposure and spread of the disease agent in the environment and to prevent laboratory infections.

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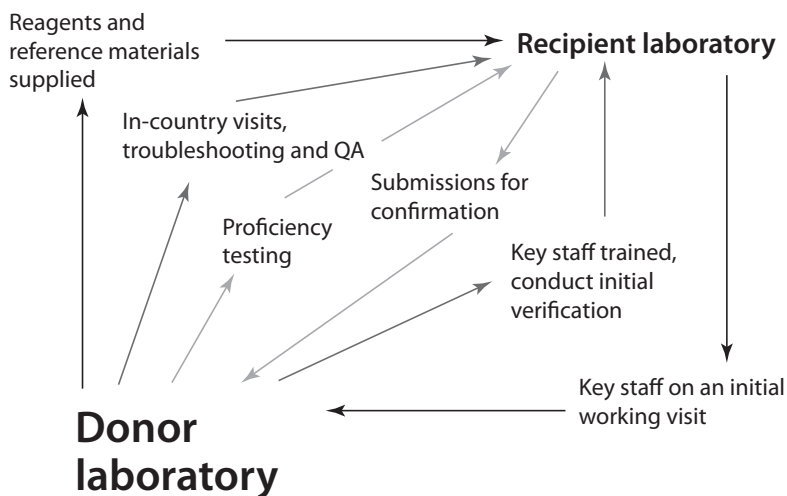


Figure 3. Inter-dependent pathways of successful technology transfer

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