Classical Swine Fever and Emerging Diseases in Southeast Asia

Vientiane, Lao PDR, 19–22 September 1999

Editor: S.D. Blacksell

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Welcome Address and Introduction

Mr Singkham Phonvisay
Director-General of Livestock and Fisheries Department

His Excellency Dr Siene Saphangthong, Minister for Agriculture and Forestry, Lao PDR, Her Excellency Karina Campbell, Australian Ambassador to Lao PDR, distinguished guests, delegates and participants: Ladies and gentlemen, I have great pleasure in welcoming all of you to this international conferernce on classical swine fever and emerging viral diseases in Southeast Asia.

WITHIN this forum, we have distinguished participants from various international organisations: ACIAR, CSIRO, EU, FAO, GTZ, IUCN, OIE and representatives of Australia, Belgium, Cambodia, China, England, Germany, Indonesia, Hong Kong, The Netherlands, Thailand, Vietnam and Laos.

A number of viral diseases including foot-and-mouth disease, classical swine fever, Newcastle disease, rabies and duck plague are still endemic in Lao PDR. Classical swine fever still causes heavy mortality, which decreases incomes and food security for many rural villagers throughout Lao PDR.

In the past 2–3 years, the Department of Livestock and Fisheries has received helpful support via ACIAR, particularly the project 1994/38, to develop diagnostic and control methodologies for classical swine fever and foot-and-mouth disease. This assistance has allowed us to upgrade our diagnostic capability and facilitate improvement in our communications with neighbouring countries and the region.

We realise that this conference will give experience, lessons and practical knowledge for our further investigation of and campaign against classical swine fever.

This is a great pleasure for us. I would now like to invite His Excellency Dr Siene Saphangthong, Minister for Agriculture and Forestry, to open the meeting.

Thank you.

His Excellency Dr Siene Saphangthong
Minister for Agriculture and Forestry

ON BEHALF of the Ministry of Agriculture and Forestry, I have great pleasure in extending a warm welcome and congratulations to all participants and guests. This international conference is very important for the livestock sector of Lao PDR and the region. I would like to express our sincere appreciation to all of you in sparing precious time to attend this conference.

Our people are striving to implement the last year of the fourth five-year development program. Based on this effort, the Ministry of Agriculture and Forestry has to coordinate and supervise some related government priority programs, namely, food security and commodity production.

The livestock subsector is one among many in food security and commodity production, and its technical programs must be promoted and implemented. Animal health is of prime importance. Classical swine fever is considered one of the most infectious and endemic diseases affecting the development of pig production throughout the country.

The strong support of ACIAR on this matter has been very helpful to us. We would like to take this opportunity to express our thanks and appreciation for such generous help.

This meeting will produce new outcomes in tackling this disease, particularly possible and efficient ways to control it. This will have great impact on food security and commodity production objectives.

I would like to extend once again my sincere appreciation to ACIAR for its support of the meeting. I would like to convey my best wishes to all of you in finding new research collaboration for closer regional cooperation, and declare the meeting officially open.

Her Excellency Karina Campbell
Australian Ambassador to the Lao PDR

Mr Sien Saphangthong, Minister of Agriculture and Forestry, Mr Singkham Phonvisay, Director-General of the Department of Livestock and Fisheries, distinguished guests, ladies and gentlemen.

THE AUSTRALIAN Government, through ACIAR, is pleased to be a partner in this conference. As you know, Australia has a long history of cooperating in the animal health field in Lao PDR.

This is the first conference in Southeast Asia to focus heavily on the very important disease classical swine fever.

I am proud to acknowledge the support of the Australian Government–funded project—the current ACIAR project aimed at improving diagnostic and control methodologies in both classical swine fever and foot-and-mouth disease—actively operational in Laos at this time.

I know Mr Blacksell, the ACIAR project scientist, has taken great steps towards establishing a first-class diagnostic facility with field support to assist in better understanding the nature of this disease, and I thank him, and his team, for their efforts thus far.

Your deliberations during this conference are important. These diseases have potentially negative impacts on the income and food security of many rural villagers in Laos. For this reason, I wish everyone involved in the conference good luck.

On behalf of the Government of Australia, let me add my words of welcome.

IMPORTANCE OF LIVESTOCK AND LIVESTOCK DISEASES IN SOUTHEAST ASIA

The Role of Pigs and Disease in Southeast Asian Livestock Systems

D. Hoffmann[1,2]

DISEASE in pigs and humans is the end product of a complex process involving the causative agent, the host and their environment. Many disciplines are necessarily involved in reaching an understanding of the disease process, the understanding essential not only to increase the productivity of pigs but also to protect human health and the broader environment.

Disease is a state of not (*dis*) being at ease (*ease*). Initially, the disturbance may be biochemical, such as impaired liver function or increased blood ammonia or blood urea. If the disease progresses, an obvious clinical sign such as poor growth rate, lack of vigour, haemorrhage, lameness or ulcers may be produced. An animal not visibly diseased is usually assumed healthy, though subclinical disease is frequently present, usually resulting in weight loss, retarded growth and the loss of the benefits of nutritional inputs.

The three possible outcomes of a disease are death, survival with disease (chronic or subclinical disease), or recovery. In all three cases, economic loss occurs. The disease outcome and the frequency of occurrence are influenced by many variables called disease determinants, which usually include one or more specific agents, and factors associated with the host animals and their environment.

The relative importance of all identifiable determinants and the interplay among them must be considered in attempting to prevent or control diseases. It is important that policy advisors are aware of the consequences and implications of making decisions not based on sound advice on disease control. The economic consequences of bad decisions can be both devastating and long term.

Disease Agents

The aetiological agents that affect pigs may be genetic, physical, infectious, chemical or nutritional.

Host determinants include age, species, breed, sex, genetic characteristics and immunological state.

Environmental determinants can be broadly divided into those influenced easily by man, such as management and nutrition, and those not easily influenced by man, such as location, cultural practices, religion and socioeconomic status.

It is impossible in this brief paper to cover all the disease determinants mentioned. Therefore, the paper focuses on the infectious agents, and refers to managerial and nutritional determinants where appropriate.

The Changing Environment

The drive in Southeast Asia to satisfy the increasing demand for animal protein has resulted in many changes to common agricultural practices. The intensification of pig production industries, combined with urbanisation, global changes and the increasing ease of travel and transport, has produced environments that may lead to an increase in

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[1] FAO Regional Animal Production and Health Officer, Asia and the Pacific

[2] The opinions and views expressed in this paper are the author's and not necessarily those of FAO.

the impact of formerly uncommon diseases and even the emergence of new diseases. These changes challenge normal control methods and indicate that new ways must be found if these emerging diseases are to be controlled. Thought is now being given to the relocation of pig production systems away from where they have developed peri-urbanly, to an area-wide integrated approach. Man-made relocations will significantly affect the disease determinants and can, if well understood, be used to reduce significantly the occurrence of disease in both pig and humans.

Emerging Infectious Diseases

'Emerging' infectious diseases can be defined as infections that have newly appeared in a population or have existed but are rapidly increasing in incidence or geographic range. Specific factors precipitating disease emergence can be identified in virtually all cases. They include ecological, environmental and demographic factors that place pigs in increased contact with a previously unfamiliar microbe or its natural host, provide potential pathogens with a novel host, or favour increased dissemination. The factors are increasing in prevalence in Southeast Asia because of the rapid development of the pig industry in the region. The increase, together with the ongoing evolution of viral and microbial variants and selection for drug resistance, suggests that infections will not only continue to emerge but also that their rates and effects will probably increase.

Zoonoses are diseases transmissible from animals to humans. One hundred and fifty are recognised worldwide. They are distributed widely, and occasional human infection occurs. However, a minor change in one of the disease determinants may provide the opportunity for a spectacular increase in prevalence. A few that could be mentioned are rabies, brucellosis, mosquito-borne encephalitis, salmonellosis and Nipah virus.

As an example, recent outbreaks of human diseases in three countries of the region were caused by zoonotic paramyxoviruses: Hendra (1994) and Menangle (1997) in Australia and Nipah (1998–99) in Malaysia and Singapore. These outbreaks were initially observed as disease in animals (Hendra, horses; Menangle and Nipah, pigs) followed by disease in humans who had contact with the infected animals. Evidence currently available suggests that fruit bats (suborder Megachiroptera, genus Pteropus) may be the natural hosts and wildlife reservoir of these paramyxoviruses. Evidence of Hendra virus infection has been found in these bats throughout their range in Australia and in Papua New Guinea, and Nipah virus infection in populations in Malaysia. While the Hendra and Nipah virus are apparently closely related, the Menangle virus is seemingly distinct. In the multi-age piggery where it was isolated, it caused widespread mortality and deformities in pig foetuses, while all other infected pigs showed subclinical infection. Eradication of the grower pigs seemingly broke the cycle of infection, but highlighted the serious and potentially devastating economic and social costs of these and future outbreaks of zoonotic paramyxoviruses. We must be able to detect, investigate and respond effectively to these and other newly emerging diseases.

Bats are probably the oldest forms of placental mammal in Australia, with fossil evidence from the Middle Miocene era, circa 15 million years ago. Some species migrate between various countries of southern Asia. Because of this past continuity of bat populations, and also because Microchiroptera had been poorly studied in Asia for rabies viruses (in contrast to the USA and Europe), St George (1989) was led to forecast that rabies (or rabies-like) viruses may already be established in bats in Australia. Thus these recently recognised diseases are known as 'emergent', but it is highly likely that they have always been present. However, once they become involved with human beings and/or domestic and productive animals, much alarm occurs.

Surveillance and Control

There is conflict of interest between the scientist who looks for evidence that a disease agent exists in a country or region, where it is not expected to be, and the regulator who

has to deal with the public health, or economic consequences, of a newly revealed situation. It does not seem to matter whether it represents a new but spreading disease or one being recognised for the first time in a stable situation. National and international reactions to the discovery of many agents in countries where they have not previously been noted all too often take no cognisance of the fact that the actual risk to humans and/or animals may not be changed. Fear and overreaction are sure to be the result when the risk is not quickly and correctly assessed—especially when that factor is compounded by sensationalised media coverage.

Ecological Changes and Agricultural Development

Micro-organisms and viruses are adapted to extremely diverse eco-niches. One of the most complex sets of adaptive characteristics concerns arthropod transmission of viruses. The arthropod-borne viruses are spectacular examples of emergence and re-emergence resulting from environmental manipulation or natural environmental change. Deforestation, amateur irrigation and the introduction of new species (usually livestock) give rise to many virus disease threats to humans and animals.

It has been predicted that the world's average temperature will rise by between 1 °C and 3.5 °C in the next 100 years. This is a faster rate of change than has been seen in the roughly 10 000 years since agriculture and human settlement began. The resulting disturbances of ecological systems may influence the incidence and distribution of infectious diseases through several mediating processes: the range, activity and biological characteristics of vectors and infective parasites; the local ecology of water and food-borne infective agents; population displacement and damage to infrastructure as a result of a rising sea level; and other socioeconomic effects.

Changes in Demography and Behaviour

The revolution in international travel and trade over the past two decades has meant that disease agents can be transported quickly between susceptible populations. The corollary is also true in that susceptible populations can move into areas where they can become infected by agents to which they have had no previous exposure.

Microbial Adaptation and Change

Outbreaks of new diseases have been documented since antiquity, but these do not necessarily involve a genetically distinct 'new' disease. More commonly, new diseases emerge because disease-causing viruses, bacteria or parasites find new pathways to previously unexposed populations.

However, the probability of interspecies transfer can be increased not only by increased contact between humans and an animal reservoir, but also by increased opportunity for viral genetic reassortment or recombination within animal or insect hosts. The complex relationships between the microbes that cause disease and the humans or animal hosts that contact the disease are constantly evolving as microbes mutate and either die or survive to reproduce. The widespread, even indiscriminate, use of broad-spectrum antibiotics has led to the evolution of bacteria that cannot now be controlled by the most common antibiotics. The common practice of adding antibiotics to feed in intensive farming establishments may well have led to the evolution of some of those bacteria.

Globalisation of food supplies is affecting outbreaks of food-borne illness, an increasing problem in human health. Examples include several outbreaks of Salmonella infection, *E. coli* O157, and Cyclospora. *Salmonella enteriditis*, phage type 4, is a worldwide problem. The organism responsible typically expresses several antibiotic-resistant genes.

It is now possible to produce a food product anywhere in the world, process it for distribution anywhere, and for it to be consumed anywhere. Thus we can expect increasing outbreaks caused by organisms previously restricted to areas of endemicity.

Some pathogens are frequently transmitted by food contaminated by infected persons. Pathogens that can cause diseases after an infected person handles food include *Salmonella typhi*, Shigella species, *Staphylococcus aureus* and *Streptococcus pyogenes*. Other pathogens are occasionally transmitted by infected persons who handle food, but usually cause disease when food is intrinsically contaminated or cross-contaminated during processing or preparation. Pathogens of this category are *Campylobacter jejuni*, *Entamoeba histolytica*, Enterohemorrhagic *Escherichia coli*, Enterotoxigenic *Escherichia coli*, *Giardia lamblia*, Non-typhoidal Salmonella, Rotavirus, *Taenia solium*, *Vibrio cholerae* 01 and *Yersinia enterocolitica*.

The full list of more than 60 organisms that can contaminate food can be found in the 'Bad Bug Book' produced by the US Food and Drug Administration.

Breakdown of Public and Veterinary Health Measures and Deficiency in Infrastructure

It is well known that conflicts within and between countries can lead to breakdown in veterinary control measures, which can lead to the re-emergence of disease. Rinderpest control in Africa and South Asia is an often-quoted example of regions where civil disturbances have contributed to re-emergence. Foot-and-mouth disease and classical swine fever control in this region are difficult to control because of uncontrolled livestock movements. Severe constraints, including widespread strife, abject rural poverty, poor or absent infrastructure (personnel, facilities, roads, communications, law enforcement), limited logistical capacity, a long rainy season, frequent climate-associated natural disasters and inadequate funding all contribute to the difficulties of controlling these diseases.

Another concern is that control and monitoring disease can move away from government control when intensification increases and vertical integration occurs. The managers of very intensive systems often become secretive about what diseases they have and what measures they are using to control them, to the detriment of good control and good practice, especially the use or misuse of antibiotics. One legitimate concern of government is to assure product quality and safety, and ways to monitor food safety must be developed. Applying such concepts as Hazard Analysis Critical Control Point (HACCP) may help, and is discussed elsewhere in these Proceedings.

Economics and Consequence of Change

The economic consequences of all aspects of animal production are not easily assessed. The long-term effect of the absence of animal fertiliser or humus on soil structure and soil fertility and the effects of mechanisation on the soil, on energy requirements or on the environment are often overlooked. For example, the lack of animals to use either waste products or by-products may well result in such practices as burning rice straw, dumping waste with potential disease hazards, or using costly waste-disposal mechanisms. The reverse is also true. Feeding waste to animals can and has caused problems, for example, feeding poultry manure to cattle has caused enormous losses due to botulism toxicity, and feeding abattoir waste to cattle caused BSE or Mad Cow Disease. Very few studies have looked at the effects on health costs of changing agricultural practices.

While it is clear that a correct balance is needed, there are cases where livestock-raising practices create problems and difficulties. Intensive units, particularly pigs and poultry, can create major environmental and health problems. In a global context, the movement of feed for livestock is itself causing massive shifts in resources which, in

the long term, create serious problems in both areas, i.e. the provider and the recipient suffering from nutrient deficit and surplus respectively. For example, the producer fails to replenish land with manure, while the recipient area suffers pollution problems seriously affecting crop production as well as water quality. For a full assessment of the interactions of livestock and the environment, see Steinfeld and others, and de Haan and others. The publications form an excellent consensus of a study supported by a series of donors and global participation.

Conclusion

Individuals and governments alike must develop a new perspective on infectious disease outbreaks in animals and humans. The protection afforded by isolation to control epidemics no longer exists.

The previously mentioned disease determinants are but some of the variables in, and constraints to, the human–animal interaction. Within one country, there may be many different interactions, the usual outcome being that one of the disease determinants becomes dominant in the process. To identify the relative importance of a disease and to decide whether control is warranted require more than a study of infectious agents. A systems approach is required, with consultation among all contributing groups. A single-discipline approach, which in this context can be described as 'tunnel vision', must be actively discouraged. The reasons for 'tunnel vision' include the current structures of some government departments and universities that promote the separateness of disciplines. Competition for limited research funds adds to the tendency for special interests to be projected and protected. To change both structures and attitudes within these organisations will take time, and is not seen as a short-term solution to the problem. An immediate and valuable contribution toward encouraging a systems approach can, however, be made by the administrators of funding bodies and their advisers. They should require that proposals seeking their support include a broad perspective of the human–animal interactions in question, and a clear intention to involve all relevant disciplines, especially when governments are planning area-wide integration of livestock.

The concept of area-wide integration (AWI) affords the opportunity to consider the epidemiology and economics of diseases when designing the system. AWI should be undertaken with a sound knowledge of disease control among other disciplines. The knowledge will influence the preferred siting of different livestock production systems not only globally but also regionally, within a country, within a catchment, and even within a farm. It will influence the type of livestock facility (housing, etc.) constructed and how it is constructed, where and how waste is treated and disposed of, where and how inputs are purchased and delivered, where and how products are processed, and, especially, management decisions. One example is the 'All-in all-out system' which has revolutionised disease control in intensive units. In many ways, the impact one can make on disease control in such a planned system is enormous. For example, if abattoirs can be sited near production facilities, animal movement across the countryside can be reduced substantially, and disease control in general made much easier. AWI offers another tremendous advantage in that the establishment of a disease-free zone (DFZ) becomes a possibility within part of a country where it has been impossible to achieve country freedom. Since the greatest constraint to the free movement of livestock is disease restrictions, the DFZ concept would free-up trade, particularly if the economic advantages were great.

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Emerging Viruses of Animals in Australia and Southeast Asia

H.A. Westbury[1]

Abstract

Australia has, since 1994, experienced a number of previously unrecognised disease outbreaks in farmed livestock and fauna. Some of these diseases, such as those caused by Hendra and Menangle viruses, were new to human and veterinary medicine; others were new although similar to diseases seen in other parts of the world, e.g: the disease caused by Australian bat lyssavirus, while others such as epidemic pilchard fish mortality were mysterious and require further study to determine the causative agent, though it is thought to be caused by a herpesvirus. Experience and knowledge gained in studying these disease are important to the Southeast Asian and Pacific regions because, as demonstrated by the Nipah virus outbreak in Malaysia in 1998 and 1999, it is possible that these viruses, or closely related viruses, could cause disease outbreaks in other countries, particularly as the natural host of Hendra, Nipah, Menangle and Australian bat lyssavirus seems to be fruit bats (Megachiroptera) that are widely distributed in the Southeast Asian and Pacific region. This paper provides an outline of the history of disease outbreaks in Australia caused by Hendra virus (HeV), Australian bat lyssavirus (ABLV) and Menangle virus (MeV) and the discovery and characterisation of these viruses.

IN AUGUST 1994, two horses died suddenly on a property in Mackay, Queensland, Australia. The owners, a veterinarian and her husband, carried out postmortems on the horses, and samples were submitted for laboratory examination. The cause of death was attributed to avocado toxicity and snakebite, respectively, based on histopathological examination, and no further tests into the cause of death were undertaken. Ten days after the death of the second horse, the husband was admitted to hospital with meningitis, from which he made an apparently successful recovery. These incidents would have been consigned to the family history except for a totally unexpected event 14 months later.

One month after the Mackay incident, 13 of 20 thoroughbred horses died or were euthanased terminally at a stable at Hendra in Brisbane, Queensland (about 800 kms south of Mackay) with the deaths occurring over a two-week period. The owner/trainer and a stable worker, both of whom had contact with the affected horses, became ill with an influenza-like disease from which the trainer died after 6 days of intensive care. A link between the illness and death and the virulent respiratory disease in the horses was not initially considered as the trainer's physician suspected *Legionella* infection as the likely cause of his illness. Similarly, the clinical signs and lesions in the horses were suggestive of acute African horse sickness, or perhaps acute equine influenza (diseases exotic to Australia), and investigations were conducted with this in mind. However, these potential diagnoses were quickly eliminated, as were bacterial diseases, plant intoxications and poisoning. A novel paramyxo-like virus was isolated from the affected horses, and, indeed, from the dead trainer. This virus reproduced the disease syndrome when inoculated into horses, and specific antibody to it was detected in the trainer and stable worker, and in all the recovered horses. The gross and microscopic lesions in the naturally and experimentally infected horses and the dead trainer were similar, and the convalescent human sera reacted strongly in immunofluorescent and immunoperoxidase tests on tissues

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from affected horses. Furthermore, this virus induced similar clinical signs and lesions in cats inoculated with the virus, and similar microscopic lesions in experimentally infected guinea pigs. The virus was therefore considered to be the causative agent of the disease and a new zoonotic pathogen. It was initially called equine morbillivirus (Murray et al. 1995) because it was more closely related to the morbillivirus genus of the virus family *Paramyxoviridae* than to the other virus groups in the family. Subsequent research (Wang et al. 1998) demonstrated, however, that it was unique and probably best categorised as the first virus of a new genus within the *Paramyxoviridae*. It is now called Hendra virus (HeV).

In October 1995, the man who assisted with the postmortems of the horses in Mackay was again admitted to hospital with clinical signs of central nervous system disease. He died following an illness of 25 days characterised by seizures and worsening paralysis. The cause of his death was not determined by extensive microbiological testing for recognised causes of such disease, and, in desperation, his physicians requested tests for HeV infection because they knew of his connection with horses and of the earlier death of the Brisbane horse trainer, even though the presenting signs of this disease were different. HeV antigen was demonstrated in his brain using immunofluorescence and immunoelectron microscopy and typical HeV nucleocapsids were seen in ultra-thin sections of neuronal cells (Hooper et al. 1996; Hyatt and Selleck 1996). The polymerase chain reaction (PCR) was used to detect HeV in cerebro-spinal fluid and brain tissue, and the nucleotide sequence of PCR product was homologous with that of HeV isolated in 1994. A significant rise in specific neutralising antibody titre to HeV was detected during the course of the disease—from 1:16 at admission to 1:5792 just before death. The virus was not isolated, perhaps because of the virus-antibody complexing.

These results incriminated HeV as the cause of the disease, especially as the microscopic lesions—vascular thrombosis and occasional multinucleated giant cells in the endothelium of blood vessels—were also consistent with HeV induced disease. Furthermore, a neutralising antibody titre of 1:4 was detected in a serum sample collected from the man soon after his recovery from the episode of meningitis in August 1994. This result suggested his source of infection with HeV may have been the horses postmortemed in 1994, even though these were presumed to have died from avocado poisoning and snake bite. Retrospective testing of stored specimens from both horses using specific fluorescent antibody and PCR tests revealed each was, in fact, infected with HeV, and, despite the absence of key tissue samples such as lung, that their histopathological lesions were consistent with HeV disease (Hooper et al. 1996).

Thus, in 1994, there were two outbreaks in Queensland of HeV disease on properties about 800 kms apart and between which there was no apparent direct or indirect contact (Douglas et al. 1997). A 'think-tank' of scientists within the Queensland Department of Primary Industries conjectured that birds or bats may be have been the source of the virus and the link between the two affected properties, so they undertook serological testing of birds and bats known to inhabit both Brisbane and Mackay.

Specific serum neutralising antibody was soon detected in bats of the four species of the genus Pteropus found in Australia and, indeed, HeV was isolated from them. Specific antibody was not detected in other fauna tested, or, indeed, in horses following an intensive structured serological survey in Queensland and elsewhere in Australia. These data pointed to fruit bats of the genus Pteropus being the natural reservoir host of HeV (Young et al. 1996) and that they were the probable source of infection for the horses, though how this occurred is unknown.

A further case of HeV disease in a horse occurred in north Queensland in 1999, the first known case since 1994, despite intensive on-going surveillance by government animal health agencies and private veterinary practitioners. This history suggests that transmission of virus from the natural host to horses is a sporadic and occasional event, a concept supported by a failure to detect HeV infection during retrospective examination of stored lung samples collected from horses with pneumonic lesions.

Nipah virus, a virus closely related to HeV, caused a dramatic outbreak of disease in humans and pigs, and some other animal species, in Malaysia in 1998 and 1999. Affected humans exhibited clinical signs of neurological disease following exposure to pigs with respiratory and/or central nervous system disease caused by the virus (Anon. 1999). Nipah virus transmits naturally and easily between pigs on a farm, between pig farms by movement of pigs, from pigs and perhaps other animals to humans only if there was close contact (as during obstetrical interventions), but not readily, if at all, between humans. Serum neutralising antibody to Nipah virus has been detected in bats and it seems likely, as with HeV, that they are the natural host of the virus. Nipah virus is related morphologically, antigenically and genetically to HeV, and is the probably the second member of the putative megamyxovirus genus of the family Paramyxoviridae but is distinguishable from HeV using serological and molecular tests (B.T. Eaton, pers. comm.).

Hendra Virus

Hendra virus was originally isolated from horse tissues using Vero monolayer cell cultures in which it induced a focal syncitial cytopathogenic effect (cpe) in about three days. The human virus was isolated in LLC-MK2 and MRC5 cell monolayers in which it induced focal cpe in about 12 days. However, the horse trainer had detectable levels of serum neutralising antibody at the time of his death and thus the amount of virus present in his tissues may have be quite small. The virus was only isolated from his kidney. HeV is remarkable in the wide range of cell culture systems in which it will grow, though Vero cells are most commonly used. It has morphological characteristics of a member of the family Paramyxoviridae, but it cannot, on morphological, biological and genomic grounds, be easily categorised into the existing genera (i.e. rubulavirus, morbillivirus, respirovirus, pneumovirus and metapneumovirus) of the family. Wang et al. (1998) suggested that HeV is the first or type virus of a new genus within the family. This suggestion is currently being considered by the International Committee for the Taxonomy of Viruses (ICTV). Nipah virus is probably the second member of this proposed new genus within the family.

Hendra virus has unique morphological characteristics that are useful for diagnostic purposes. Electron micrographs of negatively stained virus reveal pleomorphic enveloped particles containing characteristic paramyxovirus nucleocapsids. The envelope is, very characteristically, covered with 10 and 18 nm length surface projections giving the particle a unique 'double-fringed' appearance. Such a double-fringed arrangement is not a feature of previously described members of the Paramyxoviridae family. Free-lying herringbone-shaped nucleocapsids, which were 18 nm wide and had a periodicity of 5 nm were frequently seen in negatively stained preparations (Hyatt and Selleck 1996).

The virus is enveloped and consequently it is able to be inactivated by compounds that disrupt this envelope. In the laboratory, we use either 3% lysol for 3 minutes at 20 °C, 2% SDS for 2 minutes at 100 °C, 1% glutaraldehyde for 10 minutes at 20 °C, 100% acetone for 15 minutes at 20 °C, pure methanol for 10 minutes at 0 °C or gamma-ray irradiation for our diverse virus inactivation requirements, as well as formaldehyde fumigation of HeV contaminated animal rooms.

Diagnosis and Surveillance

Experience indicates that HeV disease in horses in Australia is uncommon and sporadic though this provides no grounds for complacency as there is widespread appreciation of the importance of the disease from both an animal and human health point of view. The possibility of HeV disease in horses should be considered if there is a sudden death in horses in areas and regions where fruit bats are common horse, particularly if there are premonitory respiratory disease. Clinical signs of the onset of disease are a rise in rectal temperature and increased respiratory and heart rates and, occasionally, signs of central nervous system involvement. Field veterinarians need to take stringent microbiological security precautions in handling potentially infected horses and during autopsy of such horses. These include, as a minimum, effective eye, nose and mouth protection from fomites and aerosols, complete covering of the body with overalls, and double gloving with gloves tied by adhesive tape to the cuffs of the overalls/shirts. In our institution, all in vivo and in vitro work with live virus and infected animals is done at biosecurity level 4 (BL4). The possibility of bone-stick injuries and injuries from other sharp equipment used during autopsy needs to be in the forefront of planning for such work.

The gross pathology in the lungs of affected horses is highly suggestive, but is not pathognomonic. Not all naturally and experimentally infected horses have exhibited the typical lesions of pulmonary oedema and dilation of the ventral lymphatics of the lung together with haemorrhage and froth in the trachea, bronchi and bronchioles. Histologically, syncitial giant cells in blood vessel walls particularly in the endothelium of lung capillaries and arterioles are very characteristic of the disease. These syncitial cells can also be seen in lymph nodes, spleen, brain, stomach, heart, and kidney (Hooper et al. 1997). The syncitial cells can be immunostained with specific antibody using fluorescent or immunoperoxidase staining techniques. The virus grows well in a range of cell culture systems, though Vero cells are commonly used. It induces cytopathogenic effect (cpe) of the syncitial type in about three days in Vero cells in the first passage, rarely requiring blind passaging. Electron microscopy can be used to visualise the virus by negative staining and ultrathin section of infected cell culture material, and, indeed, it can be similarly seen in tissue section from affected animals (Hyatt and Selleck 1996). The uniquie 'double fringe' of the virus can be seen in this way, and its identity further confirmed by immune electron microscopy using gold labelled probes. Polymerase chain reaction (PCR) detection and nulceotide sequence characterisation has been done using cell culture propagated virus, as well as fresh and formalin-fixed tissues from affected animals (Hooper et al. 1996). Specific antibody is detected using

ELISA or virus neutralisation tests (Murray et al. 1995; Rogers et al. 1996).

Epidemiological investigations and structured serological surveys in Queensland and other parts of the country were conducted in Australia following the detection of HeV disease horses in Brisbane and Mackay (Baldock et al. 1996; Rogers et al. 1996). Similar investigations were undertaken by the Queenslnd State Government animal health authorities following the diagnosis of the disease in a single horse at Cairns in 1999 (K.J. Dunn, pers. comm.). These studies demonstrated no spread of HeV beyond the known recorded infected premises.

A high awareness of the possibility that horses in Australia can become infected with this lethal zoonotic virus has been created among government and private veterinarians, and people who keep horses, through media and professional awareness programs. The disease is officially notifiable under legal regulations on all states of Australia and there is a legal obligation on all animals carers to report suspicions of unusual animal disease to animal disease control authorities.

Prophylaxis and Treatment

There are no vaccines to control HeV disease and no antivirals suitable for use in infected animals, even if it was financially viable to do so. The Brisbane outbreak of the disease was controlled by a stamping out program involving slaughter of all known infected horses, quarantine of premises, controls on the movement of horses within a defined disease control zone and serological surveillance to determine the extent of infection. Serological testing revealed no other known infected premises.

Likewise, serological testing was used to determine the extent of infection on the infected premises and elsewhere in Mackay and Cairns incidents. No other infected horses were identified on the premises so infection did not spread beyond the two and one affected horses on the Mackay and Cairns properties, respectively.

Hendra Virus and Bats

Six of eight susceptible bats experimentally inoculated with a dose of HeV known to infect and induce disease in horses, cats and guinea pigs developed detectable levels of specific antibody to the virus without exhibiting obvious clinical signs of disease. (Williamson et al. 1998). Only two of these had microscopic vascular lesions suggestive of HeV infection when examined 21 days after challenge. The vascular lesions observed were similar to those seen in experimentally infected guinea pigs and were able to be specifically stained using immunohistochemical techniques. No virus was isolated from samples collected at postmortem which is perhaps not surprising as they had developed specific antibody at the time of virus sampling.

Additional experimental studies also induced subclinical infection bats, including animals that were pregnant. However, the virus was isolated from the foetuses of experimentally infected pregnant bats and specific immunostaining was observed in the placenta (M.M. Williamson, pers. comm.). Taken together, these studies indicate that HeV does not induce disease in bats, at least under the conditions of the experiments, but is responsible for a subclinical infection except perhaps in pregnant animals where it might induce congenital disease following transplacental transmission. Indeed, Halpin et al. (1996) describe the isolation of HeV from uterine discharges of a bat that had miscarried twin foetuses and from three other bats.

A range of issues concerning HeV infection of bats requires further study. Little is known of the biology of the virus in bats including how it is transmitted between bats, and when this occurs, and, perhaps, most importantly how it is transmitted to its unusual host—the horse. The virus infects the four species of Pteropid bats present in Australia and it has been found across the entire geographic range of these bats. There is also serological data suggesting infection of bats in Papua New Guinea and New Britain (MacKenzie 1999) This, together with the fact that infection seems to be subclinical in bats, suggests that the virus is part of their natural microbiological experience. Why it has only apparently recently emerged is a frequently asked question for which there are diverse opinions and answers.

Australian Bat Lyssavirus

The discovery that fruit bats were the probably the natural hosts for Hendra virus prompted research to study the natural history of the virus in these bats. The Australian animal health laboratory network cooperated in this study and collected samples from bats for examination and testing for HeV. Fraser et al. (1996) noticed inclusion bodies in the neurones of a bat submitted as part of this study and conjectured that these were more likely to be associated with a lyssavirus infection than HeV.

This was in fact the case and subsequently it was found that infection with lyssavirus was widley distributed in mega- and micro-chiroptera. Genetic analysis of this lyssavirus showed that the virus was distinguishable from classical rabies virus, and other members of the lyssavirus genus, and, in fact, represented genotype 7 of the genus so it was called

Australian bat lyssavirus (ABLV) (Gould et al. 1998). It was also possible to differentiate further within the genotype and show subtle genetic differences in strains of ABLV found in mega- and microchiroptera.

A major shock occurred soon after the discovery of ABLV when a person who was a bat carer died with a 'rabies-like' disease after being bitten by an ABLV infected bat (Allworth et al. 1996). A second person also died after being bitten by another ABLV-infected bat. This caused serious concern among human public health officials and lead to recommendations about immunisation of people against ABLV for people occupationally and recreationally exposed to bats. It also lead to development of protocols for treatment, so-called post exposure therapy (PEP), of people bitten or scratched by bats. All this was possible because ABLV is more closely related antigenically to classical rabies virus (genotype 1 of lyssavirus) than to the other members of the genus, and rabies vaccine induces complete protection against challenge with ABLV.

There is still much to learn about the natural history of ABLV in bats, particularly as serum antibody to ABLV has been detected in bats with no apparent history of clinical disease. How the virus transmits between bats, and when this occurs, is not known, as is whether ABLV can infect and cause disease in other species such as dogs and cats. It is unlikely that ABLV is confined to bats in Australia or that it is the only genotype of lyssavirus naturally infecting bats in the Southeast Asian and Pacific region.

Menangle Virus

In 1997, a severe disease outbreak occurred in a breeding piggery in the Menangle district of New South Wales, Australia. The disease was characterised by decreased farrowing rates, and a diminished number of live piglets born. A high proportion of the dead piglets were mummified and stillborn and some exhibited deformities such as arthrogryposis and brachygnathia. Affected stillborn piglets frequently had severe degeneration of the brain and spinal cord, and occasionally fibrinous body cavity effusions and pulmonary hypoplasia. No disease was seen in postnatal pigs of any age (Philbey et al. 1998).

The outbreak was a real financial blow to the farm's owner. The disease outbreak was initially thought to be associated with a breakdown in the porcine parvovirus immunisation program, though this was soon demonstrated not to be the case. A novel paramyxovirus was isolated from the lungs, brain and heart of affected piglets, and serum antibody to this virus was detected in sows with affected litters and in other postnatal pigs on the property.

Specific antibody was also detected on two other farms that obtained young pigs from the affected breeding farm. No disease was detected on these properties, confirming the observations made on the index property about the lack of susceptibility to disease of postnatal pigs.

The virus was demonstrated to be unrelated to other known paramyxoviruses and it was called Menangle virus (MeV). Two piggery workers with intense occupational exposure to infected pigs were found to have specific antibody and both experienced an influenza-like disease with rash soon after the disease/infection emerged on the farms on which they worked. Serologic testing showed no alternative cause and this plus additional investigations suggested that the two men were affected by MeV. These two were the only persons in about 250 people with potential exposure to infected pigs that were found to infected or affected by MeV. The mode of transmission from pigs to humans remains unknown (Chant et al. 1998).

Subsequent studies at the Australian Animal Health Laboratory indicated that MeV was a member of the rubulavirus genus of the paramyxoviridae (M. Westenberg, D.B. Boyle and B.T. Eaton pers. comm.) and that it was capable of inducing congenital disease in susceptible pregnant sows inoculated with it at about the midpoint of gestation (H.A. Westbury unpublished data).

The gross lesions seen in the experimentally affected piglets was not as severe as that seen in the field outbreak, but microscopic lesions in the brain were qualitatively the same as those seen in naturally affected piglets, and the lesions were able to be stained with specific antibody to MeV using an immunoperoxidase technique. The lack of severity of disease expression in affected piglets may have been associated with attenuation of the virulence of the virus caused by serial passage in cell culture required for virus isolation, or to factors such as the dose of virus administered or to the route of inoculation.

The uniqueness of the disease and of MeV prompted questions about its source. Animals species (rodents, birds, cattle, sheep, cats and a dog) on or in the vicinity of the index property were tested serologically with negative results. A large colony of grey-headed fruit bats (*Pteropus poliocephalus*) as well as little red fruit bats (*Pteropus scapulatus*) roosted within 200 metres of the index property and so fruit bats were also investigated as potential sources of infection (Philbey et al. 1998).

Serum collected from fruit bats in the States of Queensland, New South Wales and Victoria as part of HeV and ABLV studies were tested for MeV antibody with positive results. Indeed specific antibody was detected in all four species of the genus Pteropus

found in Australia and specific antibody was found in bat sera collected in 1996, before the disease outbreak on the index property. Together these data pointed to fruit bats as being the source of MeV.

Thus, in the last five years, fruit bats of the Pteropus genus have been found to be the probable natural hosts of HeV, ABLV and MeV, three previously unrecognised viruses with zoonotic potential. There is a lack of knowledge concerning many aspects of these viruses, and the diseases they induce, as well as the means by which they transmit between bats, and from bats to other hosts such as horses in the case of HeV, pigs in MeV, and humans for all three.

Hendra virus and MeV are able to cause serious outbreaks of disease in farmed livestock, as has Nipah virus, a close relative of HeV. In addition, ABLV is able to cause lethal disease in humans as can HeV and Nipah virus, and the disease caused by MeV in humans is very discomforting. The disease caused by HeV in horses is very distinctive and has very characteristic gross and microscopic pathology and the virus is easily isolated.

The disease had not been recognised or described in Australia before 1994. Likewise, the disease caused by MeV in breeding sows is characteristic and had not been previously described. Similarly, the clinical signs and lesions caused by Nipah virus infection in pigs in Malaysia had not been recognised prior to the outbreak.

Why have these viruses 'emerged' in the last few years? There is no understanding of this, although speculation about poorly defined forces bringing people and their animals into closer contact with bats by various means are most frequently mentioned. What is probably indisputable, however, is the HeV, MeV and ABLV, and/or viruses closely related to them, will be found in megachiroptera, and possibly microchiroptera, in other countries in the Southeast Asian and Pacific region and that, consequently, further outbreaks of these nasty diseases could occur. Human and animal health authorities and specialists should be aware of these diseases and have means to diagnose and control them.

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CLASSICAL SWINE FEVER PATHOLOGY

Pathological Study of Experimentally Infected Chronic Swine Fever in Pigs

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Abstract

Twenty-seven 3–4 week-old pigs were divided into three groups: group 1, nine seropositive to classical swine fever virus (CSFV) pigs; group 2, nine seronegative to CSFV pigs; and group 3, control group, nine seronegative to CSFV pigs. Pigs in groups 1 and 2 were inoculated intranasally with $10^{6.5}$ $TCID_{50}$ of low virulent swine fever virus strain Kampangpetch 1/1993 but pigs in group 3 received only culture medium. Three pigs (one from each group) were sacrificed at 1, 3, 6, 9, 12, 15, 18, 21 and 24 weeks post-inoculation (p.i.). There were no differences in clinical signs and pathological findings between groups 1 and 2. CSFV antigen was detected in lymphoid organs of all experimentally-infected pigs. CSFV was successfully isolated from blood and serum samples, but not from the tissue samples. The study indicates that viral isolation from blood and serum samples can be done for confirmation of chronic swine fever.

IN LATE 1992, there were outbreaks of chronic CSF in Thailand. Clinical signs were mild with low mortality. Haemorrhagic papular dermatitis was the only gross lesion observed. Isolation of low virulent CSFV is very difficult. The objectives of this study were to investigate gross and histopathological patterns of low virulent CSFV infection and to identify suitable specimens for viral isolation.

Materials and Methods

Experimental designs

Twenty-seven 3–4 week-old weaning pigs were tested for antibody to CSFV by neutralising peroxidase linked assay (Parchariyanon et al. 1997) and divided into three groups (nine pigs each): group 1, seropositive to CSFV pigs; group 2, seronegative to CSFV pigs; and group 3 (control group), seronegative to CSFV pigs. The pigs were housed separately. Pigs in groups 1 and 2 were inoculated intranasally with $10^{6.5}$ $TCID_{50}$ of low virulent CSFV strain Kampangpetch 1/1993. Pigs in control group (group 3) received only culture medium. Clinical observation and temperature examination were performed daily. Blood and serum samples were collected weekly for haematological and virological studies. Three pigs (one from each group) were sacrificed at 1, 3, 6, 9, 12, 15, 18, 21 and 24 week p.i.

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Postmortem examination

Necropsy was performed and lesions were recorded. Organs, blood and serum samples were collected for histopathological and virological studies.

Histopathological studies

Collected samples of brain, tonsil, lymph nodes, spleen, thymus, viscera, skin and sternal bone were fixed in 10% neutral buffered formalin and embedded in paraffin wax. Thin sections (5 mm) were cut and stained with haematoxylin and eosin.

Virological studies

Collected samples of brain, tonsil, lymph nodes, spleen, thymus, liver and ileum were processed for antigen detection using indirect fluorescent antibody technique (Mengeling et al. 1963). The organs, blood and serum samples were also used for virus isolation.

Table 1. Microscopic findings of experimental pigs in group 1 and 2.*

Organs	Microscopic findings	Group 1										Group 2									
		Pig no./Weeks p.i.									Positive/Total	Pig no./Weeks p.i.									Positive/ Total
		1/1	2/3	3/6	4/9	5/12	6/15	7/18	8/21	9/24		10/1	11/3	12/6	13/9	14/12	15/15	16/18	17/21	18/24	
Brain	Perivascular cuffing	–	■	■	■		■		–		7/9	–	–	■		–	■	–		■	5/9
	Glial nodules	–	–	■	–	–	–	–	■	–	2/9	–	–	■	–	–	■	■	■	–	4/9
	Endothelial swelling	–	■		■	■	■	■	■	■	8/9	–	■	■	■	■	■	■	■		8/9
	Haemorrhages	–	–	–	■	–	■	■	■	■	5/9	–	■	■	–	–	■	■	■	–	5/9
Tonsil	Lymphoid depletion		■		■	■					9/9	■	■	■	■				■		9/9
Spleen	Lymphoid depletion	–	–	■	■	■		■	■	■	7/9	–			■	■	■	■	■	■	8/9
	Reticular hyperplasia	–	–	■		■	■	■	■	■	7/9	–	■	–	–	■	–	■	■	■	5/9
Lymph node	Lymphoid depletion	■		■	■	■	■	■		■	9/9	■		■	–	■	■			■	8/9
Peyer's patches	Lymphoid depletion	■		■	■			■	■	■	9/9	■	■	■	■	■	■	■	■		9/9
Thymus	Atrophy	–	–	–	–	–		–	–	–	1/9	–	–	–	–	–	–	–	–	–	0/9
	SSE	–	–		–		–	■			5/9	–	–				–	■		–	5/9
Bone marrow	Cellular depletion	–	–	–	●		■	■	■		6/9	–	–			■	■	■			7/9
Kidney	Glomerulonephritis	–	■	–		–	■	–	■		5/9	–	–	–	–	–	■	–	■		3/9
	Arterial degeneration	–	■	■	–	–	–	–	–	–	2/9	–	■	–	–	–	–	–	–	–	1/9
	Protein droplets	–	■	–	■	–	■	–	■	–	4/9	–	–	–	–	■	■	–	■	■	4/9
	Haemorrhages	–	–	–	■	–	–	–	–	■	2/9	–	–	–	–	■	–	–	■		3/9
Skin	Haemorrhages	–	■	■	■	■	■	■	■	■	8/9	–	■	■	■	■	■	■	■	■	8/9

* Group 3 — data not shown, p.i.= post-inoculation, SSE = Starry-sky effect
– no remarkable changes, very mild, ■ mild, moderate, ● severe

Results

Most inoculated pigs in groups 1 and 2 (eight of nine and seven of nine, respectively) had intermittent fever (39.8–40.8 °C) starting from nine days p.i.

Gross lesion observed was mild to moderate haemorrhagic papular dermatitis (about 0.5 mm in diameter) on ears and body trunks of pigs in groups 1 and 2 (8 out of 9 each) starting at 3 weeks p.i.

Microscopic lesions found in brain, lymphoid organs, kidneys, bone marrow and skin of experimentally-inoculated pigs are summarised in Table 1.

CSFV antigen was detected in lymphoid organs of all pigs in groups 1 and 2 from one week p.i. until 24 weeks p.i. Virus isolation, however, was successful from blood and serum samples, but not the tissue samples, during 3–16 weeks p.i.

Discussion

An experimental infection of chronic swine fever with low virulent strain Kampangpetch 1/1993 CSFV was reported. Observed clinical features: intermittent fever, mild skin lesions, endothelial swelling in brain and mild lymphoid depletion in lymphoid organs in agreement with studies by other investigators (Okaniwa et al. 1969; Van Oirschot 1980, 1992). In contrast, haemorrhages in kidneys, lymph nodes and urinary bladder, spleen infarction, ulcer in colon and leucopenia previously documented were not found (Mengeling et al. 1969; Okaniwa et al. 1969 and Van Oirschot 1992).

Unlike blood and serum samples from which the causative virus could be isolated successfully, the tissue samples were not suitable specimens for low virulent CSFV re-isolation. The unsuccessful re-isolation of the virus from tissue samples may be due to the much lower level of CSFV in tissues than in blood or sera.

Conclusion

The present experiment confirmed that, even in the presence of CSFV antibodies, pigs showed mild clinical signs and developed mild pathological changes when infected with low virulence CSFV. The examination of skin, brain, lymphoid organs and bone marrow for pathological lesions is very important. Samples of lymphoid organs are appropriate for the detection of the CSFV antigen, whereas blood and serum are suitable for isolation of the CSFV.

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CLASSICAL SWINE FEVER DIAGNOSIS

Diagnosis of and Emerging Diagnostic Technologies for Classical Swine Fever

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Abstract

Pestiviruses can be broadly divided into four genotypes: Classical Swine Fever (CSF), Bovine Viral Diarrhoea (BVD) I and II, and Border Disease (BD) of sheep. While there is some propensity for the different viruses to be found in their respective hosts, they are not exclusive, so any detection of a pestivirus infection in a pig will require further identification before swine fever can be unambiguously diagnosed. The clinical signs of CSF are very variable and are not pathognomonic, so laboratory diagnosis is essential.

CONVENTIONAL methodologies for the laboratory diagnosis of CSF are well documented and are provided in detail by the OIE (Anon. 1996). CSF is a list A disease and a number of OIE reference laboratories exist to provide reagents and consultancy. Initial tests include the examination of frozen sections or impression smears of tonsil, spleen, kidney and ileum for viral antigen, followed by virus isolation in a PK15 cell line or other suitable cells.

Cultures are examined for CSF virus by immunofluorescence, initially using a labelled polyclonal antibody. Further characterisation of isolates is performed using a panel of differential monoclonal antibodies. Laboratories undertaking isolation must ensure freedom of cell lines and culture media, particularly calf serum, free of adventitious pestivirus infection. In countries where the C-strain vaccine is used, its isolation must be a consideration in diagnosis and can be identified by inoculation into rabbits.

Serological tests are particularly useful in monitoring for disease in low-incidence areas and providing proof of CSF-free status. Serology is also sometimes used in tracing exercises in the event of an outbreak. The serum neutralisation test is the only serological test that can differentiate among pestiviruses. Such tests can employ fluorescent or peroxidase-conjugated antibody to visualise the virus. A number of antibody ELISAs have also been described, but current configurations cannot discriminate. The use of vaccine in a country can severely limit the value of serological tests, since no current tests can discriminate between antibodies induced by a vaccine and those induced by field infection.

Emerging Technologies

In the sphere of serology, ELISA tests are under development, designed to differentiate antibodies induced by different pestivirus genotypes. The tests will utilise recombinant proteins from the major envelope glycoprotein, E2. Along with the development of E2-based vaccines, companion tests are under development that hope to differentiate E2 vaccinal antibody from that induced by field infection, either by detecting antibody to Erns, or to E2 itself, if a deletion E2 protein vaccine is used (vanRijn et al. 1999).

A significant emerging technology for the detection of CSF is the reverse transcriptase–polymerase chain reaction (RT-PCR). This technique detects viral RNA in a number of types of sample and is very rapid and sensitive. In the past, the technique suffered from a high incidence of false positives, generated by contamination with PCR DNA. This, along with difficulties associated with scale-up, has hampered earlier introduction of this technique, but a novel one-tube method (vanRijn et al. 1999), combined with the use of a molecular probe (McGoldrick

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et al. 1999), has finally allowed this technique to enter the sphere of routine diagnosis.

In this new modification, an RT-PCR reaction is performed, followed by a second-round PCR, or nested PCR (nPCR), all within the same tube. The one-tube method involves drying the second-round reagents in a polysaccharide, trehalose, in the lid of the tube, obviating the need to open the tube. After the RT and first-round PCR, the tubes are inverted a few times and the second-round PCR is then performed. Primers which recognise all pestiviruses are used in this assay, while a fluorogenic probe confers the specificity of the reaction, providing a signal that can be read automatically using a TaqMan reader (McGoldrick et al. 1998).

The fluorogenic signal is generated by the specific action of the polymerase during successful PCR, so the amount of signal is proportional to the amount of PCR product. The presence of a signal indicates that the probe must have bound, confirming that the product of the reaction is derived from a pestivirus. Probes have been designed that allow the detection of all pestiviruses or a particular genotype, so a series of reactions can be simultaneously performed that both detect and type the pestivirus in question.

Experiments have shown the TaqMan RT-nPCR test to be up to 1000 times more sensitive than conventional virus isolation using CSF-infected blood, serum and tissues.

In the immediate future, it is anticipated that the TaqMan RT-nPCR will become widely used within Europe, where validation trials have already been undertaken and where harmonisation is now underway. With time, as it gains international recognition, it is likely to become the 'Gold Standard' for the detection of CSF and other pestiviruses worldwide.

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Considerations Regarding the Transport of Samples and Development of Diagnostic Protocols for the Detection of Classical Swine Fever Virus under Endemic Conditions

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Abstract

Many factors influence the reliability of laboratory techniques to diagnose classical swine fever virus (CSFV). These include its close relationship to other members of the pestivirus genus, such as bovine viral diarrhoea virus (BVDV) and border disease virus (BVD), and the prevailing transport infrastructure of a developing country to transport safely animal samples to the laboratory for diagnosis. Then there is the choice of the best test procedure to employ, given the problem of finding the balance between technical issues, financial considerations and the expectations of stakeholders. Against this background, two laboratory techniques for the diagnosis of CFS, the antigen trapping enzyme-linked immunosorbent assay (AT-ELIZA) and the reverse transcription polymerase chain reaction (RT-PRC), were assessed to ascertain their application to differing sets of endemic CSF circumstances.

THE SPECIFIC detection of virus or antigen is the key to outbreak diagnosis in the case of classical swine fever virus (CSFV) infection. In PR China and Lao PDR, the need for accurate CSF diagnosis in the case of suspected CSF outbreaks is essential as differential clinical diagnosis of affected animals at post-mortem between CSF as bacterial agents can make diagnosis problematic.

A problem that is apparent, especially in Lao PDR, is the minimal infrastructure for specimen collection and submission. Furthermore, high ambient temperature and a lack of specimen refrigeration facilities may result in compromised specimen quality impacting negatively in some tests. Therefore, the use of robust and reliable diagnostic technologies, given the local constraints, is essential to the diagnosis of CSF.

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In this paper, the authors discuss the relative benefits of two popular diagnostic technologies, the antigen-capture enzyme-linked immunosorbent assay (ELISA) and reverse transcription polymerase chain reaction (RT-PCR) for their suitability for diagnosis in CSF endemic situations.

Materials and Methods

Samples

In Lao PDR, samples were transported to the ACIAR project laboratory via the provincial sample submission network. Samples of spleen were collected at post-mortem and placed in a transport buffer (50% PBS pH 7.2 + 50% glycerol) in a glass or plastic tube (usually a clean, previously used, evacuated blood tube) which was subsequently placed inside a specimen transport container of locally constructed PVC plumbing fittings construction (Figure 1a, 1b). The sample was sent to the laboratory via road, post or air transport. In PR China, samples were submitted to the laboratory via a structured field network. The samples are collected post-mortem and are transported on ice to the laboratory.

Figures 1a and 1b. Collecting a spleen sample for CSFV diagnosis in Lao PDR. The PVC specimen transport container is shown on the left. (Photographs by Jim Holmes).

Diagnosis of CSF infection by the CSF antigen-trapping ELISA

The classical swine fever antigen-trapping ELISA (CSF AT-ELISA) is similar to that described by Shannon et al. (1993) with local modifications described by Blacksell et al. (1999). The assay employs three monoclonal antibodies, a pestivirus group, bovine viral diarrhoea virus (BVDV)—specific and negative, to specifically detect the presence of CSF antigen or otherwise by inference.

Sample preparation

Two grams of tissue were minced into small pieces in a 20 mL universal bottle followed by the addition of 5 mL of a solution of 1% NP-40 in PBSA. The preparation was mixed thoroughly by vortexing and allowed to stand at 25 °C for 2 hours, mixing every 10 minutes. Following incubation, the tissue preparation was centrifuged at 2000 r/min for 10 minutes and the supernatant tested undiluted. Samples not tested immediately were stored at –80 °C.

CSF AT-ELISA methodology

This method required the use of a 96 well U-bottom polypropylene microtitre plate (low protein blinding) that was used as a liquid-phase incubation plate (referred to as the LP plate) and a 96 well flat-bottom polystyrene microtitre plate (Maxisorb, Nunc, Denmark) used for the ELISA procedure (referred to as the ELISA plate). The LP plate was blocked for potential immunoglobulin binding with 5% skim milk powder (SMP) + 5% Normal Goat Sera (NGS) in carbonate buffer (blocking solution A) and incubated at 4 °C overnight. An ELISA plate was coated with goat anti-CSFV IgG at a dilution of 1 in 5000 and incubated at 4 °C overnight. Following incubation, the ELISA plate was washed 3 times with washing buffer (PBSA + 0.05% (v/v) Tween 20) with 1 minute soak between each wash. To block any potential adverse immunoglobulin binding, the ELISA plate was incubated with blocking solution A for 90 minutes at 37 °C with shaking. Following incubation, the LP plate was washed 3 times with washing buffer with a 1-minute soak between each wash. One hundred microlitres of each test sample, QC control, CSF positive and negative control samples to three appropriate wells (see Figure 2 for plate format). Add 100 µL/well of Pestivirus Group-reactive, BVDV-reactive and negative monoclonal antibodies (MAb) to the appropriate column wells (see Figure 3 for plate format). Incubate the LP plate stationary at 37 °C for 1 hour. During the incubation of the LP plate, the ELISA plate was washed 3 times with washing buffer and blocking solution added and the plate incubated for 1 hour at 37 °C. Following the completion of the respective incubations, the ELISA plate was washed by 3 washing cycles and 95 µL volumes of sample/MAb mixtures transferred from the LP plate to the ELISA plate in the appropriate format wells (see Figure 4 for plate format) and incubated for 90 minutes at 37 °C. The LP plate was discarded at the completion of this transfer step. At the completion of the incubation, the ELISA plate received a 5-cycle washing procedure and 100 µL/well of rabbit anti-mouse IgG – horseradish peroxidase conjugate diluted 1 in 1000 in PBSGT (i.e. PBSA + 1% gelatine + 0.05% Tween 20) and incubated for 60 minutes at 37 °C with shaking. At the completion of the incubation, the ELISA plate received a 5-cycle washing procedure and 100 µL/well TMB substrate was added and incubated for 10 minutes at room temperature and the reaction with 50 µL of 1M H_2SO_4 and read at 450 nm on a microplate reader.

The results were interpreted by first calculating a signal to noise ratio (S/N) for each sample thus:

$$S/N = \frac{\text{average OD450 nm with positive MAb}}{\text{average OD450 nm with negative MAb}}$$

As recommended by Shannon et al. (1993), the following interpretation was made for each sample:

S/N ratio	>2.00	Positive
	1.50–1.99	Doubtful (repeat test)
	<1.50	Negative

RT-PCR for the diagnosis of CSF infection

RNA extraction

The methodology for RNA extraction using TRIzol® reagent was essentially the same as that described by the manufacturer with some minor modifications (Christian Mittlehozer, pers. comm.). Spleen tissue from CSF-positive animals was homogenised in PBSA with 1% NP-40 to give a 20% (w/v) solution, mixed well and incubated at 25 °C for 1 hour. To clarify, the homogenate was centrifuged at 6500 r/min for 1 minute and 250 µL of the supernatant transferred to a new 1.5 mL microfuge tube. To the supernatant, 750 µL of TRIzol® reagent was added and incubated for a minimum of 5 minutes at room temperature followed by the addition of 200 µL of chloroform, vortexed for 15 seconds, incubated at room temperature for 3 minutes and centrifuged at 12000 r/min at 4 °C for 15 minutes. To precipitate the RNA, 450 µL of the aqueous phase was transferred to a new 1.5 mL microfuge tube to which 500 µL of isopropanol was added, mixed by inversion, incubated at room temperature for 15 minutes and centrifuged at 12000 r/min at 4 °C for 15 minutes. To wash the RNA

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	9	1	9	1	9						
B	2	10	2	10	2	10						
C	3	11	3	11	3	11						
D	4	12	4	12	4	12						
E	5	13	5	13	5	13						
F	6	QC	6	QC	6	QC						
G	7	+ve	7	+ve	7	+ve						
H	8	–ve	8	–ve	8	–ve						

Figure 2. Layout of samples and controls on LP plate for the classical swine fever antigen trapping ELISA.

.	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C	**Group**		**BVDV**		**Neg**		**N/U**		**N/U**		**N/U**	
D												
E												
F												
G												
H												

Figure 3. Layout of monoclonal antibodies on the LP plate for the classical swine fever antigen trapping ELISA. Group-Pestivirus group reactive. BVDV = Bovine Viral Diarrhea Virus reactive. Neg = Negative. N/U = Not Utilised.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	1	1	1	1	1	9	9	9	9	9	9
B	2	2	2	2	2	2	10	10	10	10	10	10
C	3	3	3	3	3	3	11	11	11	11	11	11
D	**Group**		**BVDV**		**Neg**		**N/U**		**N/U**		**N/U**	
E	5	5	5	5	5	5	13	13	13	13	13	13
F	6	6	6	6	6	6	QC	QC	QC	QC	QC	QC
G	7	7	7	7	7	7	+ve	+ve	+ve	+ve	+ve	+ve
H	8	8	8	8	8	8	–ve	–ve	–ve	–ve	–ve	–ve

Figure 4. Layout of sample/monoclonal antibody mixtures when transferred to the ELISA plate for the classical swine fever antigen trapping ELISA. Group-Pestivirus group reactive. BVDV = Bovine Viral Diarrhoea Virus reactive. Neg = Negative. N/U = Not Utilised.

pellet, the supernatant was carefully decanted and 1000 µL of 80% (v/v) ethanol/water was added to the resultant pellet, briefly mixed and centrifuged at 12000 r/min at 4 °C for 5 minutes. The supernatant was carefully decanted and the pellet air-dried and finally resuspended in 25 µL of DEPC-treated water and stored at –85 °C.

Description of oligonucleotide primers

Two primer sets were employed for routine CSFV diagnosis. The first primer set amplified a 288 bp region located in the 5' non-coding region (5'NCR) of the CSF genome. The primer sequences were as follows: forward primer 324, 5' –ATG CCC T/A TA GTA GGA CTA GCA – 3' (positions 108–128 bases in BVDV NADL strain), reverse primer 326, 5'– TCA ACT CCA TGT GCC ATG TAC-3' (positions 395–375 bases in BVDV NADL strain) (Vilcek et al. 1994). The second primer set was based on those described by Lowings et al. (1996) with minor modifications (P. Lowings, pers. comm.) to amplify an expected product size of 271 bp of the 5' end of the CSFV E2 gene. The primer sequences were as follows: forward primer – 5' TCR WCA ACC AAY GAG ATA GGG 3' (positions 2467–2487 in Alfort strain) and reverse primer – 5' CAC AGY CCR AAY CCR AAG TCA TC 3' (positions 2738–2716 in Alfort strain).

RT-PCR protocols

A single-step RT-PCR kit, PCR ACCESS (Promega, USA) was employed and the method as described by the manufacturer was followed. The kit allowed the RT and PCR to take place in the same reaction tube that employed a single proprietary buffer for both reactions. The RT-PCR reaction contained 4.0 µL of RNA, 1.0 µL of 10 mM dNTP, 3.0 µL of 10 µM forward primer, 3.0 µL of 10 µM reverse primer, 10.0 µLl of 5 × AMV/*Tfl* reaction buffer (composition is proprietary information), 2.0 µL of 25 mM $MgSO_4$, 26 µL of DEPC-treated water, 1.0 µL of of avian myeloblastosis virus (AMV) reverse transcriptase (5 units/µL) and 0.5 µL *Thermus flavus* (*Tfl*) polymerase (5 units/µL). The reverse transcription of RNA to cDNA took place at a temperature of 48 °C for 45 minutes followed by heating to 94 °C for 2 minutes to inactivate the AMV reverse transcriptase. The PCR immediately followed for 40 cycles of 94 °C for 30 seconds, 54 °C for 1 minute, 68 °C for 2 minutes followed by a final extension of 68 °C for 7 minutes.

To assess samples at the completion of the RT-PCR protocols, 5 µL of sample was mixed with a 5 × loading buffer (Biorad, USA) and loaded into a 2% agarose gel containing ethidium bromide (0.5 µg/mL) in 40 mM Tris-Acetate, 1 mM EDTA (TAE) buffer and subject to electrophoresis at 100 volts for 60 minutes. A 100 bp molecular size marker (Biorad Easyload) containing 10 fragments from 100–1000 bp in 100 bp increments was run in parallel on all gels. To visualise samples, the agarose gel was subjected to ultra violet transillumination.

Comparison of ELISA and RT-PCR methodologies

The RNA extraction and RT-PCR methodology was compared against the CSF AT-ELISA by assessing eight Chinese samples and 70 Lao samples. Both the E2 and 5'NCR primer sets were employed in the assessment of the RT-PCR.

Comparison of the relative sensitivity of AT-ELISA and RT-PCR methodologies to the detection of CSFV in decomposed samples

To determine the relative sensitivity of AT-ELISA and RT-PCR methodologies to the detection of CSFV in decomposed samples, a sample of experimentally-infected CSFV spleen was placed in a standard sample transport tube and subjected to ambient temperature in the shade during the month of April 1998. Samples were taken daily and processed in the usual manner for RT-PCR or AT-ELISA assessment.

Results

Sample transport

In Lao PDR, the average submission time to the laboratory was 4 days although some specimens reached the laboratory more than 7 days following dispatch. The average annual temperature in Vientiane city during 1998 was 27.4 °C (Anon. 1999) although at certain times of the year such as the hot season the temperature can be much higher. On initial examination of the samples on arrival at the laboratory, most were found to be in decomposed but acceptable condition for diagnosis. Samples that had been subjected to longer periods of transport were generally in a putrid state.

Samples submitted to the laboratory in PR China were in a generally better condition due to the refrigeration of the sample during transport.

Comparison of AT-ELISA and RT-PCR methodologies with routine diagnostic submissions

The optimised RT-PCR system of TRIzol® RNA extraction and the single-step RT-PCR with both the E2 and 5'NCR primer sets was compared against the

CSF AT-ELISA. Results are presented in Table 1 for Chinese samples and Table 2 for Lao PDR samples. From the results presented, it is apparent that the AT-ELISA was able to detect a greater number of positive samples in the Lao samples than the RT-PCR with either primer set. Overall, the 5'NCR primer set detected a greater number of CSFV positives than the E2 primer set.

Table 1. Comparison of Chinese CSF samples in RT-PCR and AT-ELISA systems

Result	RT-PCR (5'NCR)	RT-PCR (E2)	AT-ELISA
Positive	8	8	8
Negative	0	0	0

Table 2. Comparison of Lao CSF samples in RT-PCR and AT-ELISA systems

Result	RT-PCR (5'NCR)	RT-PCR (E2)	AT-ELISA
Positive	56	48	64
Negative	14	22	6

Comparison of sensitivity of ELISA and RT-PCR methodologies on decomposed samples.

The average monthly temperature during April 1998 was 29.5 °C in Vientiane Municipality (Anon. 1999). On visual inspection, at day 4 and subsequently, the spleen sample was in an obvious state of decomposition with a marked offensive odour. Results for the ELISA and RT-PCR methodologies are presented in Table 3. The AT-ELISA was able to detect antigen at least until day 9 whereas the RT-PCR was unable to detect CSFV in samples beyond day 6.

Discussion

Classical swine fever virus is a somewhat difficult virus to diagnose from a laboratory viewpoint. The CSFV is antigenically closely related to other members of the pestivirus genus, BVDV and border disease virus (BDV) and therefore must be discriminated from these related viruses before a confident result may be reported.

CSFV does not naturally lend itself to classical virological techniques such as cell culture isolation, as the agent, with a few exceptions, does not produce cytopathic effect (CPE) and the ubiquitous nature of BVDV contamination of cell lines via infected media supplements is an overriding concern.

Conventional CSF diagnostic methods in smaller laboratories have therefore relied upon the technically simpler immunofluorescence techniques employing polyclonal or monoclonal antibodies on cryostat cut sections. A more complex assay with increased sensitivity and specificity is the CSF antigen-capture ELISA initially described by Shannon et al. (1993) which has been successfully employed at regional laboratories in Thailand (Blacksell et al. 1999). The test employs a panel of monoclonal antibodies to discriminate between CSFV and BVDV antigens. The most sophisticated assay for the laboratory detection of CSFV is the polymerase chain reaction that enables the specific detection of CSFV in clinical samples (Vilcek et al. 1994). Reports of the CSFV PCR to clinical applications have concentrated mainly on primer sets with the 5'NCR (Vilcek et al. 1994).

The quality and method of sample transportation is an important factor in the overall diagnostic outcome. Samples compromised by delays in transit to the laboratory and/or high ambient temperature will decompose rapidly. Ribonucleases (Rnases) naturally present in the sample will degrade RNA rapidly making the RT-PCR less reliable or unable to generate a product.

In general, the ELISA technologies are less affected by degraded samples as only CSFV antigen is detected. Financial constraints on developing

Table 3. Comparison of RT-PCR and AT-ELISA systems when testing decomposed clinical samples. CSFV-positive spleen sample stored at ambient temperature in Lao PDR and sampled sequentially.

Assay	Days at ambient temperature								
	1	2	3	4	5	6	7	8	9
AT-ELISA (S/N)	15.2	16.8	14.2	15.4	11.2	8.8	9.2	5.3	5.0
RT-PCR (5'NCR)	+	+	+	+	+	+	–	–	–

S/N – Signal to noise ratio. Results greater than 2.0 are considered to be CSFV-positive.
5'NCR = 5' non-coding region primer set employed.

countries in general do not allow the luxury of specimen refrigeration. This factor, coupled with high ambient temperatures such as in Lao PDR, results in rapid sample decomposition. Chemical stabilisers such as using TRIzol® in the transport medium (Trevor Drew, pers. com.) is one solution to the problem but is expensive to purchase and potentially harmful to the inexperienced field operative.

In this study, we have assessed two diagnostic technologies for CSFV diagnosis. The AT-ELISA was able to reliably detect CSFV antigen in decomposed samples. The RT-PCR using the 5'NCR primer set was less reliable in amplifying CSFV genetic regions than the AT-ELISA. A nested or secondary PCR was not attempted because of the potential of cross-contamination of the sample due to limited designated PCR areas in the laboratory. A nested or secondary PCR may have provided the additional sensitivity required to make the test as sensitive as the ELISA but this must be balanced against the potential for amplifying a false positive due to a lack of PCR infrastructure.

The choice of diagnostic techniques for any infectious agent must maintain a balance between technical issues (i.e. sensitivity, specificity and methodology), financial considerations (i.e. cost per sample and overall available budget) and expectations of stakeholders (i.e. speed of testing and reporting to authorities). In a disease-free situation or regions where authorities are aiming for that achievement, technical issues and expectations of the stakeholders are of paramount importance when considering the choice of diagnostic assay. The cost of the assay may be of lesser importance given the importance placed on the 'correctness' and rapidity of the result given the potential financial losses in the case of an incorrect result. Conversely, in the case where a disease is endemic, the technical, financial and stakeholder issues take on the perspective of disease monitoring and require a different level of consideration.

Scientists have the responsibility to employ the most suitable laboratory technologies to the disease situation. There are examples of 'gold standard' assays employed at great cost to developing country institutions in terms of establishment costs, only to find later that the cost of consumables and management issues are beyond the scope of the laboratory. PCR is a case in point of a highly sensitive diagnostic technology that has important management issues especially in terms of quality control. The problem of false positives due to contamination exacerbated by lax specimen processing or a lack of basic laboratory infrastructure can have dire consequences. Furthermore, knowing the limitations of assays such as PCR is an important step in understanding the performance capabilities of a test.

While PCR may not be the most suitable assay for developing country laboratories at this point in time, it still has an important role to play in the area of molecular epidemiology. The amplification of genomic regions for future downstream applications such as sequence determination can be performed at relatively low cost and tailored to medium level throughput in a small laboratory. The important considerations of false positives due to cross-contamination of samples is still of major concern but is somewhat offset by determining sample identity following nucleotide sequencing.

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Retrospection of Research into Classical Swine Fever, National Institute of Veterinary Research, Vietnam

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Abstract

Classical swine fever (CSF) has been and still is a main theme of research in the National Institute of Veterinary Research (NIVR) of Vietnam due to its importance in the pig production of that country. Results of research conducted in the NIVR since its foundation (1968) are described. Studies of epidemiology showed a wide distribution of CSF in the country and the disease has the tendency to a chronic evolution. The presence of infection by the low virulent CSFV was demontrated by ELISA. Research into the CSF virus strain C (Chinese strain), the vaccination and the level of maternal antibodies suggested that vaccination had to be practised on piglets born to vaccinated sows when they were 35–45 days old. The maternal antibody level examined by the commercial antibody-detecting ELISA was found different from one piglet to another piglet at the same age.

THE National Institute of Veterinary Research (NIVR) of Vietnam was established in 1968. The mandate of the institution is to conduct research in the veterinary sciences in Vietnam. Its facilities are mainly located in Hanoi and a branch in Nha Trang City (centre of Vietnam).

The research themes focus on infectious and parasitic diseases in commercial animals. In addition, they are strongly oriented to the reality of the disease situation in the country.

Classical swine fever (CSF) is the most devastating disease in pig-rearing in Vietnam (Dao et al. 1985). Consequently, it has been and still is one of the main subjects for NIVR since its foundation. This paper reports the results of research into CSF conducted by the NIVR. It is worth noting that the compilation of the data to write this paper is difficult since research achievements in Vietnam in general and in veterinary sciences in particular are poorly published.

Epidemiology of CSF

The epidemiology of CSF in Vietnam was studied and reviewed by Tran and Dao (1989). In summing up CSF occurrences during 20 years (1969–89), they found that 80% of outbreaks were recorded in the period from December to March of the following year and attributed the condition to the cold climate and the active circulation of the animals and their products during that time.

On the source of the contaminant, the authors considered that CSFV existed in the location wherever there was pig-raising. This implied the ubiquity of the virus in the pig herds. Interestingly, two disease patterns were described. The first one occurred in the unvaccinated or occasionally vaccinated regions. CSF was observed in pigs of all ages. The disease was expressed in its 'pure form' and carried the endemic character. Clinical signs and lesions were identical to those that had been described widely elsewhere and the infected animals normally died.

The second pattern was characterised by secondary bacterial infections, mainly *Salmonella*, *E. coli*, *Pasteurella* and *Steptococcus*, that complicated diagnosis. This pattern was recorded in the vaccinated regions and occurred mainly in pigs of weaning age.

The infection in the sows induced reproductive failures: abortion, momification, stillborn and abnormalities. In the first quarter of 1985 in the farm of Binh Luc, 26 of 75 farrowings resulted in total loss due to CSF. The reproduction failures generalised later in the early 1990s.

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Chronic CSF and/or infection by the low virulent CSFV were indicated by field veterinarians also at the beginning of the 1990s. The disease was characterised by stunting and constipation only. This latter explains the nickname 'dry CSF' to describe this form of CSF.

Confirmation of CSFV infection by the laboratory test was reported (Nguyen et al. 1999). Other studies using the antigen-detecting ELISA (SERELISA HCV Ag Mono Indirect, Synbiotics) indicated that in the industrial pig sector, the proportion of CSFV carrier sows was as high as 20% on the studied farms. (Nguyen 1988; Nguyen and Ngo 1999). The figure could be higher than that. We have found seroconverted sows during our observation, but tested by the same antigen ELISA kit, the animals were negative (unpublished data). Low sensitivity combined with poor viraemia may explain the finding.

In addition, with the collaboration of the Australian Animal Health Laboratory (Geelong, Victoria) we have confirmed the presence of congenital CSF. This form of CSF was characterised by the birth of apparently normal piglets. However, the piglets died during the first 10 days of life.

Studies of CSFV

Virulent CSFV were isolated from diseased pigs. Two isolates were studied extensively, not the viruses themselves, but rather their pathogenicity. The two isolates were named 73A or HY (the abbreviation of Hung Yen, the name of the province where the virus was isolated) and the 73B or HT (likely, for the name of Ha Tinh province). The isolates were kept by monthly passages in the susceptible pigs. They were titrated in pigs. The 73B was found tohave a higher titre than the 73A ($10^{7.1}ID_{50}$/pig/mL of whole blood versus $10^{5.3}$ ID_{50}/pig/mL, respectively). Also, pigs inoculated with the 73B had a short (3–5 days post-inoculation) incubation time while those inoculated with the 73A had a long incubation time (6–7 days). The 73B was considered more virulent than the 73A strain and then used for virulent exposure in other studies.

The lapinised c-strain (Chinese strain) was introduced into Vietnam in 1960. This strain was also studied in the 1970s. The pathogenic stability of the strain for rabbits was confirmed after 110 passages in a total of more than 600 rabbits. The criteria used for the evaluation were incubation time, fever duration and fever intensity. On average, the fever of the inoculated rabbits began to rise 36 hours after inoculation, the duration of the fever was 23 hours and intensity was 1.5 °C higher than the rabbit body temperature before the inoculation (T.D. Nguyen, unpublished data). The avirulent nature of the virus toward the pigs was tested and confirmed as totally apathogenic for pigs of all categories, even for pregnant sows (Dao et al. 1979a). Transmission of the CSFV c-strain from sow to foetus was not found. The test used for the revelation of the condition was the inoculation into rabbits (three successive blind passages) using the spleen and the mesenteric lymph nodes of newborn piglets as the initial inoculum (Dao et al. 1979b).

The virus amount in inoculated rabbits was also studied. The spleen and the mesenteric lymph nodes contained the highest amount of the virus ($10^{4.5}$ ID_{50}/g for rabbits). Attempts to increase virus production in rabbits were not successful (T.D. Nguyen, unpublished data).

Studies of Vaccination

Maternal immunity and its influence on the immunogenic response to CSF vaccine in piglets born to vaccinated sows were investigated using 207 piglets of varying ages. The virulent exposure to the animals showed that 30-day-old piglets were 100% protected by maternal antibodies; 35-day-old piglets were 60% protected; and at 45 days old the piglets became quite susceptible. This suggested that piglets had to be vaccinated not later than at 45 days old.

Vaccinated piglets exposed to the virulent challenge developed vaccinal immunity in spite of the presence of maternal antibodies. The interesting thing was that the vaccinal immunity did not last long. Vaccination of piglets under 30 days old induced an immunity for up to two months. The 45-day-old or older piglets when vaccinated developed a solid immunity that lasted until the end of the experiment (six months post-vaccination) (Dao et al. 1990).

Based on these results, since 1980 vaccination has been recommended for piglets of 35–45 days in normal conditions and for pigs of all categories in an CSF outbreak area. Recent studies using simultaneously the antibody-detecting ELISA of AAHL (Geelong, Australia) (Shanon et al. 1993) and the ELISA kit from the Netherlands (CTB-ELISA, id-dlo, Netherlands) indicated that maternal antibodies could be detected only in piglets (446 individuals examined) under 50 days old born to vaccinated sows (98 sows). It is important to note that there was a great difference in maternal antibody level not only between litters of a same age but also between piglets of a litter. The results imply that it is difficult to get an homogenous vaccinal immunity in piglets if an active immunity to prevent the infection at early age is sought.

The anti-infection property of the vaccinal immunity was determined by exposing susceptible pigs to

the virulent challenged pigs which were previously vaccinated. The susceptible pigs became infected, having been put with the challenged pigs within 21 days post-challenge. This suggested the possibility of their infection and the excreting of virulent virus by vaccinated pigs. However, the vaccinated sows when infected by virulent CSFV did not transmit the virus to the foetuses (Dao et al. 1979b).

General Comments

Research conducted at the NIVR has responded to the problem of the existence of CSF, its distribution and its evolution in Vietnam. The research sometimes bore the character of certain diagnosis. Effectively, by clinical signs, CSF cannot be distinguished from other infectious diseases, especially infections by the low virulent CSFV. Moreover, the research by NIVR plays a guiding role in the fight against infectious diseases in the country. Research into vaccination is aimed at guiding field veterinarians in their vaccination campaigns.

Questions arising about the real situation of animal health are many. The above-mentioned results have answered some of them in a realistic fashion, and so contributed to pig industry development in Vietnam.

Differences between the above-mentioned results or conclusions and those published elsewhere may exist. The specific experimental conditions, the animals used and the experiment design could explain those differences.

However, results of the vaccination studies suggest that vaccination needs further study. The maternal antibody level is not the same in every pig, leaving some piglets unprotected either by maternal antibodies or by vaccinal antibodies. Moreover, well-vaccinated pigs could still be infected and excrete the virus, not to mention that not all pigs could be vaccinated in the vaccination campaigns. Assessment of vaccination efficacy by virulent exposure is an absolute and definite test but only valid for the exposed pigs. Furthermore, the vaccination practised in laboratories is quite different from that practised in the field. Therefore, there is an urgent need for a simple test for measuring the immunity against CSF created by the vaccination campaigns. It would help monitoring and improve the vaccination.

The fight against CSF in Vietnam might be complicated by the Bovine Viral Diarrhoea Virus. The presence of this pathogen in Vietnam remains obscure in terms of laboratory confirmation. The so-called 'immune failures' in pigs, meaning unresponsiveness to CSF vaccine, have been repeatedly reported by pig veterinary practitioners. The condition suggests an immunotolerant state that pestiviruses are well known to cause. Research in progress at NIVR is focusing on distinguishing infection in pigs from CSFV from those caused by BVDV.

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MOLECULAR EPIDEMIOLOGY OF CLASSICAL SWINE FEVER

Molecular Epidemiology of CSF: An Overview

I. Greiser-Wilke[1] and D. Paton[2]

Abstract

In combination with phylogenetic analysis, molecular epidemiology has proved to be a potent tool for tracing the origin and spread of CSF viruses after an outbreak. Exchange of data from previous studies has been greatly hampered as different genome targets for sequence determination, different phylogenetic methods for sequence analysis, and a different nomenclature for demonstrated genetic clusters were used. The aim of the project was to standardise the methodology. For this, three defined fragments of the genome (5'NTR, E2 and NS5B) were selected, and a standardised protocol for the calculation of phylogenetic trees including the nomenclature for the genetic types were established. In addition, a database accessible by World Wide Web where epidemiological and sequence data can be rapidly retrieved for comparison with the sequences of newly acquired isolates was constructed. The isolates analysed using the standardised methods showed that they could be assigned into one of three main genetic groups (1–3). In group 1, only analysis of the E2 fragment allowed us to further distinguish at least three subgroups. Most historic isolates from Europe and America, and some Asian isolates, were found in one of these subgroups. Most recent isolates from different regions in Europe and several from Asia were in group 2, where all three genetic regions analysed allowed us to distinguish three sub-groups, namely 2.1, 2.2 and 2.3. Finally, viruses mainly from Asia fell into one of the sub-groups in group 3. The viruses in three of the subgroups (3.2, 3.3 and 3.4) were from Korea, Thailand and Japan/Taiwan, respectively. A single virus isolated in the United Kingdom in the 1960s constitutes Group 3.1.

IN PAST YEARS, comparative molecular analysis has extensively been used for genetic typing of pestiviruses (Becher et al. 1997; Harasawa and Giangaspero 1998; Vilcek et al. 1999). Genetic subgroups for CSF viruses were defined (Lowings et al. 1996), and it was shown that CSF virus isolates originating from different regions e.g. in Germany (Greiser-Wilke et al. 1998), in Poland, Slovakia, Hungary or Estonia (Stadejek et al. 1997; Vilcek et al. 1997), formed relatively well-defined genetic clusters. These facts strongly suggested that in combination with epidemiological findings, genetic typing (molecular epidemiology) could become an invaluable tool for tracing the origin and spread of the virus whenever new outbreaks occur. However, comparison of results from previous studies is hampered by the fact that different regions of the genome and different statistical algorithms were used for calculating the phylogenetic trees. Therefore, it became evident that standardisation of the protocols for genetic typing and for phylogenetic analysis was necessary. In addition, to facilitate the exchange of data, a database containing the epidemiological information and the sequences from the CSF virus isolates had to be generated. The results presented here summarise those obtained in a common effort by seven European laboratories (European Commission FAIR contract PL95–0707).

Materials and Methods

CSF virus collection

Before large scale molecular epidemiology could be performed, two prerequisites had to be fulfilled. The first one was the availability of a large and representative collection of CSF virus isolates, with the corresponding epidemiological information concerning the date and geographic locality of the outbreak, and

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whether the isolate originated from domestic pigs or from wild boar.

In the Community Reference Laboratory for Classical Swine Fever in Hannover, a collection of more than 600 CSF virus strains and isolates was created, and the epidemiological data for each virus was stored in a computerised database.

Selection of target genomic regions

Although recent studies showed that almost any fragment of the virus genome can be useful for genetic typing (Becher et al. 1997; Hofmann et al. 1994; Stadejek et al. 1996; Lowings et al. 1996; Vilcek et al. 1999), only an agreement on defined regions would make new data useful for comparative studies between laboratories. Therefore, the following three genomic regions were selected (Figures in parenthesis correspond to nucleotide positions in Alfort/187* and Alfort/Tuebingen**, respectively):

- Fragments of the 5' nontranslated region (5' NTR); (190–339*; 150 bp),
- A fragment of the E2-gene (2508–2697*; 190 bp)
- A fragment of the NS5B-gene (11148–11556*; 409 bp).

The following RT-PCR primers were selected for use in future phylogenetic studies:

<u>5' NTR</u>:

Primers used for entire 5' NTR amplification of CSFV:

CSF-R411 (antisense): 5' CAC TCC CAT TGG TTT TTG TTT GT 3' (411–433*)

CSF-L001 (sense): 5' GTA TAC GAG GTT AGT TCATTC 3' (1–21*)

Sequencing primers:

obtained with primers CSF-R411/CSF-L001:

ACR408 (antisense): 5' CTC CCA TTG GTT TTT GTT TGT TTG 3' (408–431*)

ACR119 (antisense): 5' GGC TAG TCC CTC CGT TTG 3' (119–136*)

ACL072 (sense): 5' CTC CAG CGA CGG CCG AA 3' (72–88*)

Primers used for amplification of a fragment of the 5' NTR of CSFV:

CSFV-UP1 (sense): 5' CTA GCC ATG CCC WYA GTA GG 3' (84–103**)

CSFV-UP2 (antisense): 5' CAG CTT CAR YGT TGA TTG T 3' (504–486**)

Sequencing primers:

CSFV/SQ-1 (sense): 5' AGC TCC CTG GGT GGT CTA 3' (136–153**)

CSFV/SQ-2 (antisense): 5' TGT TTG CTT GTG TTG TAT A 3' (408–388**)

<u>E2 gene</u>:

Outer set

Forward 5' AGR CCA GAC TGG TGG CCN TAY GA 3' (2218–2240**)

Reverse 5' TTY ACC ACT TCT GTT CTC A 3' (2888–2870**)

Inner set (for nested PCR and for sequencing)

Forward 5' TCR WCA ACC AAY GAG ATA GGG 3' (2467–2487**)

Reverse 5' CAC AGY CCR AAY CCR AAG TCA TC 3' (2738–2716**)

<u>NS5B gene</u>:

S1 Forward 5' GAC ACT AG(T/C) GCA GGC AA(C/T) AG 3' (11128–11148**)

S2 Reverse 5' AGT GGG TTC CAG GA(A/G) TAC AT 3' (11576–11557**)

Same primers used for sequencing.

Phylogenetic methods

For phylogenetic analysis, CSF viruses were either grown in cell cultures (e.g. PK15 cells), or the RNA was extracted directly from clinical samples. After RT-PCR the products were sequenced either by cycle sequencing or by solid phase sequencing.

Calculation of bootstrapping values and phylogenetic trees

The fact that several computer programs using different algorithms were applied for phylogenetic studies further complicated comparative evaluation of results. Therefore, a standardised approach was agreed.

The sequences were first aligned using the CLUSTALW or CLUSTALX, both version 1.8, program (Thompson et al. 1994). The transition/transversion rates were calculated using the PUZZLE32, Version 4.02, program (Strimmer and von Haeseler 1996, 1997).

Bootstrapping values were calculated using the modules SEQBOOT (Random number seed: 123; 100 replicates), DNADIST (distance estimation: Maximum Likelihood; analysis of 100 data sets), NEIGHBOR (Neighbor-Joining method; outgroup: Kanagawa; Random number seed: 99; analysis of 100 data sets) and CONSENSE (outgroup: Kanagawa) of the PHYLIP (Felsenstein 1989) package. The phylogenetic trees were computed with the DNADIST and NEIGHBOR modules with the same parameters. For visualisation and printing of the trees, the TREEVIEW (Win32), Version 1.5.2, program (Page 1996) was applied. To make the trees from all genetic regions comparable, they were outgrouped to the sequence of the Kanagawa isolate. All

programs used are public domain and can be downloaded from the following World Wide Web sites:

PHYLIP package:

http://evolution.genetics.washington.edu/phylip.html

CLUSTALW and CLUSTALX (V. 1.8):

ftp://ftp.ebi.ac.uk/pub/software
(European Bioinformatics Institute, UK)

PUZZLE, Version 4.02:

http://www.zi.biologie.uni-muenchen.de/~strimmer/puzzle.html
ftp://ftp.pasteur.fr/pub/GenSoft
(Institut Pasteur, France)

TREEVIEW:

http://taxonomy.zoology.gla.ac.uk/rod/treeview.html

Sequence database

The above epidemiological data and the sequences used for genetic typing were combined in a searchable database that is accessible by WWW (Greiser-Wilke et al. 2000b). The web server in the Institute for Virology is a free server from the *Apache-Server-Project* (http://www.apache.org). The web interface was programmed in the *practical extraction and reporting language* (PERL; http://www.perl.org), a standard programming language for generating dynamic HTML code in combination with server intensive database handling. The web database and the database web interface are run on a Pentium™ based computer powered by the free operating system LINUX (http://www.linux.org). For viewing the web database, a browser (Netscape or Microsoft Internet Explorer), preferably version 4.0 or higher, is needed. The design of the pages was optimised for Netscape using a resolution of 1024 (768 pixels. The web database is localised on the home page of the Institute for Virology, and can be accessed under the address: http://viro08.tiho-hannover.de/eg/csf

Results and Discussion

Earlier studies showed that genetic typing of CSF viruses allowed them to be assigned into one of two main groups, denoted groups 1 and 2 (Lowings et al. 1996). Group 1 contained mainly historic isolates from Europe and America, whereas most recent isolates from Europe were placed in Group 2. Each of these groups had additionally been divided into a number of sub-groups, namely 1.1, 1.2, 2.1, 2.2, and 2.3. Our studies revealed that when the different genomic regions (5'NTR, E2 or NS5B) were used, similar viral relationships were observed at the group and sub-group level, although there is no clear discrimination of Groups 1.1 and 1.2 using the 5'NTR and NS5B (Björklund et al. 1999) sequences. In addition, the degree of further discrimination differed significantly between regions. The 190 nt region at the 5' end of the E2 gene and the 409 nt region of the NS5B gene both included areas of considerable genetic variability and gave good discrimination between similar CSFV isolates. The 150 nt stretch of the 5' NTR was more conserved and gave poorer resolution between closely related isolates.

The standardised protocols were used for constructing a phylogenetic tree from a data set of sequences of the E2 gene fragment. Representatives of the main European groups, historic and recent isolates from Asia and the Americas, as well as the Congenital Tremor and Kanagawa viruses, which were initially found to be extremely distinctive (Lowings et al. 1996), were included. All European, American and some Asian isolates were split into Group 1 and Group 2 viruses, but inclusion of certain Asian and American isolates made it necessary to define a new group (Group 3 with four subgroups) and a new sub-group (1.3) within Group 1.

Europe

The small number of available isolates from the period between 1920 and 1970 are all of Group 1. Most of the isolates from this period are of Subgroup 1.1, similar to Alfort 187, which is a reference strain widely used in diagnostic serology. An example of an early Subgroup 1.2 is the Brescia virus from Italy (isolated 1945). Most if not all modified live vaccines are also thought to derive from Subgroup 1.1 or 1.2 isolates made in this period. Since 1970, isolations of type 1 viruses have been very infrequent in Europe, exceptions being a Belgian isolate from 1989 (isolate Basavelde; Vanderhallen and Koenen 1997) and Ukrainian isolates from the 1990s with representatives from both Subgroups 1.1 and 1.2 (Paton et al. 2000).

Most of the CSF viruses isolated from outbreaks in Europe in the 1980s and 1990s have been of Group 2. The earliest appearance of Group 2.3 viruses was in Germany in 1982. Subsequently, these viruses have been found in many different countries, up to the present time, including Italy, Sardinia, France, Belgium, UK, Austria, Switzerland, Hungary, The Czech Republic, Poland, and The Slovak Republic. CSF viruses of Types 2.2 and 2.1 have been more restricted in their distribution. The 2.2 viruses have been found in Central Europe, in Austria, The Czech Republic, Italy, Germany, Romania and Hungary, in the period from 1985 onwards. Viruses of both the 2.2. and 2.3 subgroup have been isolated repeatedly from wild boar in different parts of Europe (Hofmann et al. 1994; Bartak and Greiser-Wilke 2000a).

The 2.1 viruses have been only sporadically reported in Europe until their involvement in a recent major epizootic in 1997 (Greiser-Wilke et al. 2000a; Widjojoatmodjo et al. 1999). They have not been isolated from European wild boar. The first European isolate was recorded in Germany in 1989, and thereafter in The Netherlands (1992), and Switzerland (1993; Hofmann and Bossy 1998). A 2.1 subgroup virus was also discovered as a contaminant of wild boar meat imported from China into Austria in 1993 (Hofmann and Bossy 1998). During 1997 and 1998, the virus is believed to have been introduced from Germany into The Netherlands, with subsequent spread to Italy, Belgium and Spain.

Asia

CSFV isolated at different times in different parts of Asia were found in all the previously defined virus groups and subgroups (Lowings et al. 1996). The historic isolate Hokkaido from 1966 from Japan is of Type 1.1, whereas the later Osaka isolate (from 1971) is the earliest available representative of Subgroup 2.3. In the 1980s, Subgroup 1.1 viruses were found in Thailand, and at the same time subgroup 1.2 viruses were found in both Thailand and Malaysia (Lowings et al. 1996; Parchariyanon et al. 2000). Subgroup 2.1 viruses were also found in Malaysia in the 1980s. A Subgroup 2.1 virus was isolated from wild boar meat imported into Austria from China (Hofmann and Bossy 1998). Subgroup 2.2 viruses have been found in Thailand in the 1990s.

In addition, isolates from Malaysia (1980s) and Thailand from the 1980s and 1990s generated a new cluster within Group 1 (Subgroup 1.3). The Asian isolates from Korea (1980s, 1990s), from Thailand (1990s), and from Japan (1870s, including Kanagawa) and Taiwan (1990s) formed a new group, displaying the Subgroups 3.2, 3.3 and 3.4, respectively. Interestingly, it was found that the originally divergent Congenital Tremor isolate from the UK (1964) formed a cluster (3.1) within this new group (Paton et al. 2000, Parchariyanon et al. 2000).

The Americas

Only Type 1 viruses have so far been reported from the Americas. Historic isolates are only available from North America and it is notable that the British and North American isolates from the 1940s and 1950s are similar Type 1.1 viruses. Type 1.1 isolates have also been identified from Brazil in the 1980s and from Mexico in the early 1990s. A virus from Honduras is rather distantly related to other Type 1 CSF viruses. Recent outbreaks of CSF in Cuba have been associated with viruses of Subgroup 1.2 (Diaz de Arce et al. 1999; Paton et al. 2000)

Conclusion

The various studies which have been performed recently clearly show that when standardised procedures are used for phylogenetic studies, genetic typing is a useful method for aiding and supporting epidemiological findings when new outbreaks occur, greatly simplifying the tracing of the spread of the disease to other geographic locations. The nomenclature of Lowings et al. (1996) has proved to be adequate, but requires revision to accommodate new groups and subgroups. While in Europe viruses of Sub-groups 1.1, 1.2, 2.1, 2.2 and 2.3 have been defined, the additional Asian Group 3 with four subgroups, and Subgroup 1.3 will have to be added (Paton et al. 2000).

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The Family Flaviviridae: A Brief Overview of the Family with Particular Reference to Members from the Asian and Australasian Regions

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THE FAMILY Flaviviridae comprises three genera; the Flavivirus genus, the Pestivirus genus, and the Hepacivirus genus. The viruses are 40–60 nm in diameter, spherical in shape, and contain a lipid envelope. All members of the family have a genome composed of a single molecule of linear positive-sense RNA, ranging in size from about 9.6 kb for hepaciviruses, to 11 kb for flaviviruses, and 12.3 kb for pestiviruses (Heinz et al. 1999). The only viral messenger RNA is the genome, and a single open reading frame (ORF) codes for a polyprotein that is proteolytically cleaved into all the virus-coded proteins. The structural proteins are located at the 5'-end of the genome, and the non-structural proteins at the 3'-end. The non-structural proteins include proteases, helicases, and polymerases. Virus multiplication occurs in the cytoplasm in association with membranes, and the progeny viruses mature in cytoplasmic vesicles.

Members of each genus are serologically related to each other, but not to members of the other genera. The most recent classification of the family is provided in the Seventh Report of the International Committee on Taxonomy of Viruses (Heinz et al. 1999), and this is used extensively below.

The Flavivirus Genus

The Flavivirus genus comprises more than 70 members. The type species is yellow fever virus from which the genus (and family) name is derived (flavus, Latin for 'yellow'). The virions are 40–50 nm in diameter, and the virion envelope contains a dense layer of surface projections about 6 nm in length. The genome length varies between 10 976 nt (Japanese encephalitis) and 10 488 nt (tick-borne encephalitis virus). The gene order is 5'-C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3'. Details of the structure and replication of flaviviruses have been reviewed by Rice (1996) and Monath and Heinz (1996).

Most flaviviruses are arboviruses, transmitted by arthropod vectors, usually either mosquitoes or ticks. Viruses replicate in susceptible species of vertebrates and arthropods, usually as alternate hosts. A few viruses have no known vector species. There are approximately 39 mosquito-borne members of the genus, 13 tick-borne members, and 17 with no known vector. The major criteria for determining the groupings of species and members within the genus are nucleotide and deduced amino acid sequence data, antigenic relationships, vector association and geographic incidence. Thus the mosquito-borne viruses have been grouped into seven groups: the Aroa virus group with four members, the Japanese encephalitis virus group with 10 members, the Ntaya virus group with six members, the Kokobera virus group with two members, the Dengue virus group with five members, the Spondweni virus group with two members, and the yellow fever virus group with 10 members (Heinz et al. 1999). Some members are defined as subspecies (e.g. Kunjin virus as a subspecies of West Nile virus, and Alfuy virus as a subspecies of Murray Valley encephalitis virus), although the interpretation of the criteria for assigning a species level, and the definition of what constitutes a subspecies, may be controversial. Of the tick-borne viruses, two virus groups have been recognised: the Mammalian tick-borne virus group and the Seabird tick-borne virus group. Perhaps a more useful indication of relationships between flaviviruses can be obtained from phylogenetic studies which tend to agree with antigenic and vector-host relationships (e.g. Kuno et al. 1998; Poidinger et al. 1996; de Zanotto et al. 1996). Two additional viruses are listed as tentative species in the genus: Tamana bat virus and Cell fusing agent virus. The latter has often been used as an out-group in phylogenetic studies.

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The most important mosquito-borne viruses are yellow fever virus which is found in west, central and east Africa and tropical South America, Japanese encephalitis (JE) which is found in east, southeast and southern Asia, and dengue viruses which are found throughout much of the tropical and subtropical regions of the world. In addition, West Nile virus has been of increasing importance with recent outbreaks in Algeria (1995), Romania (1996) and, most recently, in the New World in New York and Connecticut (1999).

The Pestivirus Genus

The Pestivirus genus contains four members (Thiel et al. 1996); Classical swine fever virus (or hog cholera virus), border disease virus (Nettleton et al. 1998), bovine viral diarrhoea virus 1 and bovine viral diarrhoea virus 2 (Baker 1995; Bielefeldt-Ohmann 1995). The type species is bovine viral diarrhoea virus 1. The virions are 40–60 nm in diameter, and the virion envelope has 10 to 12 nm ring-like subunits on its surface. Compared to the other genera, pestiviruses encode two unique gene products: a non-structural protein, N^{pro}, as the first protein of the open reading frame (ORF) which possesses an autoproteolytic activity responsible for its release from the nascent polyprotein; and a viral glycoprotein, E^{rns}, which possesses intrinsic RNase activity. The virions are composed of four structural proteins: a basic nucleocapsid protein, C, and three envelope glycoproteins, E^{rns}, E1 and E2. The genome is approximately 12.3 kb in size and consists of a single large ORF encoding a polyprotein of about 4000 amino acids. The gene order is 5'-N^{pro}-C-E^{rns}-E1- E2- p7- NS2- 3(NS2- NS3)- NS4A-NS4B-NS5A-NS5B-3'. The inclusion of NS2-NS3 in parentheses is because in noncytopathogenic bovine viral diarrhoea viruses NS2-3 is not cleaved, whereas in cytopathogenic viruses, NS3 is produced in addition to NS2-3. Molecular aspects of pestiviruses, including replication, are described in detail elsewhere (Thiel et al. 1996; Meyers and Thiel 1996; Donis 1995; Harasawa and Giangaspero 1998; Van Rijn et al. 1997).

Pestiviruses infect pigs and wild and domestic ruminants. Transmission occurs by direct or indirect contact through nasal secretions, urine and contaminated food. Congenital transmission occurs readily. Transmission may cross species between ruminants and between ruminants and pigs. The pathogenesis is complex, and infections can lead to a wide spectrum of clinical manifestations (Bielefeldt-Ohmann 1995; Thiel et al. 1996; Tautz et al. 1998). The pathogenesis and clinical aspects of classical swine fever are dealt with elsewhere in these Proceedings (Depner, these Proceedings).

Pestiviruses have a worldwide distribution. Thus, border disease virus is found in most countries where sheep production occurs (Loken 1995), bovine viral diarrhoea virus appears to be widespread in all cattle-raising countries (Houe 1995), and classical swine fever is found in South and Central America, Africa, much of Asia, and parts of Europe. The European Union is generally free of classical swine fever in commercial piggeries, but re-infections can occur from wild pigs. Molecular epidemiological and phylogenetic studies with pestiviruses have clearly defined their genetic relationships, demonstrating the occurrence of different genotypes, and have provided the tools to determine the source and spread of epidemic activity (e.g. Lowings et al. 1996; Becher et al. 1997; Becher et al. 1999; Greiser-Wilke and Paton, these Proceedings).

The Hepacivirus Genus

The Hepacivirus genus contains a single species, *Hepatitis* C virus, which occurs in six genotypic clusters (genotypes 1–6), each differing from the other by 25–35% nucleotide divergence (Heinz et al. 1999). The virions are 50 nm in diameter, and have a spherical core about 30 nm in diameter. The genome is about 9.6 kb in length and contains a single large ORF encoding a polyprotein of about 3000 amino acids. The gene order is 5'-C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-3'. Hepaciviruses differ from the other two genera in that they are unable to replicate efficiently in cell culture, and although some replication has been reported in cell lines derived from hepatocytes and lymphocytes, it has not been sufficient for further studies.

Human beings are the only natural host of hepatitis C virus. Transmission is almost exclusively by parenteral exposure to contaminated blood and blood products. There is no evidence to implicate an invertebrate vector. Hepatitis C has a worldwide distribution. Overall, it has been estimated that about 3% of the world population has been infected with hepatitis C, resulting in the chronic infection of about 170 million individuals (Heinz et al. 1999). Sero-epidemiological studies suggest that 0.1–2% of the populations of developed countries may be infected with hepatitis C, but an antibody prevalence of up to 20% has been reported from developing countries.

Extensive molecular epidemiological and phylogenetic studies have been carried out with hepatitis C virus isolates, and at least 6 genotypes have been recognised, each differing by 25–35% at the nucleotide level. The 6 genotypes can be further

divided into over 100 subtypes which differ by 15–25% at the nucleotide level, although there is a considerable overlap in the degree of heterogeneity (Heinz et al. 1999). Some significant differences in geographic distribution of genotypes and subtypes have been recognised.

Three distinct groups of viruses have also been tentatively assigned to the family Flaviviridae and probably to the genus Hepacivirus, on the basis of their genomic organisation and genetic similarity to recognised members of the family. These are GB virus A and GBV-A-like viruses, which have been reported from at least six species of New World monkeys; GB virus B, which was recovered from a tamarin monkey but with a passage history which suggests that it may have been of human origin; and GB virus C (Hepatitis G) which is a virus of human origin. All three are transmitted via blood and blood products. GB virus A does not appear to cause hepatitis in its host of origin or in other susceptible hosts; GB virus B causes hepatitis in several species of New World monkeys; and the pathogenicity and site of replication of GB virus C remain controversial. Recent work reported from Taiwan indicates that GB virus C/hepatitis G may not be associated with clinical disease, and that liver and blood mononuclear cells are not the major sites for virus replication (Kao and Chen 1999). All three viruses most closely resemble Hepatitis C in genomic organisation, although GB virus A appears to lack a complete capsid protein gene.

Members of the Flaviviridae in Eastern, Southeastern and Southern Asia and Australasia

A discussion of the incidence and geographic distribution of members of the family Flaviviridae in the Asian-Pacific region is, if only by weight of numbers and differing ecology, largely one of members of the genus Flavivirus. Members of the other two genera, while widespread in the region, do not differ markedly to incidences in the regions of the globe, except in specific genotypes or because of specific control measures. An additional reason for placing emphasis on the Flavivirus genus is the problem of serological diagnosis in the Asian and Australasian regions. Seroepidemiological studies for evidence of past flavivirus infection are extremely difficult to interpret where there has been more than one sequential infection, and indeed in many areas where people or animals have been exposed to a number of different flaviviruses over time, it is usually impossible to distinguish the different viruses. These problems are not confined to exposure to different members of the same serological group, such as the JE serological group, but represent a much wider problem encompassing all flaviviruses. Thus it is also important to be aware of other members of the genus which are known to occur in the Asian and Australasian regions.

Flaviviruses

The most important flaviviruses found in eastern, southeastern and southern Asia and Australasia are JE virus and the dengue viruses. JE virus is a member of the JE serological complex of flaviviruses which comprises 10 antigenically related members; JE virus itself, Murray Valley encephalitis (MVE), Kunjin (KUN), West Nile (WN), Alfuy (ALF), St Louis encephalitis (SLE), Koutango (KOU), Usutu (USU), Cacipacore (CPC) and Yaounde (YAO) viruses (Monath and Heinz 1996; Heinz et al. 1999). JE virus, which is responsible for approximately 45 000 cases and more than 10 000 deaths annually, is found in eastern and southern Asia (Umenai et al. 1985; Burke and Leake 1988; Vaughn and Hoke 1992), with geographic distribution from Japan, Korea and maritime Siberia in the north, through China, to the Philippines in the east, and through southeastern and southern Asia to Indonesia, India and Sri Lanka. In recent years, JE has spread westwards into Pakistan in 1994 (Igarashi et al. 1994), into Kerala State in southwestern India in 1996 (Dhanda et al. 1997), and eastwards into the Australasian region in 1995 and 1998 (Hanna et al. 1996; Ritchie et al. 1997; Johansen et al. 1998; Hanna et al. 1999; Mackenzie 1999). Much of the spread in Asia is believed to be due to deforestation for increased agriculture, and changes in land use with the huge increase in rice fields and irrigated agriculture. The major vertebrate hosts for JE virus are pigs and ardeid birds, with pigs playing a central role in virus amplification. The disease in pigs is relatively minor; transplacental transmission of JE virus is well established, leading to foetal encephalitis, abortion and stillbirth (e.g. Takashima et al. 1988). JE virus also causes hypospermia and aspermia in boars (Habu et al. 1977). JE virus does not cause disease in birds. A number of mosquito species have been shown to be competent for transmitting JE, the most important species being *Culex tritaeniorhynchus*, *Cx gelidus*, and *Cx vishnui*.

MVE, KUN, and ALF viruses are Australasian members of the JE serological complex. MVE virus is the cause of Australian encephalitis and is the most important Australasian member of the complex; KUN virus can also cause encephalitis, Kunjin encephalitis, but although the disease is generally milder than that caused by MVE virus, it can also

cause a febrile illness, sometimes with polyarthralgia and polyarthritis; ALF virus has not been reliably associated with human disease at this time (reviewed by Mackenzie et al. 1994a, b). There is evidence from isolations, human infections, and serology that MVE virus is found in Papua New Guinea (PNG) and probably elsewhere in the eastern Indonesian archipelago. KUN virus is also in PNG and parts of the Indonesian archipelago. Until the 1995 JE virus outbreak in the Torres Strait, and without good scientific reasons, the south-eastern limit of JE virus was believed to be restricted to the north and west of the Wallace Line, an imaginary line running between Bali and Lombok and separating the Oriental and Australasian zoogeographic regions, whereas MVE virus was believed to be restricted to the south and east of the line (Marshall 1988; Mackenzie et al. 1997).

Of the other members of the serological complex, WN and SLE viruses are major human pathogens. WN virus is found principally in Africa, Middle East, western Asia, and southern Europe. It spread to the New World in September 1999, causing a large epizootic in crows and other avian species, and a number of human infections. Major human epidemics have been reported in recent years in Algeria (1995) and Romania (1996), and an epidemic was reported in horses in Italy (1998). It is important to this discussion because it shares a close antigenic and genetic relationship with KUN virus, and because its geographic range overlaps with that of JE virus in India and Pakistan. It is also interesting to note that both WN and KUN viruses have been isolated in Sarawak–Borneo, the former from a wild bird (Karabatsos 1985) and the latter from *Cx. pseudovishnui* mosquitoes (Bowen et al. 1970), and it thus must occur occasionally in southeastern Asia. SLE virus is found only in parts of North, Central and South America, causing major outbreaks of human disease every five to 15 years in North America, and endemic transmission with sporadic cases in intervening years (Monath and Heinz 1996). Finally, three members of the serological complex are found only in Africa, KOU, USU and YAO viruses, but their involvement in human disease is a little uncertain although KOU virus has been isolated from patients with fever, headache, joint pains and rash, and with USU virus from a patient presenting with fever and rash (Annual Reports, Centre Collaborateur O M S de Reference et de Recherche pour les Arbovirus et les Virus de Fievres Hemorragiques, Institut Pasteur, Sengal). One virus, CPC virus, has been isolated in Brazil but has not been associated with human disease (Figueiredo et al. 1998).

The four dengue viruses, types 1–4, form a separate, closely related serological group. They are responsible for two distinct disease entities, a primary infection leading to dengue fever, and a second disease known as dengue haemorrhagic fever (DHF) which, in most instances, is the result of a subsequent infection with another dengue type. Dengue has become a major international public health concern; it is found predominately in urban and peri-urban areas of tropical and subtropical regions. DHF, a potentially lethal complication, was first recognised during the 1950s and is today a leading cause of childhood mortality in several Asian countries. Recovery from infection by one serological type provides lifelong immunity against that type but confers only partial and transient protection against subsequent infection by the other three. Indeed, there is good evidence that sequential infection increases the risk of more serious disease resulting in DHF. The global prevalence of dengue has grown dramatically in recent decades. The disease is now endemic in more than 100 countries in Africa, the Americas, the Eastern Mediterranean, Southeast Asia and the Western Pacific. Southeast Asia and the Western Pacific are most seriously affected. Before 1970, only nine countries had experienced DHF epidemics, a number which had increased more than fourfold by 1995. Some 2500 million people—two-fifths of the world's population—are now at risk from dengue. WHO currently estimates there may be 50 million cases of dengue infection worldwide every year. The epidemiological features of dengue and DHF are described in detail by Halstead (1997).

Four other mosquito-borne viruses have been found in Southeastern Asia: Tembusu, Zika, Wesselsbron and Jugra viruses. Tembusu virus was first described in 1957 from Kuala Lumpur, Malaysia, where it had been isolated from *Culex triteaniorhynchus*, *Cx gelidus*, *Aedes linneatopennis*, and *Anopheles philippinensis* (cited in Karabatsos 1985). It was subsequently isolated in Thailand from *Cx gelidus*, *Cx tritaeniorhynchus*, *Cx vishnui*, and *Cx sitiens* (P.K. Russell, pers. comm. in Karabatsos 1985; Leake et al. 1986) and in Sarawak from *Cx. gelidus*, *Cx. pseudovishnui*, and *Cx. tritaeniorhynchus* (Simpson et al. 1970; Platt et al. 1975). Tembusu virus has not been associated with human disease, but neutralising antibody to the virus has been reported in human sera from Kuala Lumpur indicating the occurrence of asymptomatic infections (Karabatsos 1985) and in Lombok, Indonesia (Olson et al. 1983). Zika virus is a relatively common virus throughout most of Africa, and has been associated with human disease consisting of fever with headache and rash (Karabatsos 1985). In Asia, Zika virus has been isolated from *Aedes aegypti* mosquitoes in Malaysia (Marchette et al. 1969), and implicated as a

cause of fever in patients in Central Java (Olson et al. 1981). Neutralising antibodies were found in human sera collected in Lombok (Olson et al. 1983). In addition, neutralising antibodies have been reported in human sera from India, Malaysia, Indonesia (Kalimantan), Philippines, Vietnam and Thailand (Karabatsos 1985). Wesselsbron virus, an important viral disease of livestock in Africa causing abortion in sheep and cattle, has been isolated in Thailand (T. Yuill and P.K. Russell, pers. comm. to Olson et al. 1981). Jugra virus was isolated in Malaysia from *Aedes* spp. and *Uranotaenaia* spp. of mosquitoes, and from the blood of the fruit bat, *Cynopterus brachyotis*.

In Australasia, the other mosquito-borne flaviviruses are Edge Hill and Sepik viruses, which have recently been reclassified into the yellow fever virus group, and Kokobera and Stratford viruses, which have been reclassified from the JE serological complex into a small group of their own. Edge Hill virus has been isolated from a range of mosquitoes trapped in north Queensland, New South Wales, and Western Australia, including *Cx. annulirostris*, *Ae. vigilax*, *Ae. normanensis*, *Ae. bancroftianus*, *An. amictus*, and *Coquillettidia linealis* (reviewed in Mackenzie et al. 1994a; Russell 1995). The major vertebrate hosts are believed to be marsupials. A seroepidemiological study in New South Wales has suggested that occasional human infections occur (Hawkes et al. 1985), and there has been one unconfirmed report implicating Edge Hill virus as the possible aetiological agent in a patient presenting with arthralgia, myalgia, and fatigue (Aaskov et al. 1993). Sepik virus was isolated from *Mansonia septempunctata*, *Ficalbia flavens*, *Ficalbia* spp., and *Armigeres* spp. trapped in the Sepik district of PNG (Woodroofe and Marshall 1971; Karabatsos 1985). High neutralising antibody titres in the convalescent serum of a New Guinea patient hospitalised with a febrile disease makes it a suspected human pathogen (Woodroofe and Marshall 1971). In addition, from its close antigenic relationship to Wesselsbron virus and from seroepidemiological studies on Lombok, there is a suggestion that it might infect livestock. Kokobera and Stratford viruses are relatively widespread in Australia, and Kokobera virus has also been isolated from PNG. Kokobera virus has been shown to occasionally cause polyarticular disease, but STR virus has only been associated with subclinical human infections on serological grounds (reviewed by Mackenzie et al. 1994a).

Tick-borne encephalitis viruses are a major problem in some parts of Asia, particularly central and northern Asia, and may be a potential problem in other areas. These include members of the mammalian tick-borne virus group, such as Russian spring-summer encephalitis virus, Kyasanur Forest virus, Omsk haemorrhagic fever virus and Negishi virus. Other tick-borne flaviviuses are less well characterised, such as Langat virus, although still classified in the mammalian group. Tick-borne encephalitis is widespread in China and in eastern Siberia. In India, Kyasanur Forest disease virus is the major tick-borne virus, but is restricted to Karnataka State. It is a prostrating human febrile disease with haemorrhagic and encephalitic manifestations and a case fatality rate of between 5% and 10% (Rodrigues 1988). Negishi virus was first isolated from the CSF of a fatal case of encephalitis in Tokyo in 1948. There was a second fatal case from Tokyo shortly after, and human cases have been recognised in China (Monath and Heinz 1996). Negishi virus is most closely related to Louping ill virus (Venugopal et al. 1992). There have also been recent examples of tick-borne viruses spreading in or from the Asian region. Thus a case of tick-borne encephalitis was observed in Hokkaido, Japan, in 1993, and virus was isolated from the blood of sentinel dogs. A serological survey indicated that the virus was prevalent in the area (Takashima et al. 1997). Subsequently, virus was also isolated from *Ixodes ovatus* ticks collected from the same area (Takeda et al. 1998). More recently, 10 human cases of infection with a flavivirus related to Kyasanur Forest disease were reported from Saudi Arabia, two of whom died (Zaki 1997). Eight of the 10 patients were working with sheep, which have been the vertebrate host and source of the infected ticks. There has been a report of the isolation of Langat virus from *Haemaphysalis papuana* Thorell in Thailand, but the relevance of this to human or animal disease in Southeast Asia remains to be determined (Bancroft et al. 1976). Two Australian tick-borne flaviviruses have been isolated from ticks removed from seabirds or obtained from nests, Samaurez Reef and Gadgets Gully viruses. The former is a member of the seabird tick-borne virus group, and the latter is a member of the mammalian group. Neither virus has been associated with disease in humans or animals.

Finally, there are at least three flaviviruses reported from Eastern and South-Eastern Asia with no known vector (Heinz et al. 1999). These include Apoi virus from Japan, Carey Island virus from Malaysia, and Phnom Penh bat virus from Malaysia and Cambodia (Karabatsos 1985). Apoi virus is a rodent virus and a member of the Medoc virus group and has only been found on the island of Hokkaido, Japan, but has been associated with human disease causing CNS symptoms and leg paralysis. Carey Island virus has been isolated from two species of fruit bat, *Macroglossus lagochilus* and *Cynopterus brachyotis*, in Peninsula Malaysia, but has not been

associated with disease. Phnom Penh bat virus has been isolated from unspecified bats from Cambodia, and from *Cynopterus brachyotis* and *Eonycteris spelaea* bats in Malaysia. It has also not been associated with human disease (Karabatsos 1985). Both Carey Island and Phnom Penh bat viruses are species in the Rio Bravo virus group (Heinz et al. 1999).

Pestiviruses

The distribution of bovine viral diarrhoea is widespread throughout the Asian and Australasian regions in cattle and buffalo. In Australia, isolation from cattle is common in most parts of the country, but very rare from sheep (P.D. Kirkland, pers. comm.). The distribution of border disease virus, however, is less well defined, although it has been reported from sheep in Australia, New Zealand, and India. In Australia, it is found relatively infrequently (P.D. Kirkland, pers. comm.). Classical swine fever virus is widely distributed throughout Asia, as indicated from the country presentations in these Proceedings, but Australia and New Zealand have been kept free through a policy of quarantine and, when necessary, slaughter of infected animals in outbreak situations. The virus has only recently been introduced to Indonesia, but is not present in PNG (P. Daniels, pers. comm.). The genotypes of classical swine fever virus and their occurrence in Asia are described by Greiser-Wilke and Paton, in this volume, and by Paton et al. (2000). All of the major genotypes or subtypes have been identified in different parts of Asia at different times.

Hepaciviruses

Hepatitis C virus is widely distributed throughout the Asian and Australasian regions, as indeed it is globally. The frequency of infections and the geographic distribution of the different genotypes and subtypes vary considerably between different regional counties in the eastern and southeastern Asian region, and also between population or patient groups within countries (e.g. Liu et al. 1992; Dusheiko et al. 1994; McOmish et al. 1994; Tokita et al. 1994; Sugiyama et al. 1995; Tokita et al. 1995; Prescott et al. 1996; Tokita et al. 1996; Greene et al. 1995; Mellor et al. 1995; Simmonds et al. 1996; Hotta et al. 1997; Kanistanon et al. 1997). Thus subtype 1b, which has a relatively high prevalence in Europe, also predominates in much of eastern and southeastern Asia, whereas subtype 3a, which is a major subtype in UK and Germany, is only found commonly in Thailand, and nowhere else in Asia. Similarly, subtype 1a, which is a major subtype in the United States, is commonly found in the Philippines, but much less frequently in other parts of Asia. Two unique subtypes, 1d and 3g, have been observed in Indonesia, and genotype 6 group variants have been described in Thailand, Vietnam and Hong Kong. Genotypes 4 and 5 are found only very rarely in Southeastern Asia.

Studies of the prevalence of GB virus C/hepatitis G in countries in southeastern Asia have found it to be low in the normal population in the Philippines, Sri Lanka (Ross et al. 1998a),Vietnam (Kakumu et al. 1998), Lao People's Democratic Republic (Bounlu et al. 1998), Thailand (Katayama et al. 1997), Bhutan, and Malaysia (Ross et al. 1998b). However, a significantly higher incidence has been found in high risk groups, such as intravenous drug users, patients on haemodialysis, recipients of kidney transplants, and patients with chronic liver disease. In addition, infection is often associated with concurrent hepatitis B or C virus infections, although co-infection did not appear to aggravate disease (Katayama et al. 1997; Poovorawan et al. 1997; Raengsakulrach et al. 1997; Kakumu et al. 1998). Genetic studies have demonstrated two Asian genetic variants, group 3 and group 4 (e.g. Kakumu et al. 1998; Wong et al. 1999; Naito et al. 1999).

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Genetic Characterisation of Classical Swine Fever Viruses from Lao PDR Collected during 1997 and 1998

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Abstract

Classical swine fever (CSF), a severe and economically important disease of pigs in Asia and Europe, is an important constraint to the future development of pig production in Lao PDR. Few Asian isolates of CFS have been included in the overall analysis of CSFV molecular phylogenetics. This paper analyses a number of isolates collected in Lao PDR during 1997 and 1998. Results are presented and discussed.

THE LAO People's Democratic Republic (Lao PDR) is a landlocked country bordered by Thailand, Vietnam, Myanmar, PR China and Cambodia. Lao PDR has a population of approximately four million people of which 64% of families are involved in pig-raising activities. Pork comprises approximately 25% of meat consumption in Lao PDR.

Classical swine fever virus (CSFV), genus: Pestivirus, family: Flavivirus, is the aetiological agent of classical swine fever (CSF), a severe and economically important disease of pigs in Asia and Europe. In Lao PDR, CSF is reported to be an important constraint to the future development of pig production. Little information is known of the epidemiology of CSF in Lao PDR or characteristics of local virus strains. To date, there have been numerous reports of antigenic and genetic characterisation of CSFV primarily from European and historical American isolates with few Asian isolates included in the overall analysis of CSFV molecular phylogenetics.

This paper analyses a 190 base pair (bp) region of the E2 gene of a number of isolates collected in Lao PDR during 1997 and 1998. These nucleotide sequences were further compared with previously published nucleotide sequences of European and Asian isolates to determine their genotype designation.

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Materials and Methods

Samples were submitted for routine CSFV diagnosis to the ACIAR project laboratory in Vientiane by district and provincial veterinary officers. Geographical and additional sample collection information is presented in Table 1 and Figure 1. Samples were assessed by CSF antigen-capture ELISA (Shannon et al. 1993) with local modifications, and CSFV-positive samples were stored at minus 80 °C awaiting further processing.

RNA extraction

Two different RNA extraction procedures were employed: (a) phenol-chloroform, and (b) TRIzol® (Life Technologies). The phenol-chloroform RNA extraction method employed was based on that described by Vilcek et al. (1994) with local modifications.

The methodology for RNA extraction using TRIzol® reagent was essentially the same as that described by the manufacturer with some minor modifications (Christian Mittlehozer, pers. comm.). Briefly, spleen tissue was homogenised in a 1% NP-40/PBSA solution to give a final 20% (w/v) solution and incubated at 25 °C for one hour. The homogenate was clarified, transferred to a new microfuge tube and 750 µL of TRIzol® reagent added and incubated at room temperature followed by the addition of 200 µL of chloroform and centrifuged. The RNA was precipitated by the addition of 500 µL of isopropanol to 450 µL of the aqueous phase

Table 1. Virus isolation and genotype designation information for the CSF viruses compared in this study.

Isolate	Month	Year	Province	Country	Genotype
CSF0307	NA	1987	NA	Brazil	1.1
CSF0306	NA	1986	NA	Malaysia	1.2
CSF0905	NA	1945	Brescia	Italy	1.1
CSF0173	NA	NA	NA	Slovakia	1.1
CSF0305	NA	1986	NA	Malaysia	2.1
CSF0014	NA	1989	NA	Germany	2.2
CSF0062	NA	1990	NA	Germany	2.2
CSF0074	NA	1990	NA	Austria	2.2
CSF0906	NA	1977	NA	Netherlands	2.2
CSF0007	NA	1984	NA	Germany	2.3
CSF0008	NA	1984	NA	Germany	2.3
CSF0084	NA	1992	NA	Germany	2.3
CSF0106	NA	1994	NA	Germany	2.3
CSF0130	NA	1993	NA	Switzerland	2.3
CSF0171	NA	1996	NA	Germany	2.3
CSF0309	NA	1974	Kanagawa	Japan	5
L12	December	1997	Champassak	Lao PDR	2.2
L61	February	1998	Khammouane	Lao PDR	2.2
L65	March	1998	Champassak	Lao PDR	2.2
L71	March	1998	Champassak	Lao PDR	2.2
L119	May	1998	Phongsaly	Lao PDR	2.1
L123	May	1998	Vientiane Municipality	Lao PDR	2.1
L168	July	1998	Bokeo	Lao PDR	2.1
L175	July	1998	Savanakhet	Lao PDR	2.2
L202	August	1998	Khammouane	Lao PDR	2.2

incubated at room temperature for 15 minutes and centrifuged at 12 000 r/min at 4 °C for 15 minutes. The RNA pellet was washed 80% (v/v) ethanol/water and resuspended in 25 μL of DEPC-treated water and stored at minus 85 °C until required.

Description of oligonucleotide primers

Primers were based on those described by Lowings et al. (1996) with minor modifications (P. Lowings, pers. comm.) to amplify an expected product size of 271 bp of the 5' end of the CSFV E2 gene. The primer sequences were:

forward primer — 5' TCR WCA ACC AAY GAG ATA GGG 3'

reverse primer — 5' CAC AGY CCR AAY CCR AAG TCA TC 3'.

RT-PCR protocols

Complimentary DNA (cDNA) was prepared by mixing the sample RNA and forward primer, incubated at 100 °C for 60 seconds then immediately placed at –20 °C for 5 minutes. During the incubation, the following cDNA synthesis mixture containing 8 units of RNasin (Promega), dNTPs (Promega), 4 μL of 5 × reverse transcription reaction buffer (Promega) and AMV reverse transcriptase was prepared and added to RNA/primer preparation. Water was added to a final volume of 20 μL and immediately placed at 42 °C for 1 hour for reverse transcription followed by 75 °C for 5 minutes to inactivate the reverse transcriptase and stored at –85 °C.

The amplification of DNA was carried out in a total reaction mixture of 25 μL that contained cDNA, dNTP, forward and reverse primers, 2.5 μL of 10 × polymerase-specific reaction buffer, $MgCl_2$, *Thermus aquaticus* (Taq) DNA polymerase and water. The reaction mixture was subjected to 35 cycles of 95 °C for 1 minute, 55 °C for 1 minute, 72 °C for 1 minute followed by a final extension of 72 °C for 5 minutes. All PCR reactions were overlayed with 25 μL of mineral oil (Promega) to prevent evaporation during thermal cycling but RT reactions were not overlayed.

To assess samples at the completion of the RT-PCR protocols, 5 μL of sample was mixed with a 5 × loading buffer (Biorad, USA) and loaded into a 2% agarose gel containing ethidium bromide (0.5 μg/mL) in 40 mM Tris-Acetate, 1 mM EDTA (TAE) buffer and subject to electrophoresis at 100 volts for 60 minutes. DNA amplicons were visualised by subjecting the agarose gel to ultraviolet transillumination.

Nucleotide sequencing

Following amplification of the DNA product, the amplicon was subjected to treatment by the standard Geneclean (Bio101) protocol to remove any

Figure 1. A geographical representation of Lao PDR representing provinces where CSFV sample collections were undertaken.

remaining primers or PCR reaction chemicals that may interfere with downstream applications. Nucleotide sequencing was performed using an ABI Prism 377-18 automated DNA sequencer at the CSIRO Division of Animal Health, Geelong, Australia.

Phylogenetic analysis of nucleotide sequence results

Lao PDR CSFV sequences were compared with sequences held on the CSFV sequence database accessed at http://viro08.tiho-hannover.de/eg/csf (Greiser-Wilke et al. 1999) (see Table 1).

The deduced nucleotide sequences and database sequences were aligned using the CLUSTALX 1.64 software package (Thompson et al. 1994). The transition/transversion ratio was calculated using the PUZZLE 4.0 program (Strimmer and von Haeseler 1996, 1997) followed by Maximum Likelihood-Neigbour Joining analysis using algorithms supplied with the PHYLIP software suite (DNADIST and NEIGHBOUR) (Felsenstein 1989). The results were bootstrapped using 100 replicates via the PHYLIP algorithm, SEQBOOT and the resultant phylogram viewed using TREEVIEW 1.2 software (Page 1996).

Results and Discussion

From the results presented, it is apparent that the Lao PDR isolates assessed fall into two distinct groups (Figure 2).

The virus isolates from Phongsaly (L119), Bokeo (L168) and Vientiane Municipality (L123) provinces clearly group with European viruses that comprise the genogroup 2.1. The Lao PDR isolates with the

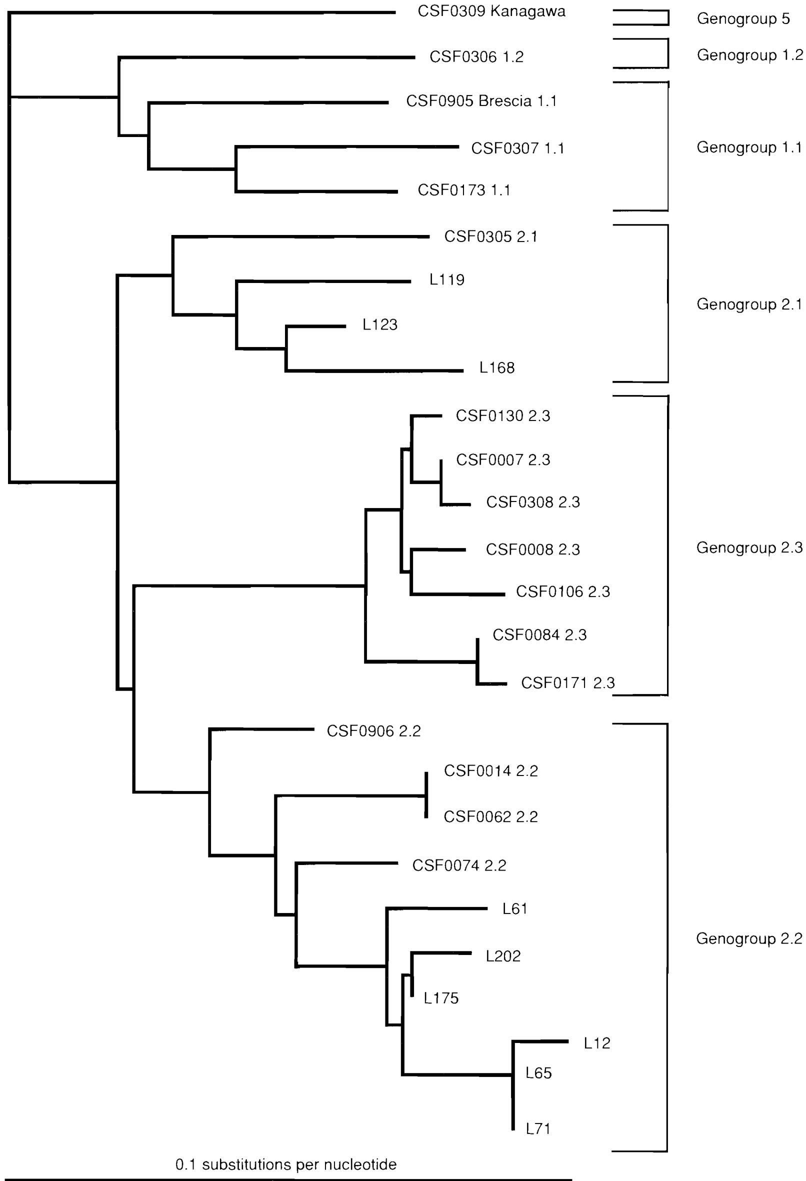

Figure 2. Phylogram of the nucleotide sequence comparison of 190 base pairs of the E2 gene of CSFV isolates from Lao PDR, Europe and Asia.

exception of L123 are both CSFV isolates from northern Lao. The northern regions of Lao PDR are very mountainous, somewhat isolated from other regions of Lao PDR. It would therefore be expected that CSF virus isolates from these areas would be distinct, given the geographical isolation.

The balance of the viruses from Champassak (L12, L65, L71), Savanakhet (L175) and Khammouane (L61, L202) provinces appear to group with viruses belonging to genogroup 2.2. All provinces where these viruses were sourced are in the southern region of Lao PDR. There appears to be a strong

geographical relationship between isolates from Champassak province and to a lesser extent with Savanakhet and Khammouane viruses. None of the Lao PDR viruses assessed in this study fall into genogroup 1 or genogroup 2.3.

Previous studies of Asian CSFV isolates have found that Malaysian isolates from the 1980s have grouped with genogroup 2.1, but to date no Thailand isolates have fallen into this group (Grieser-Wilke and Paton 1999). Thai isolates from the 1990s have been reported to group within the genogroup 2.2 (Grieser-Wilke and Paton 1999). This may account for the southern Lao isolates belonging to this genogroup, given the long common border between Lao PDR and Thailand in the southern region and the opportunity for livestock trade.

From the results presented it is evident that there is a strong geographical relationship between CSF virus isolates from the northern region (genogroup 2.1) and the southern region (genogroup 2.2) of Lao PDR. As the viruses assessed in this study were collected over only a one-year period, it is not possible to assess the level of temporal variation in Lao PDR CSFV isolates. Further work with additional virus isolates and comparison with other genetic regions is required to gain a fuller picture of the molecular epidemiology of CSFV in Lao PDR.

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Molecular Epidemiology of Classical Swine Fever Virus Isolates in Thailand

S. Parchariyanon[1], K. Inui[1], W. Pinyochon,[1] S. Damrongwatanapokin[1], P. Lowings[2] and D. Paton[2]

Abstract

Molecular epidemiology of classical swine fever in Thailand was studied by analysing the phylogenetic relationships between 20 Thai classical swine fever virus isolates and the world isolates. The results show that Thai isolates were clustered into three distinct genogroups. Ten isolates from 1991–95 form a new genogroup not reported previously. Nine isolates from 1988–93 are in the same genogroups as old US and European strains. The viruses of this group seem to have died out in Europe, but they still exist in Thailand. One isolate from 1995 is closely related to some Italian strains (c4D).

CLASSICAL swine fever (CSF) is an economically important disease for the pig industry in many parts of the world. The causative agent, classical swine fever virus (CSFV), belongs to the Pestivirus genus of the Flaviviridae family. Genetic variability of CSFV has been reported among isolates from different parts of the world (Lowings et al. 1996; Vilcek et al. 1996). Identification of the virus strains and genotypes by genetic characterisation can be used to trace the sources of infection and should improve our understanding of the epidemiology of CSF. The present report studies the phylogenetic relationship of CSFVs isolated in Thailand and the world isolates.

Twenty CSFV field strains isolated from various geographical areas in Thailand during 1988–95 were examined by comparative genetic sequence analysis. Briefly, a part of E2 glycoprotein gene of the isolated viruses was amplified by RT-PCR and directly sequenced. Sequences corresponding to Alfort strain 2508–2697 (190 base pair [bp]) were compared with those of world isolates and analysed with the PHYLIP software for phylogenetic studies (Lowings et al. 1994).

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Phylogenetic analysis of 20 Thai isolates and world isolates proposes that CSFVs can be divided into three major genogroups instead of two as reported previously (Figure 1). Thai isolates were clustered into all three genogroups, termed genogroups 1, 2 and 3.

Group 1 contains old US and European strains and is divided into two subgroups, 1A and 1B. Subgroup 1A is represented by Alfort strain and includes three Thai isolates from 1988–93. Subgroup 1B is represented by Brescia strain and Malaysian isolate (VRI 4167) of 1986. It includes six Thai isolates 1988–91, five of which are closely related to the Malaysian strain.

Group 2 contains recent European strains and is divided into three subgroups, 2A, 2B and 2C. Subgroups 2A and 2C are represented by Malaysian isolate (VRI 2277) and UK 86/2, respectively. No Thai isolates were found in subgroups 2A and 2C. Subgroup 2B is represented by Italian isolate (c4D) of 1991. One recent Thai isolate is closely related to this Italian isolate. No Thai isolates before 1995 are found to be in this group.

Group 3 is a new genogroup not reported previously. Ten Thai isolates belong to this group. No isolates from any other part of the world are reported to be in this group.

The gene of E2 glycoprotein (gp55) which elicits neutralising antibodies and induces a protective immunity is one of the most variable parts of the

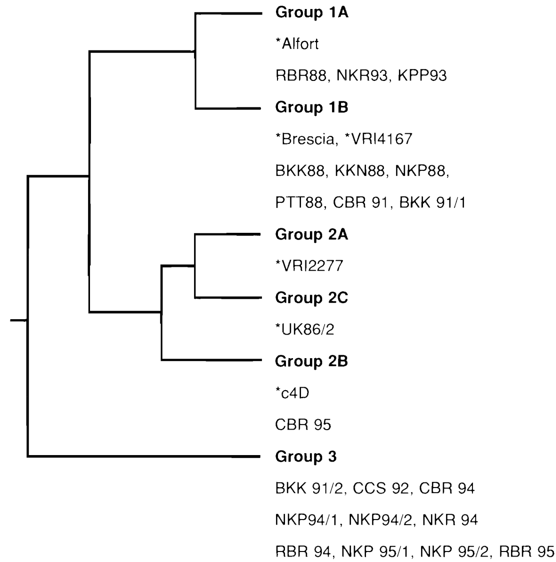

Figure 1. Phylogenetic relationships among six subgroups of CSFV based on 190 bp of E2 gene. Strains marked with * are representative of each subgroup published previously (Lowings et al. 1996).

pestivirus genome. Phylogenetic analysis of the E2 gene of 115 CSFV world isolates has shown that CSFV can be divided into two major genogroups and five subgroups, termed 1A, 1B, 2A, 2B and 2C (Lowings et al. 1996).

A new and third genogroup of CSFV was found to exist in Thailand. This genogroup, termed group 3, has examined 10 of 20 Thai isolates. It indicates that group 3 is the dominant genogroup in Thailand. The present study showed the evidence of a distinct lineage of CSFV in Thailand, and emphasises the need for global virus characterisation in order for molecular epidemiology tracing of CSFV to be fully effective.

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CONTROL STRATEGIES AND LOCAL IMPACT OF CSF

Approaches to CSF Control Using Vaccination

Annette Bonde Larsen[1]

Abstract

Classical swine fever (CSF) is still an important problem for pig production worldwide and even in some declared free areas in Europe massive outbreaks have been experienced. In the Netherlands, traditional live vaccines have been shown to be effective to control clinical disease, and also for eradication if vaccination is combined with stamping-out infected herds. Due to the need for CSF vaccines for emergency vaccination in CSF-free areas, that do not interfere with diagnostic procedure, marker vaccines as well as a discriminative diagnostic ELISA kit have been developed. Vaccination with Porcilis® Pesti has shown to be highly protective against severe challenge. There are different approaches to the control of CSF. For control in endemic-infected areas, traditional live vaccines are preferably used, whereas the modern marker vaccines are more suitable as an aid to eradication in herd areas or at country level and as a new tool for eradication in CSF-free areas.

THE FIRST OUTBREAK of classical swine fever (CSF) was reported in 1833 in Ohio and the disease became widespread in the 1860s. Since then, CSF has been an important problem for pig production all over the world. Even today, many parts of the world put much effort into controlling and eradicating the disease. Some areas such as North America are declared free of CSF.

Most countries in the European Union (EU) vaccinated intensively in the 1970s and 1980s in order to control and eradicate CSF. In 1991, a non-vaccination policy was implied in EU, which means that vaccination against CSF is banned and that stamping-out is used in outbreaks. However, there have been some major outbreaks in Germany and Belgium, the latest in the Netherlands, where many pigs were killed.

During the last epidemic in the Netherlands, 11 million pigs were killed, 700 000 CSF-infected, 1.1 million preventive-eradicated and 9.2 million animal welfare in standstill (quarantined) areas. The main source of re-infection in EU is swill-feeding, but CSF-infected wild hogs are also considered to play a role.

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Control of CSF Using Traditional Vaccines

The vaccines used for a long time to help control CSF are live attenuated vaccines based on either the C-strain, the Thiverval, or the Japanese GPE, strain. Those vaccines are highly efficacious and safe (Aynaud 1988).

In the Netherlands, it has been shown that CSF can be eradicated from enzootic areas by strict mass vaccination combined with stamping-out infected herds (Terpstra and Robijus 1977).

In 1973, in three enzootic areas in the Netherlands, compulsory mass vaccination was applied. Initially, all animals more than two weeks old were vaccinated once with an attenuated live C-strain vaccine. During the initial vaccination campaign, approximately 2–3 weeks, movement of pigs was prohibited. The initial mass vaccination was followed by a single vaccination of young stock at 6–8 weeks of age and of all stock introduced from outside the areas. In two of the areas, vaccination was stopped after one year, in the third area, after three years. CSF outbreaks decreased quickly after the campaign began and clinical disease disappeared after five months (see Table 1).

No clinical disease due to residual virus was seen in the two years follow-up after vaccination terminated. Finally in 1979 the Netherlands was declared CSF-free for the first time.

Table 1. Monthly outbreaks of CSF before and after the start of the mass vaccination campaign.

	Monthly outbreaks of CSF in emergency areas in the Netherlands											
	Months prior to vaccination						Months after vaccination					
Area	*6*	*5*	*4*	*3*	*2*	*1*	*1*	*2*	*3*	*4*	*5*	*6*
I	4	7	8	14	16	10	7	2	1	—	—	—
II	1	4	14	50	91	89	20	10	3	3	—	—
III	5	3	4	3	5	14	3	2	3	—	—	—

(Source: C. Terpstra, TvD, 1977, 102, 106–112)

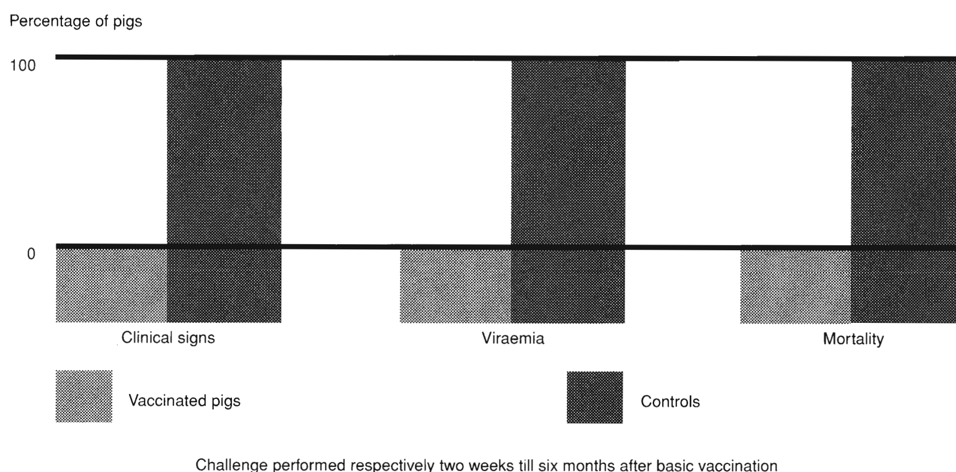

Figure 1. Results of challenge with heterologous virulent CSF virus.

Based on experience in Europe, the most important factors to control CSF are vaccination of *all* pigs in *all* herds with a highly efficacious vaccine supplemented by the introduction of only vaccinated animals and good biosecurity measures.

Control of CSF Using Modern Marker Vaccines

The main reason for applying the non-vaccination policy in EU was international trade—to be declared free of CSF and to maintain the CSF-free status. This is not possible when traditional live vaccines are used because of interference with serological monitoring. It was interesting to develop marker vaccines against CSF which could be used in case of outbreaks in non-infected areas. These vaccines are inactivated subunit vaccines based on the E2 protein of CSF virus. They allow vaccinated animals to be differentiated serologically from field-infected animals.

Intervet, in cooperation with Professor Thiel at the Federal Research Centre for Virus Diseases of Animals in Giessen (Germany), developed a marker vaccine against CSF that, together with a discriminating diagnostic test, can be used as a strategic tool for controlling the spread of CSF.

Porcilis® Pesti is an inactivated subunit vaccine containing 120 ELISA units (EU) of E2 protein in a water-in-oil adjuvant. Pigs with maternally derived antibodies can be vaccinated from the age of six weeks onwards. In a naïve population, vaccination can be given at a younger age. The basic vaccination consists of two doses of 2 mL at a 4-week interval. Revaccination with a single dose is recommended at 6-month intervals.

The efficacy of the vaccine has been shown in challenge studies where pigs were vaccinated twice in a 4-week interval. Vaccinated pigs and unvaccinated controls were challenged with at least 300 (to 1000) LD50 of the heterologous CSFV-strain Alfort 187 (reference challenge strain obtained from Hannover, CSFV Reference Lab.) by the intramuscular route (*Ph. Eur.*). Pigs were challenged 2–26 weeks following the second vaccination.

Protection could be demonstrated from two weeks after the second vaccination onwards for at least half a year. All control animals died or were killed, when moribund 7–10 days after challenge. In contrast, all vaccinated pigs survived (Figure 1).

Furthermore, a discriminative ELISA kit has been developed by Dr A.G. Bommeli. This ELISA is based on the detection of antibodies against the Erns protein of CSF virus, a structural component of all CSF viruses. Porcilis® Pesti, however, contains only the E2 protein and therefore induces only E2-specific antibodies. A serum sample negative for Erns is therefore originating from either a non-infected pig or a pig vaccinated with Porcilis® Pesti, and a sample positive for Erns derives from a field-infected animal or an animal vaccinated with traditional live vaccines.

It is now discussed intensively within the EU whether such marker vaccines should be used as an additional tool in the eradication of CSF, when new outbreaks occur. There is great pressure from countries which have experienced large outbreaks where millions of healthy pigs were killed and destroyed due to welfare reasons. Furthermore, CSF marker vaccines might be used as an aid in eradication at herd/area or country level because of the discriminative advantages and the high level of protection induced after vaccination.

Conclusion

There are different approaches to controlling CSF, depending on the starting-point in the area or country concerned. For endemic-infected areas or countries, the best approach is to use a good live vaccine to control clinical disease. By combining a strict vaccination scheme with stamping-out infected herds, classical swine fever virus can be eradicated from the area.

Modern marker vaccines will be more suitable as an aid for eradication at herd/area or country levels and as a new tool for the eradication of outbreaks in free areas.

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Monitoring CSF Antibody Levels in Vaccinated Swine

Shanyi Naichun, Li Chundi[1], Zhang Yinguo[1], Zhang Wendong[1], Wan Jiaju, Gao Huafeng, Liu YinWu, Li Xinrong and Du Jian

Abstract

Applying indirect agglutination, swine in different regions and at different ages have been investigated for serology; the test has been carried out by injecting different vaccines and immunising with doses widely used. The swine were divided into several groups, and the serum collected periodically. This method was used to detect serum antibody levels. To analyse and compare antibody levels of swine in Yunnan Province, piglet maternal antibody levels, immunising doses and immunising titres were surveyed.

CSF IS A dangerous disease for swine and a serious menace to the pig-raising industry. It is one of the 16 contagious diseases attributed 'A' in the health laws of OIE.

CSF prevalence and morbidity has changed in recent years, its epidemicity becoming periodic, wavy and of limited outbreaks, compared with past years. It currently appears as atypical CSF, mild CSF, and so-called 'high fever with no reasons'. Clinical symptoms are ablivited obviously, death rate has decreased, and its pathology change is not typical. There is subclinical infection, placental transmission, geneogenous trembles of newborn piglets and symptoms of pregnant sows carrying the virus. It is hard to explain why immunisation is not effective in the regions and pig farms are affected.

Injecting vaccine is the key control measure to prevent CSF—what is the antibody level of vaccinated swine using different vaccines, the maternal antibody level of piglets, elementary immunisation antibody levels and secondary immunisation antibody. CSF serology diagnostic reagents for indirect agglutination testing are produced in the Yunnan Tropical and Subtropical Animal Virus Disease Laboratory. The reagents have strong specific reactions, and agglutination with negative serum and other antigens of contagious disease cannot happen. The serum of vaccinated swine was collected to investigate antibody levels from seven different regions (Kunming, Qujing, Yuxi, Honghe, Dali, Linchang and Lijiang in Yunnan Province) to discover the correlation of antibody levels between vaccinated sows and newborn piglets, and how best to change basic immunisation for piglets.

[1] Yunnan Tropical and Subtropical Animal Virus Disease Laboratory

Materials

1. Reagents for indirect agglutination tests were produced by YTSAVDL.
2. Positive serum was produced by YSTAVDL, antibody titre dilution 1:2048.
3. Negative serum was collected from non-vaccinated swine in Qujing and Kynming.

Method

The antibody level of CSF-vaccinated swine was surveyed in the main regions of Yunnan Province. Swine (4675) were abstracted from Kynming, Qujing, Yuxi and Honghe. Serum was isolated and conserved below minus 20 °C, detecting the antibody level of vaccinated swine using IHA diagnostic agents of the same serial number.

To investigate piglet maternal antibody level, 15 healthy sows were selected from Honghe region and vaccinated using C-strain vaccine including a standard immunising dose, four times the dose, and six times the dose. Sow antibody levels at 30, 60 and 90 days were taken, and piglet maternal antibody levels at 15, 30 and 45 days.

In the investigation of antibody changes after basic immunisation, healthy piglets 7 days old were selected in Kunming and their maternal antibodies detected. Twenty piglets with maternal antibody titre

equal to 1:8 or 1:16 were selected and divided randomly into three groups, injected with a standard dose of cattle body reaction vaccine, a standard dose of cell cultural vaccine and four times the dose of cell cultural vaccine. According to each group number at 7, 14, 28, 35 and 42 days and from the second month to eleventh month, each month's serum was collected and the antibody titre detected. If the result was greater than a dilution of 1:16, it was judged qualified.

Results

The IHA reagents for CSF serology diagnosis are strongly specific. Agglutination reaction with positive serum occurs, and there is no reaction when negative serum or serum infected by other contagious diseases are detected.

Investigating the antibody levels of vaccinated swine using the indirect agglutation test in the main regions of Yunnan province indicated that 3887 of 4675 vaccinated swine, about 83.14%, had enough antibodies to prevent CSF. The results were different in different regions—1305 specimens in Kunming (positive rate 66.8%), 335 specimens in Qujing (83.5%), 370 in Yuxi (74.6%), 812 in Honghe (87.9%), 218 in Linchang (88.9%), and 1396 in Lijiang (93.4%).

The sows were vaccinated using one, four and six times the immunising dose of cell culture vaccine. Their antibody levels increased with the increased dose. The maternal antibody levels of these sows' piglets were investigated. Of the sows vaccinated using once the immunising dose, at 15 days, 8 of 10 of their piglets had sufficient antibody to prevent CSF. At day 30, 4 of the 10 piglets had sufficient antibody. At 45 days, 2 in 10 had sufficient antibody to prevent CSF. If the sows were vaccinated using four times the immunising dose, at 15 days, 18 in 20 piglets had sufficient antibody, at 30 days, 11 in 20, at 45 days, 7 in 20. If the sows were vaccinated using six times the immunising dose, at 15 days, 18 in 20 of their piglets had sufficient antibody, at 30 days, 12 in 20 piglets, at 45 days, 8 in 20.

The positive rate of elementary immunisation antibody in piglets (see Table 3): the results indicate at 7 days after immunisation with the standard dose of cattle body reaction vaccine, the positive rate can reach 85.7%, maintained for two months, and three to five months later can reach 71.4%. At 7 days after immunisation with a standard dose of cell culture vaccine, the positive rate of antibody can reach 87.5% and be maintained for one-and-a-half months. After two to three months, the positive rate is 75%; for four to five months, 62.5%. When the piglets were vaccinated using four times the dose of cell culture vaccine, at 7 days all had enough antibody to prevent CSF and it could be maintained for six months.

Summary

Reagents for the indirect agglutination test for CSF are strongly specific and cannot conjugate with CSF negative serum or other disease serum. The test can be used to detect in serum firstly, piglet maternal antibody, and secondly, the antibody of swine vaccinated by CSF vaccine. Thirdly, it can be used to monitor the changing of the CSF antibody.

In seven main regions of Yunnan province, 4675 vaccinated swine were investigated. About 83.14% of the total have sufficient antibody to prevent CSF. The antibody level of vaccinated swine is high and reliable in Yunnan.

Newborn piglets can acquire maternal antibody by sucking colostrum. Sows vaccinated with different immunising doses transfer different antibody levels to their piglets, piglet maternal antibody increasing with the growth of the immunising dose. As the piglets age, the maternal antibody level decreases and eventually disappears. Most have insufficient to prevent CSF infection 45 days later.

All piglets vaccinated with four times the dose of cell culture vaccine can be protected at 7 days and the antibody maintained for six months, so using this vaccine and dose, the swine produce high titre antibody of long durations. At the same time, the immunising capacity was strong.

Table 1. Investigating vaccinated swine antibody levels.

Number Region	Antibody	IHA dilution of vaccinated swine					Result (%)	
		1:128	1:64	1:32	1:16	<1:16		
Kunming	1305	539	308	48	34	376	71.9	(29/1305)
Qujing	335	215	50	10	5	55	83.5	(280/335)
Yuxi	370	142	72	38	26	92	75	(278/370)
Honghe	812	210	297	168	39	98	87.9	(714/812)
Dali	239	86	57	25	20	51	78.6	(188/239)
Linchang	218	98	51	29	16	24	88.9	(194/218)
Lijiang	1396	973	134	127	70	92	93.4	(1304/1396)
Sum	4675	2263	969	445	210	788	83.14	(3887/4675)

Table 2. Piglet maternal antibody levels.

Vaccinated sow				Piglets		IHA dilution of material antibody					Result (%)
No.	Vac. type	Dose	IHA	Number	Age	1:64	1:32	1:16	1:8	1:4	
5	cell cultural vaccine	1		10	15	2/10	4/10	2/10		2/10	8/10 (80)
					30		2/10	2/10	4/10	2/10	4/10 (40)
					45		2/10	2/10	6/10	2/10	2/10 (20)
5		4		20	15	6/20	6/20	6/20	2/20		18/20 (90)
					30	4/20	2/20	5/20	5/20	4/20	11/20 (55)
					45		4/20	3/20	8/20	5/20	7/20 (35)
5		6		20	15	6/20	6/20	6/20	2/20		18/20 (90)
					30	4/20	2/20	6/20	5/20	3/20	12/20 (60)
					45		4/20	4/20	8/20	4/20	8/20 (40)

Table 3. The positive antibody rate of piglets after basic immunisation.

Group	Age Dose			Positive rate detected by IHA method									
	Number Type			0	7	14	21	35	42	60	90	120	160
A	Type A	10	1	0/10 0.0	6/7 0.857	6/7 0.857	6/7 0.857	6/7 0.857	6/7 0.857	6/7 0.857	5/7 0.714	5/7 0.714	5/7 0.714
B	Type B	10	1	0/10 0.0	7/8 0.875	7/8 0.875	7/8 0.875	7/8 0.875	7/8 0.875	6/8 0.75	6/8 0.75	5/8 0.625	5/8 0.625
C		10	4	0/10 0.0	10/10 1.0	10/10 1.0	10/10 1.0	10/10 1.0	10/10 1.0	10/10 1.0	10/10 1.0	10/10 1.0	10/10 1.0

Note: Type A represents cattle body reaction vaccine, type B represents cell cultural vaccine.

Table 4. Monitoring piglet antibody.

A — Test group: A Vaccine type: cattle body vaccine immunising dose: one

Date No.	7 days	14 days	21 days	28 days	35 days	42 days	60 days	90 days	120 days	150 days
45	8	4	16	16	16	16	16	16	16	16
48	64	64	64	32	32	32	16	8	4	4
49	256	128	64	32	32	16	32	8	8	8
50	128	128	32	16	16	16	32	32	32	32
74	16	16	16	16	16	16	16	16	16	16
75	16	16	16	16	16	16	16	16	16	16
79	64	32	16	16	16	16	16	16	16	16

B — Test group: B Vaccine type: cell cultural vaccine immunising dose: one

Date No.	7 days	14 days	21 days	28 days	35 days	42 days	60 days	90 days	120 days	150 days
17	8	4	4	8	8	8	8	4	4	4
18	16	16	16	16	16	16	16	16	8	8
51	64	128	64	16	16	16	8	4	8	8
53	64	32	64	32	32	16	16	32	16	16
54	32	64	64	32	32	32	32	32	32	32
55	64	128	64	32	32	16	16	16	16	16
56	16	16	32	16	16	16	16	64	128	64

If the IHA dilution is equal to or more than 1:16, it can be judged positive.

Controlling Pig Diseases in Vietnam and Lao PDR in Village Smallholder Systems

T. Gibson† and I. Wilkie‡

Abstract

This paper outlines recent experiences of two developmental projects in remote areas of northern Vietnam and western Laos in attempting to reduce the high death rate of village pigs by Swine fever vaccination programs. In general, the programs have not been successful even after due attention to maintaining an effective cold-chain to the village. Swine fever was definitely confirmed by laboratory analysis as present in sick pigs in the two villages in Vietnam from which samples were taken. In Laos, it was only infrequently confirmed as present in sick pigs from samples submitted from many villages. The results could be explained by the presence of fatal diseases additional to swine fever in the project areas. However, more basic research is required to reduce the high death of village pigs. Such research is beyond the ability of developmental projects which must rely on continuing advice from research projects such as the Animal Health Project.

THIS PAPER reflects experience as short-term consultants to two separate projects: Ky Son Pilot Scheme, UNDCP, in Ky Son District, Nhge An Province, Vietnam, and the (German) GTZ Bilateral Aid Project, the rural development project in Bo Keo Province, Lao PDR. Ky Son is in the central-northern highlands of Vietnam and adjoins the mountainous Lao Province of Xieng Khouang. Bo Keo is in western Laos and is the Lao province of the well-known 'Golden Triangle', those adjoining areas of Laos, Thailand and Burma where much opium was once grown. Villages in the Ky Son Project area range 150–1300 m amsl, those of the Bo Keo Project 350–650 m.

Both projects have many similarities: both are area development projects of which livestock development is one of several major components; both are largely concerned with ethnic minority peoples where the Hmong group is common to both; both have an opium replacement or rehabilitation component; and both are in areas comparatively remote from modern services including electricity, all-weather roads and veterinary services.[1]

This paper outlines the process by which both projects have attempted to address and control the problem of pig diseases in remote ethnic minority villages. Nearly all pigs are the native black 'sway-back' pig, mostly free-ranging, although there has been much official encouragement in recent years for villagers to pen livestock. No purchased feed is normally given to pigs. Feed obtained from scavenging is supplemented by products in and near the villages, such as coarse rice-bran, maize (when available), tubers of yams (wild and cultivated yams, especially *Xanthosoma sagittifolium*), cassava (especially in Ky Son), and sweet potato, green papaya fruit and a wide range of vegetative material such as banana stems, leaves of cassava (less in Bo Keo), sweet potato, coco yam (*Colocasia esculenta*) the fibre tree 'bo sa' (*Broussonetia papyrifera*?),

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[1] In both project areas, infrastructure and services are more developed in the larger towns and, in Bo Keo, in villages adjacent to the Mekong River. In these more advanced areas, pig production is usually more successful and vaccination more regularly conducted. This paper refers only to the more remote villages of major interest to both projects.

pumpkin shoots and weeds (especially *Amaranthus* spp.). Husbandry of pigs in the project villages is similar to that described by Visitpanich and Falvey (1980) for the nearby highland ethnic minority peoples of northern Thailand.

The data on which development has been based are largely anecdotal and difficult to confirm, but this report illustrates the problems of development in such remote areas and the great need for more reliable data such as can be obtained from the Animal Health Project.

Initial Surveys

At the commencement of the consultancies (1997–98), rapid surveys were conducted in many villages.[2] Surveys were conducted mainly by discussion in informal villager meetings. There was a generally consistent pattern in replies. Villages experienced regular, often yearly or biennially, epidemics in which the reported death rate of pigs varied from about 20% to almost 100% during any one epidemic.[3] The most common signs of disease described were red spots or red rashes on the skin (thus leading many villagers to believe that mosquito bites were the cause of disease), red spots on the intestines and red meat, and pus discharged from the corners of the eye. Inappetance and a dejected appearance were almost universal. Fever was regularly mentioned and salivation and diarrhoea were sometimes mentioned. Swollen throats with fever were occasionally mentioned. Abortion was very rarely mentioned.

Officials or village para-vets had conducted vaccination campaigns irregularly in some villages.[4] In Ky Son, pasteurellosis vaccine obtained from the Government Laboratory in Hanoi was administered and in Bo Keo, only swine fever vaccine from the Government Laboratory in Vientiane. Villagers were equivocal as to the effectiveness of pig vaccination; in Ky Son, villagers were more adamant that vaccinated pigs died at least at the same rate as unvaccinated pigs. Villagers generally believed that all animal vaccinations are largely ineffective (most especially for poultry). This belief in the general ineffectiveness of animal vaccination[5] has led to the failure of official attempts to develop self-funding regular vaccination programs based on a local para-vet system in these remote villages.

Villagers sometimes administered antibiotics to sick animals but (for pigs and poultry especially) stated that antibiotics were generally unsuccessful in preventing death. The only effective means the villagers knew to prevent pig (and poultry) death was isolation; pigs are sometimes located in remote fields, which has the disadvantage of requiring extra labour for care-taking (feeding, guarding). Sometimes villagers reported that simply keeping pigs in pens in villages (where they are isolated from other pigs) is effective in reducing the death-rate but this method has the disadvantage of requiring much more prepared feed than that required for free-ranging pigs.

At the time of the surveys, local officials reported that an occasional sample from a diseased animal had been sent to a laboratory for diagnosis, but they were generally unaware of the results of the testing.

Veterinary officers in both countries generally stated that swine fever was the major cause of pig death.[6] In Ky Son, some local officials believe that pasteurellosis and leptospirosis are important in pigs. The conclusions appear to be based on the interpretation of field observations and, at least in the case of Ky Son, diagnoses made many years ago.[7] In Ky Son, local officials did not attempt swine fever vaccination in remote villages because of the difficulty in maintaining the cold-chain to such villages.

As a result of the initial survey, the following conclusions and recommendations were made (among others):

1. pig production has great potential to contribute to village household protein intake and cash income;
2. village pig production is severely constrained by the high death-rate of pigs, which does not seem to be controllable under present practices;
3. the cold-chain for vaccines from the manufacturing laboratory to the village pig should be improved;

[2] See Gibson (1997), (unpubl.) Consultancy Report, GTZ-RDP in Bo Keo and Gibson (1998), (unpubl.) Consultancy Report, Ky Son Pilot Scheme, UNDCP, Hanoi.

[3] For the hills of northern Thailand, Visitpanich and Falvey (1980) found that the average incidence of disease epidemics was once every 1.6 years with a mean mortality rate of 74%.

[4] The official reported yearly rate of vaccination of pigs varied 30–70% but the informal surveys suggested that actual vaccination rates in the remote villages were much less.

[5] This is in contrast to the general belief by villagers that human vaccination is successful.

[6] Visitpanich and Falvey (1980) reported that 'swine fever is the most serious disease in the highlands (of northern Thailand)' but provided no supporting evidence.

[7] Swine fever was first identified in Vietnam in Ky Son in 1964; leptospirosis was recorded in Ky Son in 1970 (B.Q. Huy in Gibson [1999] [unpubl.] Consultancy Report, Ky Son Pilot Project, UNDCP). Pasteurellosis was recorded in pigs in Vietnam in 1988 (Townsend et al. Veterinary Microbiology, 63: 205–215). Villagers sometimes accord death to the ingestion of rat poison often placed around houses and fields.

4. the quality and appropriateness of pig vaccines should be investigated;
5. the precise cause of most pig deaths should be investigated;
6. vaccination demonstrations should be made in some villages using the improvements listed above.

Workshops were held to address the recommendations, and the activities discussed in the following section initiated.

Recent Activities

Cold-chain improvements

Swine fever vaccine is particularly vulnerable to heat. There was great doubt as to whether the vaccine, even if potentially effective, reached the village in acceptable condition. The route from the manufacturing laboratories to the district towns where the vaccines are stored before distribution to the villages is long and tortuous. Vaccines were observed leaving laboratories on several occasions without being packed on ice or stored in suitable insulating containers. The cold-chain is dependent on officials remote from the end-users, with little incentive or the means to ensure an adequate cold-chain.

At the district (or provincial) holding centres, the electricity supply is irregular such that on some occasions it can reasonably be expected that the vaccines will reach room temperature before being re-cooled. And finally, even if vaccines are placed in ice before transport to villages, the remoteness of the villages often means that the ice is melted some days before actual vaccination.

The projects have addressed these problems by directly obtaining and transporting the vaccines from the manufacturing laboratories to the project centres and storing vaccines in project refrigerators with access to supporting power supplies. Previous attempts to supply solar, kerosene or gas refrigerators to districts have not been very successful due to the inability to repair the refrigerators when needed and an insufficient volume of vaccine sales to pay for the fuel.

Improvements in vaccination technique

An important problem in vaccinating free-ranging pigs is the difficulty in satisfactorily restraining pigs during vaccination. The method usually employed is to attract pigs to a feeding trough and to inject suddenly before the pig can react. This often results in no or insufficient vaccine entering the pig, but such 'vaccinations' are usually recorded as vaccinated animals.

Pig catchers have been tried but have not been successful to date. In some of the pig demonstrations mentioned below, pigs were individually caught at feeding troughs and vaccinated. This is a labour-intensive and time-intensive operation.

Another potential problem is the transmission of disease using the same needle for all pigs in a village. The projects are supplying more needles to veterinarians and are emphasising more regular changing of needles.

Quantitative village surveys

Noong De, Ky Son

The first village in which the improvements were demonstrated was Noong De.[8] On 7 October 1998, 32 pigs were vaccinated and ear-marked. Some pigs were free-ranging, some penned. On 24–25 November, a complete survey was conducted of all 66 households in the village[9] (see Table 1).

The results were unexpected and disturbing. In the seven weeks since vaccination, more vaccinated pigs died (34%) than unvaccinated pigs (26%). No pigs were reported as dying within the first two weeks after vaccination. Thus increased susceptibility to swine fever in vaccinated pigs exposed to swine fever shortly after vaccination cannot be an explanation for the results. Signs of death were reported by villagers as similar in both vaccinated and unvaccinated pigs. The most common signs were red skin

[8] Needles were not frequently changed in this demonstration.
[9] The survey was conducted by Mdm. Mai of the District Veterinary Office, Mr Tuan, Project Field Extension Officer, Mr Vu, District Agricultural Extension Officer and Mr Thoong, village para-veterinarian.

Table 1. Summary of pig death rates since 7 October 1998.

	Vaccinated on 7 October, 1998			Not vaccinated on 7 October 1998		
	Total vaccd	Alive on 25 Nov.	Died since 7 Oct.	Total not vaccd	Alive on 25 Nov.	Died since 7 Oct.
Total no.	32	21	11	256	190	66
Average (%)	100	66	34	100	74	26

spots and eye discharge. Salivation, fever, throat swellings and red intestines were reported in some dead pigs (not all in the same pigs); diarrhoea was not common.

It is possible that some irregular swine fever vaccination had been conducted in this village in previous years, as it is easily accessible by an all-weather road.

Nga Ba, Ky Son

In November, 1998, 20 pigs were vaccinated with both pasteurellosis and swine fever vaccines and were ear-tagged. By March 1999, 5 unvaccinated pigs (of about 50 unvaccinated pigs) had died whereas no vaccinated pigs had died. It is understood that at the time of reporting, many more unvaccinated pigs have died but no vaccinated pigs. In April 1999 two weaner piglets were said by a villager to look sick and he expected them to die within a couple of days. The piglets showed no obvious signs of disease. Both were slaughtered and both had severe button ulcers on the inside of the large intestine (suggestive of classical swine fever). Samples from both pigs were analysed at the National Veterinary Research Institute in Hanoi and both were positive.[10]

It is most unlikely that any swine fever vaccination had ever been previously conducted in this village, which until the last year or so was very difficult to reach by vehicle.

Nam Tin, Bo Keo

There are five villages in the Nam Tin area. Pigs were vaccinated and most vaccinated pigs ear-tagged at the end of May to early June 1999. Every household was interviewed at the end of August 1999.[11] Gross results are presented in Table 2, and summary results in Table 3.

On average, there was no benefit from vaccination. About 11% of pigs died within three months after vaccination whether vaccinated or not. It is

[10] Acknowledgment is due to Dr Nguyen Tien Dung and Dr Wicher Holland for assistance.

[11] Vaccination was conducted by Mr Khammy, Provincial Veterinary Officer and village para-veterinarians. The survey was conducted by Mr Soubanh, Field Extension Officer, Rural Development Project.

Table 2. Swine fever vaccination results in Nam Tin area.

Number present at vaccination				Number sold since vaccination		Number eaten since vaccination		Number died since vaccination	
Large pigs		Small pigs							
Vac.	Not vac.	Vac.	Not vac.	Vac.	Not vac.	Vac.	Not vac.	Vac.	Not vac.
Nam Tin Village: total 38 households, 31 with at least one pig at time of vaccination.									
16	7	9	21	2	2	2	1	0	4
Phou Van Neua village: total 71 households, 65 with at least one pig at time of vaccination.									
32	44	7	162	2	5	8	33	2	28
Phou Van Tai village: total 91 households, 80 with at least one pig at time of vaccination.									
28	71	0	152	6	17	4	42	12	38
Nam Tong Neua village: total 30 households, 28 with at least one pig at time of vaccination.									
10	65	0	90	0	13	1	21	1	13
Nam Tong Tai village: total 27 households, 26 with at least one pig at time of vaccination.									
8	35	0	62	1	6	1	13	2	11

Table 3. Summary data, pig deaths at Nam Tin in three months after vaccination.

Village	Total number		Death rate (%)		Sold + eaten (%)	
	Vac.	Not vac.	Vac.	Not vac.	Vac.	Not vac.
Nam Tin	33	34	8	15	27	11
Phou Van Neua	51	272	4	10	26	18
Phou Van Tai	50	320	24	12	36	26
Nam Tong Neua	12	202	8	6	10	22
Nam Tong Tai	12	127	16	9	25	56
Totals and averages	158	955	13	10		

notable that there has been appreciable irregular swine fever vaccination in this area for some years.

Villagers tend to increase sales of pigs or to slaughter pigs (especially sick pigs) when epidemics reach a village. There is no evidence from the data presented that the death rate results were distorted by villagers selling or slaughtering sick, unvaccinated pigs; in fact more vaccinated pigs were sold or slaughtered than unvaccinated pigs (results for Nam Tong Neua and Nam Tong Tai should be ignored because of the very low rate of vaccination in both villages).

Nam Khok, Bo Keo

Pigs were vaccinated with swine fever vaccine in early June 1999 and every household surveyed in early September 1999[12] (see Table 4).

The death-rate in vaccinated pigs was 24% within three months of vaccination and 21% in unvaccinated pigs. This village is a more progressive village near the Provincial Headquarters, and irregular swine fever vaccination may have been practised for some years.

Laboratory analyses

In addition to the two samples sent from Nga Ba, Ky Son and positively identified as swine fever, another one sample was sent to the National Veterinary Research Centre, Hanoi, from the Ky Son District capital of Moung Xen in June 1999. This sample also returned positive for swine fever.

This is in contrast to the situation in Bo Keo, where, in the last two years, 20 samples from dead pigs were sent to the Animal Health Project in Vientiane. Only two of the 20 samples returned positive for swine fever. Most samples were collected from pigs in more accessible areas near the Mekong River, where some irregular swine fever vaccination has been conducted for some years.

[12] Pigs were vaccinated by Mr Khemsuk of the Provincial Livestock Office and surveyed by Mr Khammy of the same Office.

The swine fever and pasteurellosis vaccines used in the Noong De demonstration were sent to the National Centre for Examination of Veterinary Products in Hanoi. Both vaccines were reported to be effective.[13] It has also been reported that an NGO, Concern, has also tested swine fever vaccine in Bo Keo, but the results are not known at the time of reporting.

Conclusions and Recommendations

Results obtained so far are equivocal. In most instances, vaccination with swine fever vaccine with or without pasteurellosis vaccination does not appear to be beneficial. Much credence can be given to villager opinion that pig vaccination is ineffective. It is possible that prior to project intervention, pig vaccination would have been less effective than that reported above, as the projects went to considerable effort to ensure that the cold-chain was not broken.

The results obtained so far can be explained by the presence of both swine fever and other fatal epidemic diseases in the project areas. Swine fever vaccination may be effective when correctly stored and administered before the occurrence of a swine fever epidemic. But the death of pigs is still important from other as yet unknown diseases.

Swine fever vaccination appeared to be most effective in the most remote village where previous swine fever vaccination was unlikely (Nga Ba, but pasteurellosis vaccine was also injected in this village). It would be of interest to ascertain whether there is a difference in the prevalence of swine fever between villages related to the degree of previous swine fever vaccination. It would be of interest to monitor the death rate of penned and free-ranging pigs.

[13] See H. D. Phan in Gibson (1999), [unpubl.] Consultancy Report, Ky Son Pilot Project, UNDCP. The swine fever vaccine was tested by injection into rabbits whose temperatures were monitored. The pasteurellosis vaccine was tested by injection into rabbits which were then challenged with a known virulent strain of pasteurellosis.

Table 4. Swine fever vaccination results, Nam Khok village, Bo Keo.

Number present at vaccination				Number sold since vaccination		Number eaten since vaccination		Number died since vaccination	
Large pigs		Small pigs							
Vac.	Not vac.	Vac.	Not vac.	Vac.	Not vac.	Vac.	Not vac.	Vac.	Not vac.
96	33	49	79	60	0	0	0	24	21

Tentative recommendations for development projects in remote areas of south-east Asia include the following.

1. Continue close association with research projects such as the Animal Health Project in an attempt to identify the cause of pig diseases and effective control measures.
2. Continue subsidising vaccination demonstrations in villages with ear-tagged pigs and with detailed monitoring until such time as the pig vaccination can be shown to be consistently effective.
3. Retrain para-veterinarians (and officials) in improved vaccination procedures (cold-chain requirements, effective restraint methods, and effective vaccination hygiene).
4. Assist in providing the means for para-veterinarians to deliver vaccines in good condition to village pigs — provide bicycle transport, adequate cool-boxes, adequate needles).
5. Privatise the vaccine supply chain as much as possible so that vaccines reach the pigs in good condition. In Vietnam, this could be done by the semi-private vaccine-producing laboratory in Ho Chi Minh City opening agencies in cities near remote areas (Vinh City for Ky Son); if vaccination is effective it will be economical for village para-veterinarians to travel to the agencies to purchase vaccines. In Bo Keo, it could be done by village para-veterinarians purchasing directly from Thailand; there does not seem to be any easy way reliably to send vaccines from Vientiane to Bo Keo under consistently cool conditions.
6. Obvious research priorities for swine fever vaccines include:
   - the development of heat-resistant strains as per the heat-resistant Newcastle Disease strains developed for poultry under ACIAR auspices; and
   - the development of heat markers on vaccine bottles, as occurs with some human vaccines.

Reference

Visitpanich, T. and Falvey, L. 1980. A survey of the highland pig industry. Thai Journal of Agricultural Science, 13, 259–267.

Monitoring CSF Maternal Antibody in Piglets from Vaccinated Sows

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Abstract

Classical swine fever (CSF) is a contagious disease causing losses to the pig industry. Immunisation using CSF vaccine is a key control measure. Pregnant sows vaccinated with attenuated virus vaccine transmit antibody to piglets sucking colostrum, but it can be affected by the vaccine virus. Before the active immunisation function can be acquired, the piglet may be infected. This paper discusses monitoring of CSF maternal antibody for piglet as the basis for an immunity procedure.

IMMUNISATION using CSF vaccine is a key control measure against classical swine fever (CSF). Much research reports that pregnant sows vaccinated with attenuated virus vaccine produce enough antibody that piglets sucking colostrum receive the antibody to prevent CSF infection. But the maternal antibody can be affected by the vaccine virus.

If the vaccine is injected too late, the immunity efficiency is maintained, but the maternal antibody level may become lower or disappear. Before the active immunisation function is acquired, the piglet may be infected by CSF virus.

Monitoring CSF maternal antibody for the piglet is the basis of a reasonable immunity procedure. Different immunity and vaccine injecting procedures are currently used at pig farms. To guide husbandry production, it is necessary to research the technology of CSF immunity. Ten sows and their 80 piglets' antibodies have been tested at pig farms using an indirect aggulation test.

Materials and Methods

The swine tested

The swine tested were primiparous sows aged six months. The CSF antibody was tested twice using the indirect agglutation test before the experiment. Sows with antibody levels lower than dilute 1:2 were selected for the test. Fifteen days before mating, 10 sows were divided into two groups. One group of five was injected with attenuated virus vaccine cultivated by testicle cell. Two of the five were injected with the standard vaccine dose, two were injected with twice the standard vaccine dose, and one of the five with four times the standard vaccine dose. The other group of five was injected with CSF cattle body reaction vaccine, two with the standard vaccine dose, two with twice the standard vaccine dose, and one with four times the standard vaccine dose.

Vaccine

- CSF attenuated virus vaccine cultivated by calf testicle cell, Serial No. 9810, produced by Yunnan Biota Medicine Factory.
- CSF attenuated virus vaccine cultivated by cattle body reaction, Serial No. 9805, produced by Haerbin Veterinary Research Institute.

Materials for CSF indirect aggulation test

- CSF indirect aggulation test antigen: 5 mL per bottle.
- Standard positive serum 2 mL per bottle.
- Standard negative serum 2 mL per bottle.
- Dilute solution, 10 mL per bottle.

These reagents were offered by Lanzhou Veterinary Research Institute.

Serum samples

All serum collected from swine for testing was recorded according to ear number. After piglets were

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born, serum was collected and isolated at day 1, day 7, day 14, day 21, day 28, day 35 and day 42. Recorded detail included time, sow's number, serum number and piglet's number.

Collecting a 5 mL blood sample from the vena cava was the most practical means of isolating the CSF virus and serological testing at the laboratory.

Operating method

Testing was according to the indirect aggulation test procedure.

Results

Detecting sow antibody

Of the 10 sows, one was diseased and one died in Caesarean birth (the piglets could not suck colostrum). Their antibody level was determined two months before the experiment, a half month before mating and 48 hours after accouchement (see Table 1).

Table 1. Sow antibody lever detected by IHA.

Sow No.	2 months before test	Half month before mating		48 hours after accouchement	Memory
		Vaccine	Results		
1	1:4	A 1 mL	1:64	1:32	
2	1:2	A 1 mL	1:64	1:64	
3	1:2	A 2 mL	1:128	1:64	
4	1:8	A 2 mL	1:32	1:32	
5	—	A 4 mL	1:64	1:32	
6	1:2	B 1 mL	1:16	1:16	
7	1:4	B 1 mL	1:32	1:32	C
8	1:2	B 2 mL	1:64	1:32	
9	—	B 2 mL	1:2	1:8	D
10	1:2	B 4 mL	1:32	1:16	

Note: A = cell cultured vaccine was injected; B = cattle reaction vaccine was injected; C = died in Caesarean birth; D = diseased.

Piglet maternal antibody level detected at different ages

Sows were immunised using CSF attenuated vaccine cultured by testis cell and cultivated in cattle body, piglet serum collected and its maternal antibody at different ages calculated.

Piglet maternal antibody level decreased or disappeared with age (see Table 2).

Vaccinated sows and newborn piglets which did not suck colostrum

No. 7 sow had difficulty in calving. The antibody of her 13 piglets (born by Caesarean operation) was determined at day 2 and day 7. All results were negative.

In eight vaccinated sows, two had serum antibody level dilute 1:64, five had level dilute 1:32; one dilute 1:16. The geometric mean is dilute 1:32.

Table 3 shows piglet antibody level, determined after the piglets sucked colostrum. It indicates that even though the sows were immunised at the same time or had the same antibody level, even in piglets born to the same sow, the maternal antibody demonstrated was quite different.

Discussion

Because the No. 7 sow died, the 13 piglets born to it had no colostrum and the results of IHA are all negative. Other reports agree that IgG cannot pass placental circulation into the piglet body. It is therefore feasible to inject CSF vaccine before sucking, as the vaccine antigen will not conjugate with the maternal antibody.

Of newborn piglets acquiring maternal antibody by sucking, the level can reach dilute 1:256 at day 7, and the half-period is about 15 days. As age increased, the antibody level became lower. Its positive rate is 45% at day 21, 33.8% at day 28,

Table 2. Piglet maternal antibody level.

Age	Number	Positive number	Negative number	Antibody lever detected by IHA					
				1:8	1:16	1:32	1:64	1:128	1:256
48 hours	83	61	73.5	36	16	9	0	0	0
Day 7	80	76	95.0	0	6	9	17	14	30
Day 14	80	57	71.3	2	14	7	10	18	0
Day 21	80	36	45.0	9	15	8	4	0	0
Day 28	80	27	33.8	15	10	2	0	0	0
Day 35	80	15	18.8	11	4	0	0	0	0
Day 42	80	6	7.5	6	0	0	0	0	0

Table 3. The absorption of maternal antibody by newborn piglets.

Ear No.	Total No. piglets	*Time	IHA dilute	Piglet antibody level after sucking colostrum									
				48 hours					Day 7				
				1:16	1:32	1:64	1:128	1:256	1:16	1:32	1:64	1:128	1:256
1	11	4	1:64	0	3	8	0	0	0	0	1	6	4
3	10	4	1:128	2	4	4	0	0	1	1	4	6	0
5	8	4	1:32	1	3	4	0	0	1	2	4	1	0
6	12	4	1:16	0	7	5	0	0	0	4	2	3	2
10	10	4	1:32	0	4	2	4	0	0	0	5	2	3
	51			3	21	23	4	0	2	7	16	18	9

Note: Time means the gap between immunity and accouchement; IHA means sow antibody level following birth.

Table 4. Piglet antibody to different doses.

Dose	Item	Age				
		Day 7	Day 14	Day 21	Day 28	Day 35
Standard dose	Total	9	8	8	8	8
	Average	1:64	1:128	1:32	1:8	1:2
Twice standard dose	Total	10	11	9	9	8
	Average	1:32	1:256	1:64	1:32	1:2
Four times standard dose	Total	12	8	8	7	5
	Average	1:64	1:256	1:64	1:32	1:4

18.8% at day 35, and 7.5% at day 42. The possibility of infection by CSF virus increases with age. After 14 days, some piglet antibody levels reached only dilute 1:4 or 1:2, and a few had no antibody detected by this method.

Table 3 shows that the relationship between the maternal antibody level and the antibody acquired by the piglet is not correlated. The maternal antibody absorbed by the piglet is decided by how much specific IgG exists in the colostrum and what time the piglet sucked. So there exist differences among the piglets. The sows were vaccinated at the same time and had the same antibody level. Piglets born to the same sow show obvious differences in acquired maternal antibody. The reasons may be as follows.

First, each sow has different lactogenesis. Second, lactogenesis is different among the udders of the same sow, and the udder is stereotype for each piglet. Third, the performance of sucking and absorbing is different among piglets.

Table 4 suggests it is unnecessary to add an unlimited vaccine dose, as even a standard dose acts well.

Acknowledgments

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Classical Swine Fever Virus Sero-Epidemiology in Savannakhet Province of Lao PDR

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Abstract

An extensive survey took place in Savannakhet province of Lao PDR in 1999 to determine the prevalence of classical swine fever virus (CSFV) antibodies in vaccinated and unvaccinated pigs, using a simple random sampling (SRS) technique. The exercise was also used in training mechanism for local staff in survey techniques. In this paper, details of the technique and its results are presented and discussed.

SAVANNAKET is a province located in south/central region of Lao PDR sharing common borders with Thailand in the west and Vietnam in the east. A total of 15 districts comprising 1543 villages are located within the province. As of 1998, the human population of Savannakhet was 671 758 people with pig numbers reported as 187 400 head (Anon. 1999).

In March of 1999, a survey to determine the prevalence of classical swine fever virus (CSFV) antibodies in Savannakhet province of Lao PDR was undertaken. The primary objectives of the survey were to:

- determine the overall sero-prevalence of CSFV antibodies in the study area;
- determine the sero-prevalence of CSFV antibodies in unvaccinated pigs in the study area;
- determine the sero-prevalence of CSFV antibodies in CSFV vaccinated pigs in the study area;
- determine the level of CSFV vaccination in the study area;
- provide training for local staff in survey techniques.

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Four districts, Xaybuly, Khantabuly, Outhoomphone and Atsaphangthong, located close to the western border with Thailand, were chosen for the study.

Materials and Methods

Sampling parameters

Using the structured surveillance methodology of Cameron (1999), a single species survey of pigs in the Savannakhet province was undertaken. The location of Savannakhet province is shown in Figure 1. The selection of the survey parameters was chosen following the application of a two-stage sampling process that employed the simple random sampling (SRS) technique (Cameron 1999). The two-stage sampling process was employed to break the sampling into two steps:

- the selection of the villages; and
- the selection of animals within the selected villages, which provided flexibility to optimise survey accuracy and cost by the selection of the appropriate numbers of villages and animals.

To give an accurate estimate of CSF sero-prevalence in the province, the Survey Toolbox software (Cameron 1999) and the following parameters were employed.

Estimated prevalence, variance and costs were elucidated from results of a survey undertaken in Vientiane Municipality in March 1998. (Livestock

Figure 1. Location of Savannakhet province and survey district area in Lao PDR.

figures supplied by the Savannakhet provincial livestock office).

Estimated prevalence:	*21.1094%*
Within village variance:	*0.129570*
Between village variance:	*0.87995*
Ave population per village:	*40*
Total number of Villages	*407*
Cost per village:	*$20.00*
Cost per animal:	*$2.00*
Confidence:	*95%*

From the Survey Toolbox software it was determined that a random sample of a total 45 villages in the four districts of the province would be selected and that a 9.77% (rounded upwards to 10.00%) of pigs would be randomly selected for sampling in each selected village.

Logistics and strategy

A single team comprising one Animal Health Division member, one provincial staff member, one district staff member and a Village Veterinary Worker (VVW) undertook the survey over a 30-day period. One vehicle was required to undertake the work.

The villages to be sampled in the survey were informed approximately four weeks prior to the visit that a survey team would come to the village on a certain date. The afternoon prior to the visit, the appointment was confirmed ensuring that all livestock owners in the village would be in attendance. In the morning of the visit, a village interview was held, usually at the village headman's house that was attended by all livestock owners. A record of all pigs was made by the survey team including names of owners, species and age of animals. The Random Animal Section component Survey Toolbox software suite (Cameron 1999) was then used to randomly select 10% of the pigs in the village for sampling, the animals identified and recorded on a 'bleed sheet' and subsequently bled.

Classical swine fever complex-trapping-blocking ELISA

The classical swine fever complex-trapping-blocking ELISA (CSF CTB-ELISA) method developed by CSIRO Division of Animal Health, Geelong, Australia and the Elizabeth Macarthur Agricultural Institute (EMAI), Camden, Australia was employed to determine the antibody levels to CSFV in the survey sera.

The CTB-ELISA required the use two microtitration plates, a 96 well U-bottom polypropylene microtitre plate which was used as a liquid phase incubation plate (referred to as the LP plate) and a 96 well flat-bottom polystyrene microtitre plate used for the ELISA procedure (referred to as the ELISA plate) (Nunc, Denmark). The LP plate was blocked for potential immunoglobulin binding and incubated overnight at 4 °C. An ELISA plate was coated with goat anti-CSFV IgG at a dilution of 1 in 5000 and incubated overnight at 4 °C. Following incubation, the ELISA plate was washed 3 times followed by a blocking step.

The LP plate was divided into 3 equal sections (columns 1–4 [section A], 5–8 [section B] and 9–12 [section C], Figure 2). Eighty microlitres of serum dilution buffer (PBSA + 0.1% Tween 20 + 5% Normal Goat Serum (NGS)) was added to the four columns of section A of the serum dilution plate followed by the addition of the 20 μL of test serum. For every test, each plate contained a quality control (QC) titration. A QC serum was titrated in 2-fold steps from 1:5 to 1:160 (Figure 2). On the LP plate, 40 μL of the diluted sera was transferred to section B and 20 μL of the diluted sera to section C (antigen-free dilution), see plate format for details. CSFV antigen was diluted 1:5 in a 1% NP40 (v/v) in PBSA. Forty microlitres of the diluted antigen was transferred to all wells of section B serum dilutions and 20 μL of 1% NP40 solution without antigen was

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	9	17	QC	1	9	17	QC	1	9	17	QC
B	2	10	18	QC	2	10	18	QC	2	10	18	QC
C	3	11	19	QC	3	11	19	QC	3	11	19	QC
D	**Section A**		20	QC	**Section B**		20	QC	**Section C**		20	QC
E	5	13	21	QC	5	13	21	QC	5	13	21	QC
F	6	14	22	QC	6	14	22	QC	6	14	22	QC
G	7	15	23	Neg	7	15	23	Neg	7	15	23	Neg
H	8	16	24	Neg	8	16	24	Neg	8	16	24	Neg

Figure 2. Layout of samples and controls on LP plate for the classical swine fever complex-trapping blocking ELISA.

added to all wells of section C serum dilutions and incubated for 1 hour at 37 °C with shaking. Following incubation of the ELISA plate, 100 µL/well of blocking solution A was added to all wells and incubated for 1 hour at 37 °C with shaking.

For each plate, 340 µL of anti-p80 monoclonal antibody (MAb) was required which was adsorbed and diluted prior to use. The 340 µL of anti-p80 monoclonal antibody was mixed with 340 µL of Normal Pig Serum (NPS) and incubated at 37 °C for 15 minutes. Following incubation of the MAb/NPS mixture, 667 µL of the MAb/NPS mixture was added to 1333 µL of PBSA + 0.1% Tween 20. Forty microlitres of the diluted MAb/NPS mixture was added to each of the section B antigen dilution wells and 20 µL added of the diluted MAb/NPS mixture to each of the section C antigen-free dilution wells and mixed gently and incubated at room temperature for 30 minutes. At the completion of the incubation, the ELISA plate received a 3-cycle washing procedure and 50 µL of the serum/antigen/MAb/NPS solution transferred in duplicate to the ELISA plate (Figure 3) from each of the section B antigen serum dilutions on the LP plate. To one well on the ELISA plate (adjacent to serum/antigen/MAb/NPS dilutions), 50 µL from section C antigen-free dilutions on the LP plate were transferred to the ELISA plate and incubated for 1 hour at 37 °C with shaking. At the completion of the incubation, the ELISA plate received a 5-cycle washing procedure and 100 µL/ well of rabbit anti-mouse IgG – horseradish peroxidase conjugate at 1:1000 (Zymed, USA) in PBSGT and incubated for 60 minutes at 37 °C with shaking. At the completion of the incubation, the ELISA plate received a 5-cycle washing procedure and 100 µL/well TMB substrate was added and incubated for 10 minutes at room temperature and the reaction stopped with 50 µL of 1M H_2SO_4 and the optical density (OD) read at 450 nm using a microplate reader (Labsystems, Finland).

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	1	1	9	9	9	17	17	17	QC	QC	QC
B	2	2	2	10	10	10	18	18	18	QC	QC	QC
C	3	3	3	11	11	11	19	19	19	QC	QC	QC
D	4	4	4	12	12	12	20	20	20	QC	QC	QC
E	5	5	5	13	13	13	21	21	21	QC	QC	QC
F	6	6	6	14	14	14	22	22	22	QC	QC	QC
G	7	7	7	15	15	15	23	23	23	Neg	Neg	Neg
H	8	8	8	16	16	16	24	24	24	Neg	Neg	Neg

Figure 3. Layout of sample/antigen/monoclonal antibody mixtures when transferred to the ELISA plate for the classical swine fever complex-trapping-blocking ELISA. Columns 1–2, 4–5, 7–8 and 10–11 contain antigen and columns 3, 6, 9 & 12 are antigen free.
QC = Quality control serum titration. Neg = Negative.

Determination of samples containing anti-immunoglobulin (anti-Ig) or rheumatoid-like factor activity is by the presence of color in the antigen free well. If the anti-Ig factor result exceeds the negative control (i.e. OD max) antigen-free percentage inhibition result by greater than 25%, the sample result was considered invalid therefore discounted.

Determination of the CSFV antibody status of the sample was accomplished by calculation of the percentage inhibition (PI).

$$PI = 100 - \frac{\text{Mean Sample OD}}{\text{Mean Negative Control OD}} \times 100$$

Interpretation of the result was as follows. Samples with a PI <15% were considered negative, >15% but <25% were considered suspect and samples with a PI >25% were considered CSFV antibody positive.

Calculation and presentation of survey results

Survey results to determine the overall and district sero-prevalences were calculated using the Survey Toolbox software and maps were constructed using Arcview 3.0 software (ESRI, USA).

Results and Discussion

The villages sampled, pig numbers and the numbers of pigs selected in village are presented in Table 1 and overall seroprevalence results for each of the districts are presented in Table 2. Overall, in the four districts studied, 11.94% of the pigs were CSFV antibody positive. In Khantabouly district, 16.28% were positive, Xaybuly district 19.23% were positive, Outhoomphone district 7.98% were positive and Atsaphangthong district 3.53% of the pigs were CSFV antibody positive. A map indicating the percentage CSFV sero-prevalence in each sampled village is presented in Figure 4. In the study area, of the 204 pigs surveyed, 195 of the pigs were unvaccinated for CSFV. Overall, 12.05% of the pigs were CSFV antibody positive. In Khantabouly district, 17.44% of the pigs were positive, Xaybuly district, 16.35% of the pigs were positive, Outhoomphone district 7.98% of the pigs were positive and Atsaphangthong district 3.53% of the pigs were CSFV antibody positive.

With regard to CSFV vaccinated pigs in the study area, of the 204 pigs surveyed, only 9 (4.44%) of the pigs were vaccinated for CSFV of which 4 (44.4%) gave an antibody response greater than the CSF

Table 1. Survey village names and locations. Pig population and the number of pigs selected using randomisation techniques are also presented.

Village	District	Pig Pop.	No. selected	Village	District	Pig Pop.	No. selected
Ban Nongden	Khanthabouly	23	2	Ban Nalaikhok	Atsaphangthong	72	7
Ban Congkan	Khanthabouly	16	2	Ban Sisaphanxai	Atsaphangthong	22	3
Ban Dangdhamdoun	Khanthabouly	19	2	Ban Dongmarkhon	Atsaphangthong	59	6
Ban Yang	Khanthabouly	74	7	Ban Lhaofigth	Xaybuly	20	2
Ban Phosy	Khanthabouly	18	2	Ban Nhaoneua	Xaybuly	53	6
Ban Phonesavang	Khanthabouly	100	9	Ban Nhonsesavane	Xaybuly	103	10
Ban Nalehao	Khanthabouly	17	2	Ban Manilad	Xaybuly	18	2
Ban Ladsavangxai	Khanthabouly	35	4	Ban Veuneneua	Xaybuly	46	5
Ban Nhonsavath	Khanthabouly	47	5	Ban Hathsaishohongthai	Xaybuly	19	2
Ban Phonesemthai	Khanthabouly	145	15	Ban Nhongyang	Outhoomphone	14	2
Ban Dongkhamluang	Khanthabouly	29	3	Ban Pharkhayna	Outhoomphone	60	6
Ban Namphouthai	Khanthabouly	41	5	Ban Khokneua	Outhoomphone	46	5
Ban Muangklaineua	Khanthabouly	103	10	Ban Thampha	Outhoomphone	12	2
Ban Phonsomehong	Khanthabouly	82	9	Ban Nakhemekang	Outhoomphone	15	2
Ban Phonesykeo	Khanthabouly	26	3	Ban Phonnakhaune	Outhoomphone	28	3
Ban Phonethan	Khanthabouly	51	6	Ban Nhonehang	Outhoomphone	23	3
Ban Phalai	Atsaphangthong	14	2	Ban Nakhilekngai	Outhoomphone	24	3
Ban Nongsay	Atsaphangthong	19	2	Ban Nakham	Outhoomphone	31	3
Ban Naphangkhok	Atsaphangthong	40	4	Ban Phonesavang	Outhoomphone	26	3
Ban Cheilamonthai	Atsaphangthong	87	9	Ban Phonethoune	Outhoomphone	47	5
Ban Tabongphet	Atsaphangthong	28	3	Ban Dongmargneve	Outhoomphone	8	2
Ban Nhongpaksong	Atsaphangthong	74	7	Ban Manivingthai	Outhoomphone	3	2
Ban Nhonhang	Atsaphangthong	73	7	Total		1910	204 (10.68%)

Table 2. Results of overall and unvaccinated CSFV antibody prevalence in survey districts of Savannakhet.

District	Overall Prevalence (%)	Overall Variance	Overall 95% CI	Unvaccinated Prevalence (%)	Unvaccinated Variance	Unvaccinated 95% CI
Khantabouly	16.279	0.000006	15.797–16.762	17.442	0.000008	16.873–18.011
Xaybuly	19.231	0.000005	18.777–19.685	16.346	0.000006	15.870–16.823
Outhoomphone	7.982	0.000001	7.750–8.314	7.982	0.000001	7.750–8.314
Atsaphangthong	3.525	0.000012	2.841–4.210	3.525	0.000012	2.841–4.210
Overall	11.923	0.000002	11.659–12.187	12.049	0.000002	11.754–12.344

CI = Confidence Interval.

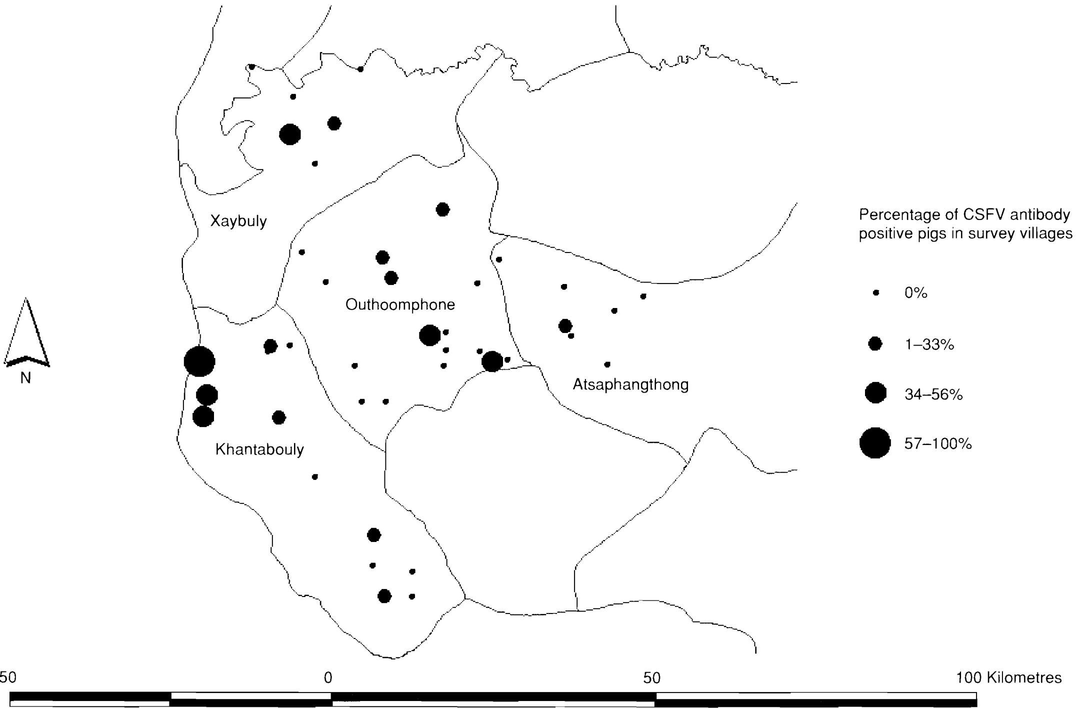

Figure 4. Geographical representation of the prevalence of CSFV antibodies in villages in the survey area.

CTB-ELISA screening dilution of 1:8. The overall percentage of CSFV vaccination was very low (Figure 5) as vaccination was only practiced in 2 of the 45 (4.44%) villages surveyed. Reasons for low levels of participation in vaccination programs in Lao PDR may be due to a number of reasons. Sodarak (1998) suggested that diminished credibility of vaccines to unexplained livestock deaths, problems with vaccine cold chain resulting in compromised vaccines, erratic supply of vaccines, low levels of interest in pig vaccination and a lack of training of VVWs contributed to the low overall vaccination numbers.

Given the overall low level of CSFV vaccination, there was still a significant level of CSFV antibodies observed in unvaccinated pigs. The majority of these antibodies were observed in pigs under 6 months of age (Figure 6) accounting for a total of 4.93% of the

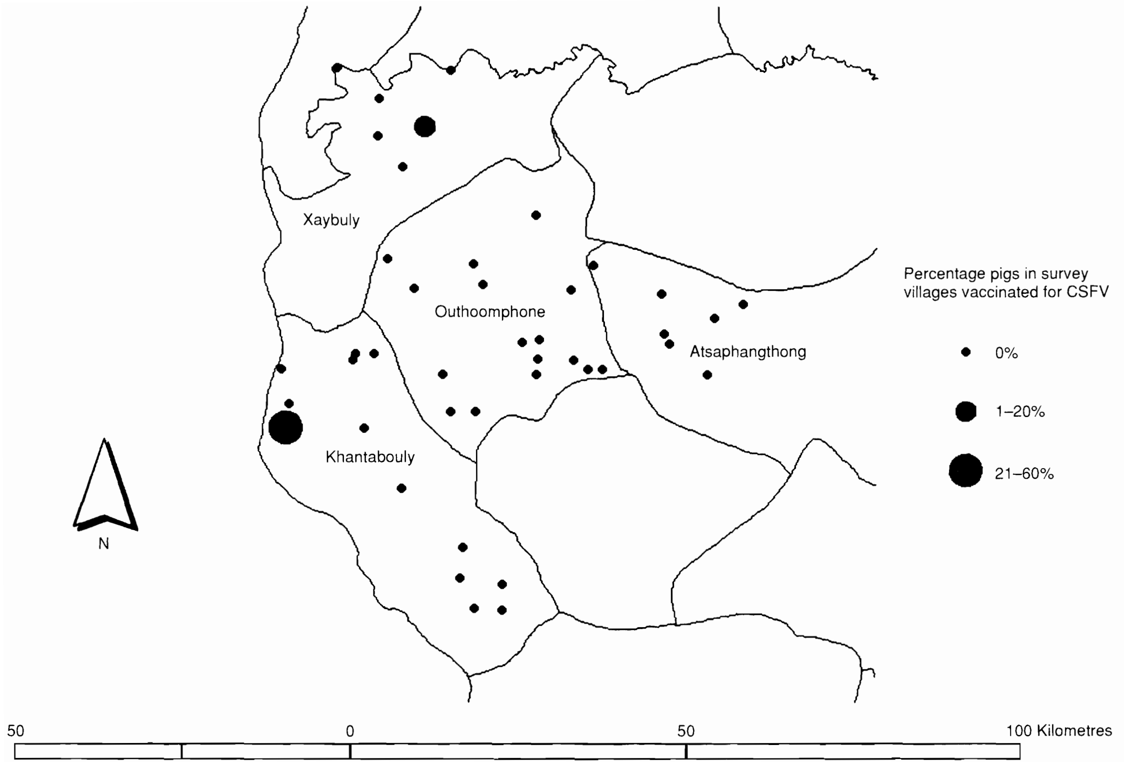

Figure 5. Geographical representation of the percentage of pigs in the survey villages that are CSFV vaccinated.

total unvaccinated CSFV positive pigs. Nevertheless, in the age range 9–10 months, there was a higher than expected number of unvaccinated CSFV-seropositive animals (3.45%) that may be accounted as CSFV persistently infected animals.

To ensure that a representative sample of animals was assessed during the serological surveillance, randomisation techniques were employed to provide confidence in the serological prevalence results. Cameron (1999) investigated the use of active surveillance techniques.

Methodologies were employed for the planning and analysis of the serological surveys. To calculate the number of villages and the proportion of animals from each village, Survey Toolbox (Cameron 1999) was used to perform requisite calculations following the input of necessary parameters.

The SRS technique is considered to be the most appropriate sampling technique when there is a reliable sampling frame for the villages in a district (as is the case in Lao PDR) but the livestock population data are unreliable (also the case in Lao PDR) (Cameron 1999). During the survey, we found the Survey Toolbox software suite and randomisation methodologies for disease surveillance to be suitable for such undertakings in Lao PDR.

Acknowledgments

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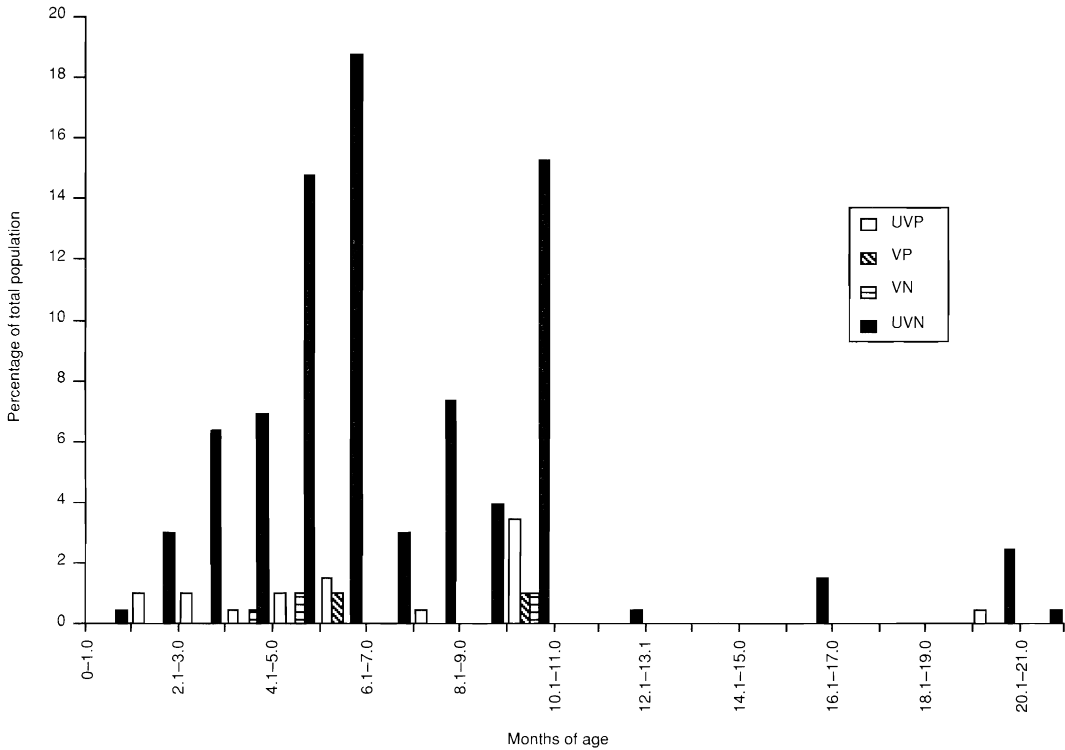

Figure 6. A graphical representation of pig age versus CSFV immune status relative to vaccination.
UVP = Unvaccinated Positive. VP = Vaccinated Positive.VN = Vaccinated Negative. UVN – Unvaccinated Negative

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Clinical, Pathological and Diagnostic Aspects of Classical Swine Fever

K.R. Depner[1] and S. Porntrakulpipat[2]

Abstract

Chronic CSF virus infection has been defined as a lethal disease with a duration of 30 days or more. In adult animals, CSF virus often induces only transient infections with few mild clinical and pathological signs. CSF virus has the ability to cross the placental barrier and to infect foetuses. The laboratory diagnosis of CSF is based on detection of viral antigen, isolation of virus, demonstration of virus-specific antibodies and detection of viral RNA. In the case of virus isolation or antigen detection, the advantages of monoclonal antibodies of different specificity are used to allow an unambiguous differentiation between field and vaccine strains of CSF virus, or between CSF virus and other pestiviruses.

CLASSICAL swine fever (CSF) is a fatal viral disease affecting pigs and wild boar. The infection causes major economic losses especially in countries with an industrialised pig production system. Eradication programs of the past decades have been successful to a great extent in North America, Australia and some European countries.

The causative agent—the CSF virus—is an enveloped RNA virus and belongs to the Pestivirus genus of the *Flaviviridae* family. It is related to bovine viral diarrhoea (BVD) virus of cattle and border disease (BD) virus of sheep. This relationship may have severe diagnostic consequences as cross-reactions do occur and can lead to false positive results.

The CSF virus is relatively stable in moist excretions of infected pigs and fresh meat products, including ham and salami type sausages. It is readily inactivated by detergents, lipid solvents, proteases and common disinfectants.

The clinical course is determined largely by a variety of host factors. However, the severity of CSF virus infections has often been related solely to the virulence of the virus involved. Highly virulent, moderately virulent as well as avirulent CSF virus strains or isolates have been described. This classification was made by taking into account mortality rates as well as epidemiological and clinico-pathological findings. However, viral virulence alone does not determine the course of the disease. The age of the pig at the time of exposure, its immunological reactivity as well as the dose of the infecting virus were shown among other factors to be of utmost importance. In addition, variations in nutrition and immunological or biochemical polymorphism of the infected pigs might be important factors.

CSF has been categorised according to the time of infection as pre and post-natal infection or according to the clinical course as peracute, acute, subacute, chronic and late-onset form. However, it has to be kept in mind that under field conditions on a herd basis several forms of CSF are occurring simultaneously and the overall clinical picture might be rather confusing. The chronic and the late onset diseasse especially, which are persistent infections, are often difficult to diagnose clinically and pathologically. These forms are mainly responsible for the perpetuation and survival of the CSF virus in the pig population.

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Pathogenesis of CSF

Under natural conditions, the mode of entry of CSF virus in the animal is the oronasal route and the tonsils are the primary site of virus replication. CSF virus was demonstrated in the tonsils as early as seven hours post exposure. No virus has been detected in other tissues of the oral cavity within 24 hours following oral exposure. It is interesting to note that, after intramuscular and subcutaneous application, CSF virus was found most consistently and in a continuously high concentration in the tonsils.

The virus initially infects epithelial cells of the tonsillar crypts and subsequently spreads to the surrounding lymphoreticular tissue. From the tonsils, CSF virus is transferred via lymphatic vessels to the lymph nodes draining the tonsillar region. After replication in the regional lymph nodes the virus reaches all other organs of the body via the blood. High titres of virus have been measured in spleen, bone marrow, visceral lymph nodes and lymphoid structures lining the small intestine. As a result of virus multiplication in lymphoid tissue and in circulating leukocytes and mononuclear cells, the virus titer in the blood during viraemia might be very high. The virus probably does not invade parenchymatous organs until late in the viraemic phase. Generally, CSF virus titres in lymphoid tissues are higher than in parenchymatous organs. The spread of virus throughout the pig is usually completed in 5–6 days.

It has been shown that CSF virus affects the immune system. A main characteristic is generalised leukopenia which is usually seen before the onset of fever. From immunofluorescene studies, there is ample evidence that the CSF virus has a distinct affinity to cells of the lymphoreticular organs. In these organs, the virus causes severe depletion of lymphocytes affecting both B cell and thymus-dependent areas. Thymus and bone marrow atrophy, as well as generalised depletion of lymphocytes, have been recorded wherein a B lymphocyte deficiency was prominent.

Postnatal Infection

Peracute, acute and subacute form

The severity of the postnatal infection (Figure 1), which is the most common form, depends mainly on the animal's age at the time of exposure, its immunological reactivity as well as the virulence of the virus. Today, the peracute form of the disease appears to be the exception and acute and subacute forms are seen predominantly.

Pigs with peracute CSF show little more than a rise in body temperature to about 41 °C before they die 2–5 days after exposure to the virus. In these cases, post-mortem changes are essentially those of shock with pulmonary congestion and edaema and congestion of the liver and gastro-intestinal tract. Usually, there is little evidence of haemorrhage.

In acute cases, early symptoms like high body temperature (>41 °C), inappetence and apathy can be seen after an incubation period of about 3 to 7 days (Figure 2). The progression of the disease is marked by conjunctivitis, nasal discharge, intermittent diarrhoea, swollen lymph nodes (Figure 3) and muscle tremor. The terminal stage is characterised by 'typical' skin cyanosis mainly on ears, nose, tail and abdomen and skin haemorrhages of different grades (Figures 4, 5) predominantly over bone protuberances. Sick animals show weakness of the hind legs (waving or staggering gait) which is often followed by a posterior paresis. Leukopenia and thrombocytopenia are common findings. The predominant post mortem findings consist of haemorrhagic diathesis and swollen, haemorrhagic lymph nodes. However, the severity of pathological lesions can vary widely (Figures 6, 7).

Animals which do not die within four weeks of infection either become convalescent developing high titres of neutralising antibodies or they become chronically ill remaining persistently infected with CSFV.

Chronic CSF

The chronic CSF virus infection has been defined as a lethal disease with a duration of 30 days or more (Figure 8). Based on the clinical signs, three phases of chronic CSF can be distinguished. In the first phase of illness, anorexia, depression, elevated temperature, and leukopenia are present. After several weeks, the appetite and general appearance of the pigs improve and the temperatures decrease to normal or slightly above normal values. Leukopenia usually persists. This general clinical improvement is characteristic for the second phase of illness. In some animals, low titres of neutralising antibodies can be detected during this phase which is about one month after infection. In the third phase, pigs again become anorectic and depressed. Intermittent fever is noticed while the body temperature seldom rises above 41 °C. Growth retardation, wasting and diarrhoea are the most evident signs. Such pigs have skin lesions, and frequently stand with arched backs (Figure 9). Pigs with chronic CSF may survive for more than 100 days. Secondary bacterial and parasitic infections are frequently involved. Thus, the clinical picture may often be uncharacteristic and misleading. There is little evidence of petechial haemorrhage in chronic CSF. In the large intestine, button ulcers are

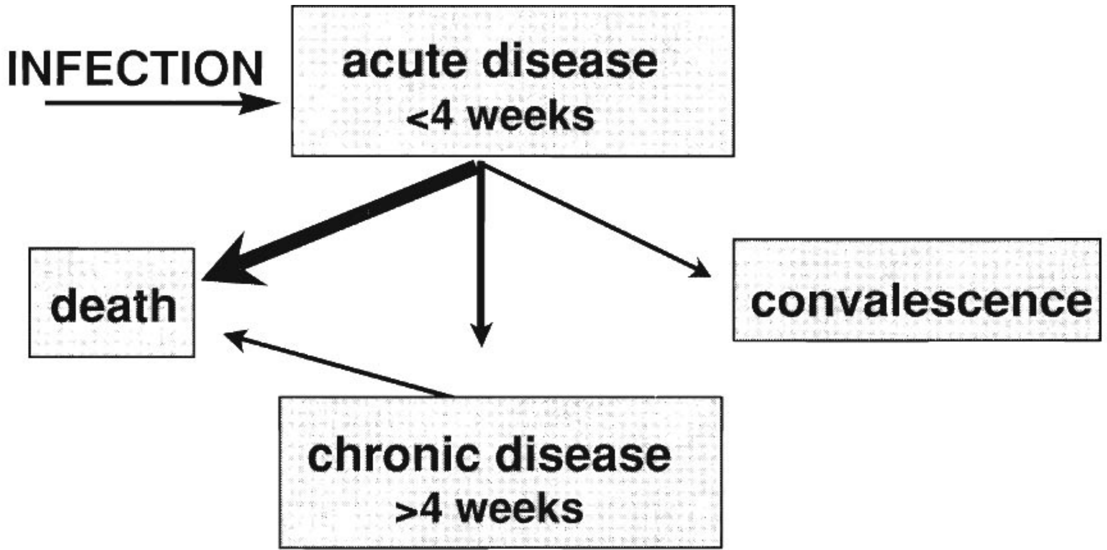

Figure 1. Clinical course of postnatal CSF.

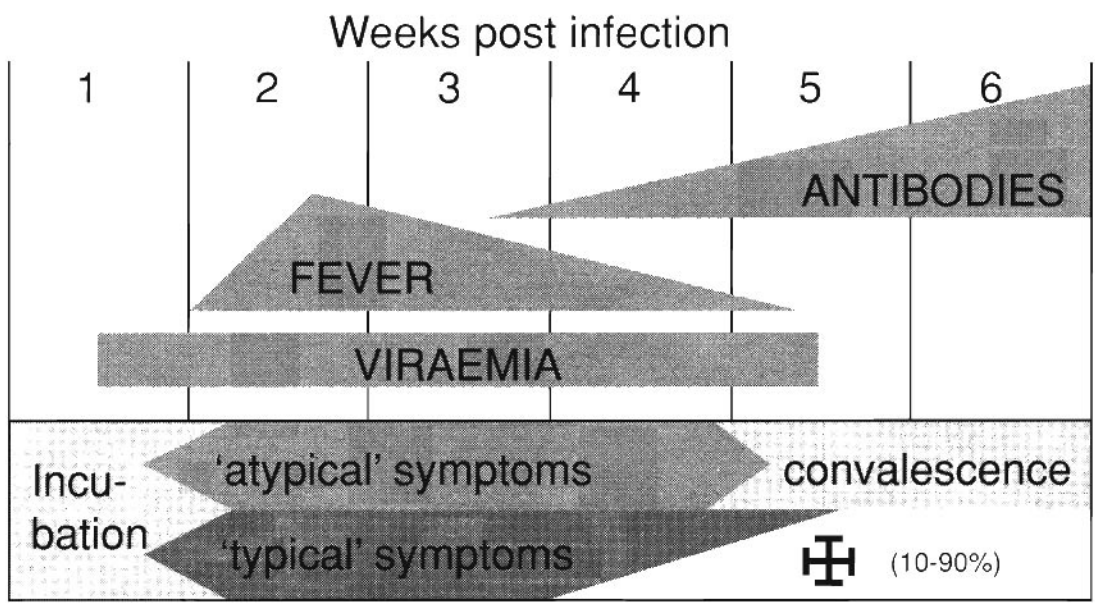

Figure 2. Acute course of CSF.

Figure 3. Acute CSF, swollen inguinal lymph nodes.

Figure 4. Acute CSF, Map-like confluent haemorrhages.

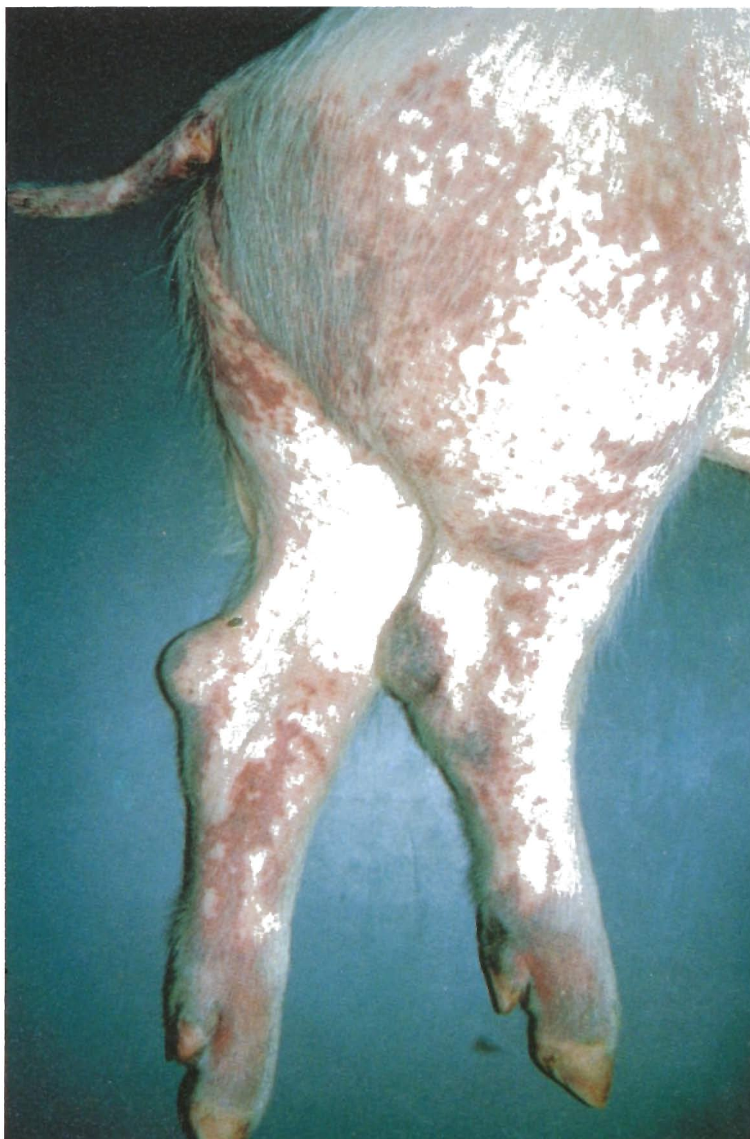

Figure 5. Acute CSF. Map-like confluent haemorrhages.

Figure 6. Acute CSF, haemorrhages on different organs.

Figure 7. Acute CSF, epiglottal haemorrhages.

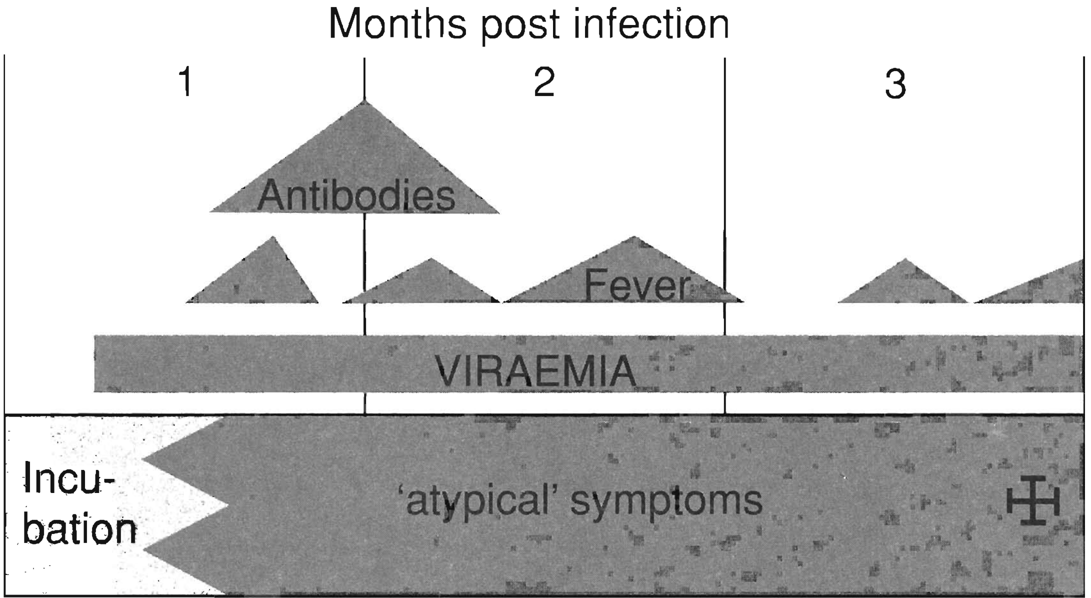

Figure 8. Chronic course of CSF.

Figure 9. Pig with chronic CSF.

occasionally seen which are followed by more diffuse diphtheroid-necrotising enteritis (Figure 10). The lymph nodes show only hyperplasia.

Although the chronic form of CSF is a rather rare event, it might play an important role in the spreading of the infection since these persistently infected pigs continue to excrete CSF virus.

Prenatal Infection and Late Onset Form

In adult animals, CSF virus often induces only transient infections with few mild clinical and pathological signs. In seronegative pregnant sows, reproductive failure frequently occurs. CSF virus has the ability to cross the placental barrier and to infect the foetuses. The outcome of transplacental infection depends on the gestational stage and the immunocompetence of the foetus. The infection can result in embryonic or foetal death leading to abortions, teratogenic malformation of the foetuses, mummification, stillbirth, neonatal death as well as in clinically healthy offspring persistently infected with CSF virus (Figure 11). Stillborn and/or mummified animals may be present along with viraemic and non-infected piglets in one litter (Figure 12). Early infections of pregnant sows (before day 41 of gestation) usually result in abortions and stillbirths, whereas later infections (before day 85 of gestation) yield viraemic animals. The development of the immune system enables the foetus to combat infections after about day 85 of gestation. Inoculation of virus after day 85 of pregnancy usually yields normal, non-viraemic animals. However, viraemic animals can also be induced with low virulent virus strains when foetuses get infected during the late days of pregnancy or even shortly after birth. That is probably equivalent to the chronic form in postnatal infection.

Only persistently viraemic piglets which became infected in utero develop the *late onset* disease. This form of infection is not always clinically and pathologically evident. It is characterised by an initial, relatively long period during which viraemic piglets remain free of disease. Colostrum-derived antibodies can only be detected for a short time compared to non-viraemic litter mates. Not until a few weeks after birth the piglets develop mild anorexia and depression, conjunctivitis, dermatitis, diarrhoea, and locomotive disturbances leading to posterior paresis. Lesions characteristic of acute CSF, particularly petechial haemorrhages, are not present. Body temperatures are normal. Growth retardation is the most common finding (Figure 13). These viraemic piglets are permanently shedding virus until they die. They can play a key role in the spread of CSF virus within the pig population. Most pigs survive (unrecognised) for several weeks, but all eventually die. The longest survival time for a viraemic piglet has been reported to be 11 months.

Diagnosis of CSF

A tentative diagnosis of CSF can be made in the field if a thorough history is obtained and clinical signs and necropsy lesions are carefully observed. Important points in the history are recent introduction of pigs into the herd, swill feeding, fertility problems (e.g. abortions, still births) or high mortality rate.

In acute CSF infection, a marked leukopenia is associated with the initial rise in body temperature. The leukopenia persists until death or recovery and is found in chronic CSF infection as well. Tonsils, mandibular lymph nodes and spleen are the most appropriate tissues for virus detection.

The laboratory diagnosis of CSF is based on detection of viral antigen, isolation of virus, demonstration of virus-specific antibodies and detection of viral RNA. In case of virus isolation or antigen detection, the advantages of monoclonal antibodies of different specificity are used to allow an unambiguous differentiation between field and vaccine strains of CSF virus, or between CSF virus and other pestiviruses.

For antigen detection the direct immunofluorescence test (FAT) is widely used. The test is both rapid and reliable, and is employed to detect CSF virus in cryostat sections of tonsils, spleen, lymph nodes, kidney or ileum. The result can be obtained within 2 hours after the specimens reach the laboratory.

Isolation of CSF virus in cell cultures is regarded as the most sensitive method but more time consuming than FAT. Virus isolation can provide confirmation in cases where the DIF is suspicious. The result can be obtained 3–5 days after the specimens have been processed. The buffy coat and homogenised lymphatic tissues are the materials of choice for virus isolation.

For large-scale screening, antigen-capture enzyme-linked immunosorbent assays (ELISA) have been developed. These ELISA have a good specificity and require less-specialised facilities and can be performed much more rapidly than virus isolation. However, their sensitivity is below the sensitivity of FAT or virus isolation on cell culture. Reliable results are obtained only with samples from pigs already manifesting clinical signs (e.g. fever). Therefore, the use of antigen ELISA has to be linked to the clinical examination of the animal. It makes no sense to use antigen ELISA for screening healthy pigs. Due to lower sensitivity, the antigen-capture ELISA can be used only on a herd basis.

Figure 10. Chronic CSF, button ulcers.

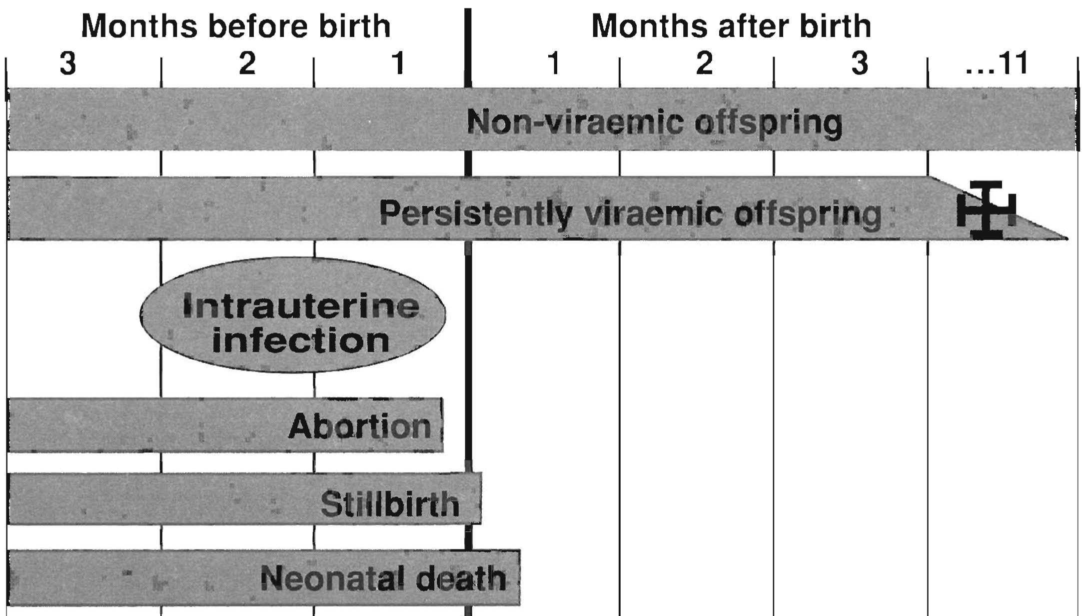

Figure 11. Congenital CSF.

Figure 12. Mummified foetuses.

Figure 13. Persistently infected piglets (late onset of CSF).

The development of molecular biology has contributed to the introduction of highly sensitive and new diagnostic approaches. Reverse Transcription Polymerase Chain Reaction (RT-PCR) is nowadays a standard method for CSF diagnosis. The sensitivity and specificity of RT-PCR is claimed to be comparable to that of virus isolation or even better. RT-PCR, however, is at present not suitable for screening large numbers of samples.

In regions were vaccination against CSF is not applied, antibody detection is a useful and complementary diagnostic tool for epidemiological investigations and for suspected farms where the usual virus detection procedures have failed. Several tests are available for the detection of CSF antibodies. The *fluorescent antibody virus neutralisation test* and the *neutralising peroxidase-linked assay* are the most commonly used techniques.

Although the neutralisation tests are considered to be the most sensitive and specific tests, they are time consuming and not suitable for automated systems and large-scale screening. To achieve these purposes, several ELISA techniques using specific monoclonal antibodies have been developed and are commercially available. The tests are essentially based on two formats: non-competitive ELISA and competitive or blocking ELISA. The sensitivity of the ELISA is regarded to be below the sensitivity of the neutralisation tests. Therefore, the ELISA can be used only on a herd basis. The ELISA technique requires less-specialised facilities and can be performed much more rapidly due to automated systems than the neutralisation test. Large numbers of sera can be examined within a short period of time. All ELISA systems are subject to occasional difficulties in interpretation. It is therefore essential to have access to a neutralisation test for investigation of sera which gave inconclusive ELISA results.

Acknowledgment

The authors wish to thank the Community Reference Laboratory for CSF, Hannover, Germany, for providing most of the Figures (Figures 2–10).

Strategies for Epidemiological Surveys in Developing Countries

Angus Cameron[1]

Abstract

Accurate information on the health of livestock populations is important to enable veterinary decision-makers to target appropriately disease control activities. This information is most commonly collected by passive surveillance techniques, which suffer from a number of weaknesses. Active surveillance, or the use of structured epidemiological surveys, can overcome these weaknesses, but only if appropriate survey design and sampling procedures are used. This paper examines the requirements for epidemiological surveys in developing countries, and discusses the costs and benefits of techniques for collecting data truly representative of the population under study.

LIVESTOCK diseases continue to have a major impact on the livelihoods of the rural population in many developing countries around the world. Not only epidemic diseases like haemorrhagic septicaemia, foot-and-mouth disease, Newcastle disease of poultry and classical swine fever of pigs, but chronic and subclinical diseases such as parasitism have a serious impact on livestock production. In countries like Lao PDR, a large proportion of the GDP is derived from agriculture, and a significant proportion of that is from livestock. Decreased production due to disease may therefore hamper national development and the prospects of international trade. More important, however, is the impact of disease on the individual smallholder farmers. Livestock may be a source of food (meat, eggs, milk), fuel and fertiliser (dung), draft power, and a form of savings. Death of livestock or decreased production due to disease can have a devastating impact at the individual farmer level.

Controlling livestock diseases in developing countries has the potential to bring improved security and poverty alleviation to the rural population through decreased losses and increased production from livestock. The task of livestock disease control in developing countries is hampered by problems of inadequate resources, funding, skills, transport and communication infrastructure and farmer awareness, etc. These problems, particularly the often severe lack of funds to carry out extensive disease control activities (e.g. vaccination programs) mean it is imperative that available funds are utilised as effectively as possible. Wastage of resources in any situation is regrettable, but when resources are critically scarce, all efforts must be made to ensure that wastage is minimised.

The way to avoid wastage of resources for livestock disease control is to target available resources to areas of need, and in such a way that they have the greatest possible beneficial impact. Targeting the use of available resources appropriately is the responsibility of the veterinary services managers (decision-makers) and requires a wide range of information, which may include:

- what diseases are present;
- where they are present;
- what the impact of these diseases is; and
- what control strategies are available to combat them.

The quality of the decisions, and therefore the efficiency with which resources are targeted, depends on the quality of the information on which the decisions are based. To ensure appropriate decisions, there are criteria which information used by decision-makers should meet, ideally:

- precise—the information on the level of disease or other factors should not be broad approximations. They should be not only reasonably precise, but also of known precision, so that we can say, with

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a certain probability or confidence, in what range the true value lies;
- unbiased—the information should be close to the true value;
- population-based—the information should relate to, and be representative of, the population as a whole, not just a portion of it;
- timely—information should be up to date, reflecting the current situation; and
- relevant –pertaining to current problems.

The collection of this information is often done incidentally, or as an afterthought, leading to information of less than ideal quality. There is also often resistance among veterinary services to expending resources on the collection of information, based on the attitude that it takes resources away from the core work of the organisation (e.g. extension, clinical services or vaccination programs). However, without the appropriate information, other activities may be being conducted in an inefficient or indeed wholly inappropriate way, and achieving little benefit.

A simple example of the need for appropriate information is provided by a foot-and-mouth disease vaccination program. Different serotypes may be responsible for the disease. Vaccines are serotype-specific, offering no cross-protection for other serotypes. It is not uncommon to have multiple serotypes present in the same area. One type of information required by decision-makers is which serotype or serotypes are causing the disease. Commonly the decision is based on a limited number of samples which may not be representative of the entire population. This could lead to the selection of a vaccine that fails to offer protection against one or more of the serotypes causing disease.

This provides an example some of the problems in the most common approach used in all countries to collect information about animal health—*passive surveillance*. In passive surveillance, a report or piece of information on livestock diseases is initiated by somebody other than the veterinary services, such as the livestock owner. The owner, in response to noticing a disease in his animals, notifies the local veterinary services. This may result in an investigation, samples being collected, and a report or laboratory submission being made. In passive surveillance, veterinary authorities have little control over where reports originate or how often they are made, as they depend on the observations of people outside the veterinary services.

Passive surveillance systems are useful to gain a general picture of important diseases present, to respond to disease outbreaks, and to identify the incursion of new diseases. However, information collected through a passive surveillance system is usually inadequate for the purposes of informed decision-making and targeting resources, because it does not meet the criteria listed above, and commonly suffers from the following problems.

- Under-reporting—many relatively minor or chronic diseases may never be reported, as farmers believe that they are normal or that nothing can be done about them. Even with more significant epidemic diseases, reporting levels may be as low as five per cent due to communication difficulties or lack of public awareness. Under-reporting means that the true level of disease and the true impact of the disease cannot be calculated.
- Slow reporting—passive disease reports are often very slow to travel from the farmer to the decision-makers. A major disease outbreak may be virtually over before veterinary authorities are informed. This makes control, and indeed investigation, very difficult.
- Bias—various parts of the population are much more likely to report disease than others. This includes farmers closer to major centres with good transport and communication infrastructure, as well as richer and better educated farmers. As a result, the pattern of disease reports does not reflect the true distribution in the population. It may lead to incorrect conclusions, for example, that disease is more common among larger herds or in certain intensively farmed areas.
- Unable to calculate rates or prevalences—many measures of disease require a knowledge of the underlying population. Passive surveillance provides only information about reported disease events, but gives no information on the population in which the disease events occurred. This makes it impossible to compare the level of disease in one area with that in another, making targeting disease control activities more difficult.

Epidemiological Surveys

Active surveillance describes data collection in which the veterinary authorities initiate the activity. Because the collection of information is controlled by the people who will use the information, it is possible to ensure that its quality is high and that it provides the type of information needed for informed animal health decision-making. One of the most common and useful forms of active surveillance is the use of epidemiological surveys.

Surveys are structured data collection exercises that aim to gather information that can be used to answer particular questions. Surveys can be designed and executed by veterinary services staff, and so the way in which data are collected, and its quality, can

be closely controlled. Surveys generally have quite a specific objective, such as to determine the prevalence of a particular disease, to establish whether a disease is present in a country or province, or to measure the impact of a disease.

Surveys may produce data of high quality that meets all the criteria for the provision of useful information listed above. However, to achieve it, they must be carefully designed and executed. Poorly designed surveys can suffer from many of the same problems as passive data collection systems. In fact, a poorly designed survey can cause more damage than inaccurate passively collected data, for two reasons. Firstly, surveys cost money and occupy staff time and resources. If the information derived from a survey does not reflect the true situation, and does not improve the ability of decision-makers to manage livestock disease problems, then money and resources have been wasted. Secondly, it is widely recognised that information from surveys is more reliable than information collected through passive surveillance. If a poorly designed survey produces an incorrect result (one which does not reflect the real disease situation in the field), decisions made and actions taken on the basis of those results could well fail to achieve useful disease control, and result in a further wastage of scarce resources.

The desirable characteristics of a disease survey for developing countries are that the survey is:

- rapid—surveys that require long periods of time for data collection are often too expensive to implement, and provide results that are out of date by the time they are available;
- inexpensive—surveys require money to run. If a survey is to be used in a developing country, its cost must be kept as low as possible, while still meeting the need for quality information;
- precise—information should not be too approximate. Precision is largely related to the sample size which, in turn, is related to the cost and duration of a survey. It may often be appropriate to design a less precise survey in order to make it faster and cheaper;
- unbiased—this is one of the most important characteristics. The results of the survey must be correct, in that they reflect the true situation in the field. Any significant biases have the potential to completely undermine the value of the survey, and to result in poor decisions. The most significant cause of bias in livestock surveys in developing countries is through inappropriate sampling techniques; and
- practical—the best-designed survey is of no use in a developing country if it cannot be practically implemented. Survey designs must be a compromise between collecting the best possible quality data and working within the constraints of the local environment.

A range of survey designs for specific purposes has been developed to meet the needs of developing countries (Cameron 1999). Various aspects aim to ensure practical application, that surveys can be conducted quickly and at low cost. However their most important aspect is the techniques used to ensure that the survey results are correct—that is, that they are not biased.

Many surveys measure a population characteristic, such as disease prevalence. For any given population, there is a true value for this characteristic, which the survey aims to estimate. We cannot know the true value unless we examine every animal in the population, so every survey that examines only a sample has a degree of uncertainty in the result (the variance) related to the sample size. If a particular survey was repeated many times using exactly the same design, it is likely that a range of different results would be obtained. Bias is a theoretical value that measures the difference between average of the results from many identical surveys (the expected value) and the true value in the population. In a well-designed survey, these values are the same, and there is no bias.

One of the most common situations in which bias occurs is when the sample selected is not representative of the population as a whole. Consider this hypothetical example. A serological survey may be used to assess the effectiveness of classical swine fever vaccination programs. The survey design dictates that 40 blood samples are required to calculate the proportion of pigs with protective antibodies against the disease. The collection of blood samples from pigs is a tiring and noisy exercise, but is much easier when there are good facilities and staff willing to help. The survey team therefore elects to visit a number of larger piggeries for the blood collection, as it will be faster and easier. Once analysed, the survey concludes that the level of protection afforded by the current vaccination program is adequate, and that no further public education programs are required to increase the uptake of vaccination. Unfortunately in the area being studied, the larger piggeries make up a minority of the pig population, as there is a large number of smallholder or backyard pig farmers. The uptake of vaccination on the larger piggeries is quite high, but very few smallholder farmers are able to afford it. In this example, the use of an inappropriate sample results in an incorrect conclusion from the survey. In turn, a decision is made (not to attempt to increase public awareness) which could lead to a failure better to control the disease.

To avoid this sort of problem, it is necessary to ensure that the sample selected for the survey is truly representative of the population in question. There is, in fact, only one reliable way to do this—the use of formal random sampling techniques. Random sampling techniques (probability sampling) are those in which each member of the population has a known, non-zero probability of being selected in the sample. In practice, random selection can be achieved through the use of random number tables, computer-generated random numbers, cards, dice or coins, etc. One of the prerequisites for most random sampling techniques is that a reasonably accurate sampling frame, listing every member of the population, be drawn up.

Another reason to use formal random sampling procedures is that all formulae used to calculate survey results, from the simplest average to complex confidence intervals, are based on the assumption that random sampling was used. The use of these formulae when random sampling is not used will produce invalid results.

As in broader survey designs, a number of practical, simple approaches to sampling frame generation and random sampling have been developed, suitable for application in both developing and developed countries. The principles of these techniques have been widely known to researchers and scientists for many years, but in many fields of research, including veterinary epidemiological surveys, the use of formal random sampling techniques is the exception rather than the rule.

Why should this be so? In some cases, it is due to either a lack of understanding of the need for such procedures, or a lack of knowledge of practical approaches to the implementation of random sampling. However, more often it is due to the impression that random sampling techniques are too difficult to achieve in the field, or more specifically, that the increased time, effort and expense involved in using them is not warranted.

Survey Costs and Information Benefits

This impression is based on an informal assessment of the costs of using random sampling versus its benefits in terms of improved information quality. Both sides of the equation are somewhat intangible, and depend on the specific survey being undertaken. However, to gain a better understanding, it is worth examining the components of this cost–benefit analysis.

The costs of mounting a survey to collect data on livestock diseases may be divided into a number of components. Using the hypothetical example of a serological survey, the cost components may include:

- laboratory infrastructure establishment—serological surveys require laboratory facilities to analyse serum samples collected. Laboratory facilities to undertake this analysis may be established for a variety of reasons, but disease surveillance must be counted amongst them. A portion of the (usually very high) establishment and maintenance costs for the diagnostic laboratory should be allocated to the survey activity;
- laboratory staff salaries—the salaries of staff responsible for analysing specimens should be taken into account for any survey;
- survey planning—involves staff time and other costs incurred in planning survey design, coordinating field activities, and obtaining any necessary data such as sampling frames;
- transportation—the cost of transporting field survey teams from site to site, as well as transporting specimens to the laboratory for analysis;
- field equipment—equipment necessary for field work, including animal restraint, cold chain, etc;
- survey team training—the cost of any training required to ensure that field survey teams undertake the survey work as intended;
- field staff salaries—salaries for field staff during the period of the survey;
- field consumables—for example, needles, blood tubes, serum tubes;
- laboratory tests—the cost of analysing the specimens to obtain the serological result;
- data management—the cost of staff time in recording and processing data, as well as any equipment required for data management (e.g. computers);
- data processing and analysis—staff time required to analyse data and report survey results.

The most significant of these costs are usually related to the laboratory (the cost of analysing specimens, and establishing and maintaining the laboratory), transportation, and staff salaries.

When contrasting a survey that aims to collect unbiased data by using formal random sampling techniques with one that does not, the increases in cost are due only to increases in the time required for:

- survey planning (usually small, as the only extra planning task is finding an acceptable sampling frame);
- training staff (again small, as random sampling techniques can be taught in a matter of hours); and
- field specimen collection time.

The only significant increase in time and cost is due to increased time required to collect specimens in the field. In the case of a village survey, this is

related to the development of a sampling frame, and the increased time taken to move between randomly selected livestock owners, to sample their animals. Depending on the precise situation, this may not result in any increase in cost. If survey teams collect specimens from one village per day, the salaries of field staff will need to be paid for the full day, regardless of whether the samples are collected in 3 hours (without the use of random sampling) or 5 hours (using formal random sampling techniques). Even when random sampling procedures do increase the actual cost of field work, the increased cost as a percentage of the total survey costs (taking all other components listed above into account) is generally relatively small.

If the costs of random sampling are small, what then are the benefits? The benefit is increased quality of information, but it is here that the cost–benefit analysis strikes a problem. While survey costs can be clearly quantified, it is very hard to put a value on increased quality of information. The question of the value of collecting more information for making decisions on livestock disease control has been examined (Ramsay et al. 1999). However, the question at hand is the incremental value of improved quality information. Another problem facing the assessment is that it is impossible, after collection, to distinguish poor quality data from good quality data.

The real benefit of improved quality data through better survey procedures lies in two areas. Firstly, lack of bias means that the result of the survey will be correct. Secondly, users can have *confidence* that the result is correct. Potentially biased surveys conducted using non-random sampling procedures may at times produce an answer that is close to the truth, but, it is impossible to know when this is the case, and when it is not. We can therefore have little confidence in the result. The results of surveys using random sampling are not only free from sampling bias, but we can quantify how confident we are with the use of a confidence interval.

Perhaps the best way to assess the benefit of improved quality information is to examine two further hypothetical examples. Firstly, consider a survey to demonstrate freedom from disease for the purposes of international trade. In such a survey, biased sampling may lead to an incorrect conclusion that a country or zone is free of disease when disease is in fact present. The consequences of this error can be significant, especially if it results in the spread of a pathogen to an importing country. It will result in the loss of trading opportunities and loss of international reputation, and a delay in the opportunity to eradicate the pathogen.

As a second example, consider a survey designed to determine which major factors are associated with the spread of classical swine fever. The results will be used to help design appropriate control programs. A village survey is conducted to look at the incidence of disease and a range of farm-level factors. Lack of random sampling means that a biased sample of farms is examined. It includes mostly farms close to the centre of the village, with a close relationship with the village leader (who is asked to advise which farms to survey). As a result, larger and better managed farms are selected. A low level of disease is found amongst farms surveyed, and careful examination of the data reveals that there may be a slight breed predilection, with farms with native breeds of pigs being more susceptible. In light of these results, the recommendation is made that the use of European breeds of pigs will help decrease the impact of the disease. In reality, in this hypothetical village, there is a relatively high level of CSF, but it mostly occurs in smaller farms at the edge of the village. It is mainly spread by swill-feeding (a practice which none of the larger farms use). The breed predilection is in fact due to the fact that more native pigs are reared unrestrained, while European breeds are generally penned. The unrestrained pigs are able to spread the disease through the village.

If better survey techniques had been used in this example, the role of swill-feeding and confining animals would have become apparent. This is because smaller farms would have had the same chance of being included in the sample as the larger farms, and the sample would have been representative of the population as a whole. Failure to identify these two key risk factors means that inexpensive and simple control measures, such as cooking all swill and confining pigs, were not identified, and an opportunity to decrease the impact of disease was lost.

In both these examples, poor survey procedures will not necessarily lead to an incorrect conclusion every time, but they run the risk of providing an incorrect conclusion. In many situations the implications (and potential costs) of an incorrect decision are extremely significant.

Conclusion

Improved animal health information plays an important role in combating disease in developing countries. One constraint to the collection of improved quality information through active surveillance is the lack of consideration of good survey design. The use of formal random sampling techniques may add to the cost of surveys, but the extra cost is rarely significant when considering the overall cost involved in mounting a survey. On the other hand, the benefits of improved quality of

information and the increase in confidence that we have in information usually far outweighs any small increase in cost.

There is a need, therefore, for all involved in livestock disease surveys, both in developing and developed countries, better to understand the implications of survey design and sampling procedures, and to work toward maximising the quality of information collected. Surveys of any sort cost much time and money. Why risk wasting money and effort by getting an incorrect or biased result?

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COUNTRY REPORTS

Classical Swine Fever in Thailand

S. Damrongwatanapokin, W. Pinyochon, S. Parchariyanon, K. Inui[1]

Abstract

Classical swine fever (CSF) is a contagious viral disease that causes great economic loss to the swine industry in Thailand. The disease is considered endemic in the pig population and outbreaks apparently occur in spite of extensive CSF vaccination. Control of CSF is an immediate necessity and to control it in Thailand, it is essential to understand the current CSF status and to identify underlying problems associated with CSF outbreaks. Study of the molecular epidemiology of CSF virus isolates, study of a chronic strain of CSFV, improvement of diagnostic technology, and serological surveys for monitoring the vaccination program are carried out in order to provide a scientific basis for the development of CSF control programs in Thailand.

PIG NUMBERS in Thailand were about 8 million in 1999. Table 1 shows pig numbers from 1988 to 1997. In 1997, there were more than 10 million pigs, but there was decline to about 7 million in 1998 due to an economic crisis in the country. The pig-raising industry in Thailand comprises about 8000 farms (4000 are fattening pig farms). The number of sows in production is about 800 000. Farm size varies, with about 2000 farms of more than 300 sows and 2000 farms of fewer than 300 sows. The highest density pig-raising area is Region 7.

Table 1. Pig numbers in the past 10 years.

Year	Pig numbers
1988	5 740 000
1989	6 015 000
1990	7 350 000
1991	8 202 000
1992	8 333 000
1993	8 569 000
1994	8 479 000
1995	8 562 000
1996	8 708 000
1997	10 139 040
1998	Data not available

Note: Data from epidemiology section, NIAH.

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In Thailand, CSF was first reported in 1950 (Kongsamak 1980) in Bangkhen area near Bangkok. Since then, CSF has gradually become enzootic, and was declared a notifiable disease in 1954.

Diagnostic Technology

Rapid and accurate diagnosis is the key factor for the control of CSF. Methods for viral antigen detection, virus isolation and antibody detection have been improved and standardised, and are now routinely conducted at the central and regional veterinary diagnostic laboratories.

Immunoperoxidase staining techniques and neutralising peroxidase-linked assays (NPLA) were developed by Parchariyanon et al. (1997) for viral and antibody detection instead of the immunofluorescent staining technique, and are routinely performed for the diagnosis of CSF.

Molecular techniques to detect viral RNA such as the reverse-transcriptase polymerase chain reaction (RT–PCR) have been introduced to support these conventional methods. The combination of these methods will increase the speed and accuracy of diagnosis.

Molecular Epidemiology of CSFV Field Isolates in Thailand

Genetic characterisation was carried out with 78 field virus isolates collected over the past decade.

The results strongly suggested that:

1 there was a new introduction of CSFV in 1996, possibly from Europe; and

2 this strain was responsible for most outbreaks in the field in the later years. It was also shown that:
3 Thailand has a unique group of CSFV that is genetically different from any isolates of other parts of the world.

The molecular epidemiology database of Thai isolates was established, which will facilitate tracing the source of infection in future CSF outbreaks.

A Study of Chronic Strain of CSFV

A low-virulent strain, which causes a chronic type of the disease, was identified from the field. The pathogenesis of this strain was extensively studied in the experimental pigs to understand its importance in the field.

The results suggest that:

1 this strain could cause prolonged and mild disease in piglets;
2 it is difficult to isolate the virus and to detect the antibody due to the low level of virus replication and immune responses; and
3 this strain could produce vertical transmission in pregnant sows.

The finding indicates that infection with this strain may spread unnoticed in the field and complicate diagnosis.

Serological Survey for Monitoring the Immune Response of Vaccinated Pigs

A total of 1519 serum samples collected in 1998 was tested for CSFV neutralising antibody titre by NPLA. The sera were collected from gilts and sows (parity 1–5) from 26 farms with no history of CSF outbreak during the past two years.

The results indicated that:

1 immune status of individual pigs varied even at the same farm with the same vaccination;
2 the average titre of vaccinated pigs is about 1:128; (3) 35 of 1519 pigs had no detectable antibody response, and most were the sera collected from gilts that were vaccinated once at 6–7 weeks of age.

Thus, two CSF vaccinations are strongly recommended in young animals to override the interference of passive immunity and to achieve good immune responses.

Control of CSF in Thailand

At present, no strict regulation is placed on farms where the disease is diagnosed. Culling and massive vaccination are the common control measures upon outbreaks. Approximately 30 million doses of CSF vaccine are used annually for prevention and control of the disease.

All registered vaccines are live attenuated vaccines and most of them belong to Chinese strains. Lapinised Chinese strain produced by the Department of Livestock Development, Pest-Vac (Fort Dodge), Pestiffa (Merial) and Coglapest (Sanofi) are the four major vaccines mostly used in the field. There are more than 10 kinds of CSF vaccine currently being used.

Although all these strains have been proved safe and effective in the literature, the experiences of the country or the regions where successful control of CSF has been achieved suggest the use of a single strain for the control and eventual eradication of CSF.

It has the advantages that rather uniform immune response is expected and that the quality of vaccine will be much easier to control and standardise. The possibility of such action should be fully considered.

Future Plan

Methods for genetic analysis and the database of molecular epidemiology have been established. They have proved to be very useful for the differentiation of field isolates and to facilitate tracing the source of infection.

More recent CSF Thai isolates will be characterised. Cooperation of countries in the region will establish in Southeast Asia a database of CSF isolates which will provide the epidemiology of CSF outbreak in this region and in other parts of the world. Study of the pathogenesis of a low-virulent strain suggests the perpetuation of the virus in the field.

More research for the detection and elimination of infected animals is recommended. Vaccination has clearly proved of economic benefit, but it is not able to eliminate CSFV unless accompanied by strict sanitary and removal measures. Setting up an effective vaccination program in the presence of a field virus on a farm is difficult, since infection may occur when maternal immunity drops and immunisation by vaccination has not yet developed.

Seromonitoring of those farms is needed to achieve the most effective vaccination program. In addition, good zoosanitary measures and strict control of the movement of animals must be in practice for the effective prevention and control of CSF.

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The Distribution and Control Strategies of Classical Swine Fever in Indonesia

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Abstract

Large areas of Indonesia are infected with classical swine fever (CFS) and urgent measures need to be implemented to control the spread of infection and to prevent it from reaching disease-free provinces. However, efforts to control and/or eradicate CFS from the islands are likely to fail if movement of pigs and pig products cannot be adequately controlled, or laboratory facilities are not provided.

INDONESIA is an agricultural country in Asia encompassing more than 17 000 islands, and covers a land area of 1.9 million square km, extending from east to west along the equator. The country consists of 27 provinces, 296 districts, 3625 sub-districts and about 67 033 villages. The population of the country is about 202 million, and 62.2% live on Java Island, which in terms of land area, accounts for only 7% of the country. Agriculture (forestry, food crops, fisheries, estate crops and livestock) is the most important sector of the Indonesian economy.

While a monetary crisis has affected Indonesia since 1998, the livestock sub-sector, as an integral part of the agriculture sector, has not been seriously affected, particularly those farms with local commodity-based resources. However, the monetary crisis has caused the bankruptcy of some commercial farms, especially those whose feed commodity-base was highly dependent on imported sources (i.e. commercial poultry farms). The numbers of commercial chickens decreased significantly from 755.9 million in 1996 to 360 million in 1998 for broilers, and from 78.7 million in 1996 to 48.2 million in 1998 for layers.

Even though the majority of the human population in the country is Moslem, pigs represent the third-largest animal group after cattle and goats, and are found throughout Indonesia. However, they are concentrated in eight provinces outside Java Island, particularly in the eastern islands where the majority of the human population is not Moslem (North Sumatra, West Kalimantan, North Sulawesi, South Sulawesi, Bali, East Nusa Tenggara, Irian Jaya and East Timor) (see Figure 1). Pigs in some provinces are reared traditionally, extensively as a status symbol and are not economically valued, while in others they are reared semi-intensively for business.

During the monetary crisis, pig numbers were not seriously affected but decreased significantly during the outbreak of classical swine fever (hog cholera) in 1996. At present, pig numbers have almost returned to normal after the outbreak, except in North Sumatra. Table 1 shows the pig population by province, 1994–1998.

Distribution of Classical Swine Fever

Indonesia was categorised as a country free of classical swine fever (hog cholera) (CSF) until 1993. The introduction of CSF to Indonesia began 1994–96 when the death of thousands of pigs was reported repeatedly in North Sumatra, Jakarta, Bali, Central Java and North Sulawesi. The eruption of the outbreak of CSF caused Indonesia to become a country infected with the second contagious disease listed in OIE List A disease beside Newcastle disease (ND).

The disease gained entry into North Sumatra in July 1994 and subsequently spread as far as East Timor over the next four years. The outbreak was first reported in the districts of Dairi and Simalungun and spread to other districts such as Deli Serdang, Asahan, North Tapanuli and Karo (Anon. 1996, 1998). There were reports at that time that pigs had

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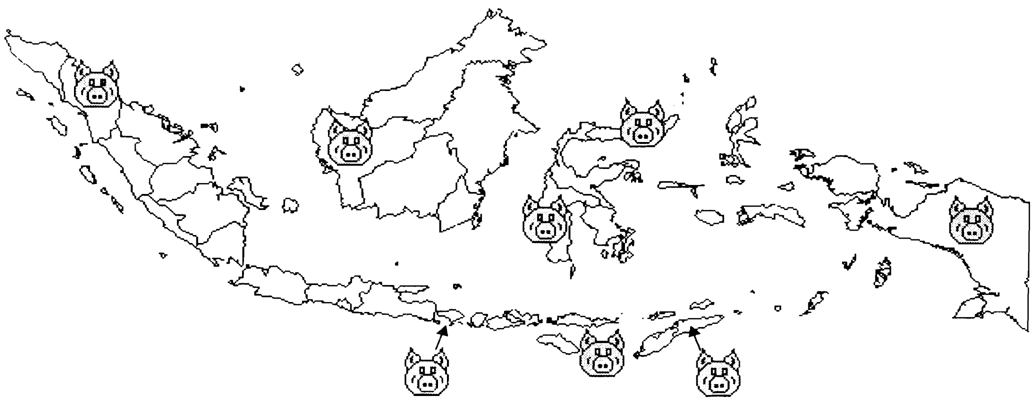

Figure 1. Concentration of pig population in Indonesia.

Table 1. Pig population by province (1994–98).

No.	Provinces	Year				
		1994	1995	1996	1997	1998*
1	Aceh	1 725	508	459	322	594
2	North Sumatra	2 387 849	920 998	948 235	976 277	1 005 175
3	West Sumatra	32 640	46 283	46 733	46 955	47 179
4	Riau	217 394	250 796	364 046	525 088	850 438
5	Jambi	11 829	9 881	8 254	16 192	21 851
6	Bengkulu	1 004	1 029	1 065	1 103	1 142
7	South Sumatra	200 655	203 879	77 209	72 564	78 761
8	Lampung	72 893	72 127	75 560	72 280	77 932
9	Jakarta	41 750	8 600	8 464	0	0
10	West Java	49 649	42 314	53 060	25 550	21 285
11	Central Java	139 548	132 594	120 393	100 532	83 947
12	Yogyakarta	8 573	8 735	7 985	6 741	6 301
13	East Java	56 656	62 622	54 510	54 610	53 518
14	West Kalimantan	911 686	923 752	616 130	331 786	737 740
15	Central Kalimantan	129 363	136 560	143 805	145 838	158 934
16	South Kalimantan	12 654	12 347	11 075	11 075	7 062
17	East Kalimantan	97 611	106 882	114 335	116 170	118 029
18	North Sulawesi	519 106	555 672	500 100	505 051	303 031
19	Central Sulawesi	138 581	149 667	173 212	226 670	247 954
20	South Sulawesi	520 048	554 759	574 674	575 061	575 448
21	Southeast Sulawesi	13 300	14 017	16 563	18 523	19 820
22	Bali	1 056 318	1 079 831	1 073 062	1 131 283	1 136 442
23	West Nusa Tenggara	23 889	22 508	23 850	26 153	28 130
24	East Nusa Tenggara	1 406 074	1 537 982	1 589 060	2 229 134	3 204 543
25	Maluku	91 539	95 979	100 308	109 335	119 175
26	Irian Jaya	407 328	426 665	517 165	532 680	548 660
27	East Timor	308 385	343 169	377 898	375 866	383 382
	Indonesia	8 858 047	7 720 156	7 597 210	8 232 839	9 836 473

Note: * preliminary figures.

been introduced to the North Sumatra area from the northern Malaysia Peninsular where the disease is known to occur. At that early stage, there was confusion about the exact cause of the major losses in North Sumatra, and definite diagnosis was not made until January 1995.

In the intervening 6–12 months, infection had spread to the province of Riau and West Sumatra. The disease occurred in Bengkalis district of Riau in April 1995 and in Padang area of West Sumatra in August 1995. From early 1995, vaccination using Chinese 'C' strain was being practised in Sumatra, although not yet officially authorised by the Indonesian Government.

By February 1995, pigs had been transported from Sumatra to the Jakarta area and the result was that the disease was introduced into Java. Despite major pig mortalities in Kapuk of the Jakarta area, the definite diagnosis of CSF was not made until June 1995, around 12 months after the disease had first occurred in North Sumatra. Due to further movements of pigs and pig products, CSF infection rapidly spread to Central Java in June 1995. High pig mortality was reported in the districts of Sukoharjo and Sragen in Central Java (Syafriati 1998). Vaccination of pigs against CSF was officially adopted in July 1995, some six months after vaccine was imported and first used in the field. By September 1995, CSF infection in pigs had also been seen in West Kalimantan.

In eastern Indonesia, the CSF outbreak was firstly reported in Denpasar district of Bali in October 1995. High pig mortalities in Bali starting in early May 1994 were previously identified as caused by streptococcosis (*Streptococcus zooepidemicus*, group C beta haemolytic). Losses in pigs in some herds and areas in Bali were very high and it was reported that mortality in some piggeries reached almost 80%. The disease then spread into eight districts and became endemic. Severe losses compromised pig production on the island. By this time, vaccination of pigs had been widely adopted by farmers and veterinary authorities as the only viable measure. The disease was then under control with the adoption of mass vaccination with a booster system.

Toward the end of 1995 and early 1996, CSF infections were seen in North and South Sulawesi. The disease was reported in the districts of Menado and Minahasa in North Sulawesi, as well as in Luwu and Tator of South Sulawesi, within the period January to August 1996 (Anon. 1997). In these two areas, vaccination appears to have been used effectively in trying to contain the disease.

In August 1997, an outbreak of pig disease was reported in the district of Dili, East Timor. The disease then spread into the districts of Liquisa and Aileu, and subsequently to other districts in East Timor such as Ermera, Viqueque and Lautem. Epidemiological evidence points to infection reaching the Dili area by way of pork products imported from Bali. Vaccination of pigs adopted in the six infected districts in East Timor brought the disease under control.

In mid-1997, there were reports of unexplained deaths of pigs in the district of East Sumba and possibly in the district of East Flores, East Nusa Tenggara. However, field investigations failed to reveal any concrete evidence that CSF infection was actually present on these islands. An occurrence of pig disease was then reported in the district of Kupang, East Nusa Tenggara in March 1998, and it was found serologically positive to CSFV infection. Serological positive sera were also reported from other districts in East Nusa Tenggara such as Timor Tengah Utara and Timor Tengah Selatan (Santhia et al. 1997, 1998).

Table 2 shows the distribution and number of pig deaths due to CSF in Indonesia.

Table 2. The distribution and number of pig deaths due to CSF in Indonesia.

Province	Year of occurrence	Population infected (head)	Number of deaths (head)
1. North Sumatra	1994–1995	?	± 1 000 000
Riau	1995	?	?
West Sumatra	1995	3330	619
Jakarta	1995	39 500	± 23 000
Bali	1995–1996	?	± 350 000
Central Java	1995–1996	?	± 20 000
West Kalimantan	1995	?	± 30 000
North Sulawesi	1995–1996	37 068	22 237
South Sulawesi	1995–1996	?	86 615
East Timor	1997–1998	89 259	17 607
East Nusa Tenggara	1998	?	?

Note: ? = data not available.

Diagnosis of Classical Swine Fever

Field cases have been confirmed by Indonesian laboratory testing in Sumatra, Java, Bali, Kalimantan, Sulawesi and Timor. The work for examining and monitoring the CSF situation on these endemic islands was carried out by the Research Institute for Veterinary Science (RIVS) Bogor, Veterinary Drug Assay Laboratory (VDAL) Gunung Sindur and seven regional diagnostic laboratories (Disease Investigation Centres Region I–VII). All laboratories mentioned above have the capability of diagnosing CSF infections in pigs, although the methods of testing for CSF are varied.

Disease Investigation Centre (DIC) Region VI Denpasar and Region VII Maros handle almost all of the animal disease diagnostic work for the eastern islands area of Indonesia. DIC Region VI Denpasar had developed a rapid and accurate method of diagnosing CSF infections using the detection of specific CSFV antigens in cryostat tissue section by an immunoperoxidase-staining (IPX) technique. Virus isolation using tissue-culture pig kidney cells (TC: PK15 cell line) done by RIVS Bogor and VADL Gunung Sindur has shown valid results. RIVS Bogor is currently establishing research activities aimed at developing polymerase chain reaction (PCR) technology for CSFV diagnosis.

Based on the strengths and weaknesses in diagnostic capabilities for CSF infections in each of these laboratories, a recommendation has been made on the diagnostic techniques most appropriate for the Indonesian laboratory system. It should be used and further developed in the future as presented in Table 3.

Control Strategies for Classical Swine Fever

The control of CSF has been placed as a national priority beside those for rabies and brucellosis. Based on the case report and epidemiology picture of the disease, the country is divided into three different areas of status, that is, infected, suspected, and free areas. The division of disease status area is to facilitate the implementation of control programs for the disease. At present, 10 provinces are officially declared as infected areas, 11 provinces as suspected areas, and six provinces as free areas as (see Table 4).

Considering the current state of the Indonesian economy and the limited resources available, the only course available is to control infection in pigs in the endemic areas through the use of efficacious vaccines. Internal controls on the movements of pigs and pork production should be examined carefully to prevent the spread of infection to islands not yet infected.

Two types of vaccines were available to pig producers in the CSF endemic areas. The first is a Brazilian tissue culture adapted live vaccine, based on the Chinese 'C' strain vaccine virus manufactured in France ('Pestiffa'). A second vaccine is the Japanese Kitasato vaccine based on guinea pig adapted (attenuated) live virus. Both vaccines had

Table 3. Laboratory techniques for examining CSF infections.

Laboratory method	Technique	Type of specimen	Reference lab
Histopathology	H & E	Spleen, brain and all organs	—
Isolation	TC: PK5	Spleen	RIVS or VADL
Identification	FAT	Spleen, ileum, lymph node, tonsil	DIC Region VI Denpasar
	IPX	Spleen, ileum, lymph node, tonsil	DIC Region VI Denpasar
	AC-ELISA	Spleen, whole blood	DIC Region IV Yogyakarta
	CTB-ELISA	Sera	DIC Region IV Yogyakarta
	CediTest ELISA	Sera	DIC Region IV Yogyakarta
	NPLA	Sera	VADL
	FAVNT	Sera	VADL

Note: AC-ELISA = Antigen capture ELISA.
CTB-ELISA = Complex trapping blocking ELISA.

Table 4. Provinces categorised as free, suspected and infected.

Area/province		
Free	Suspected	Infected
1. South Sulawesi	1. Aceh	1. North Sumatra
2. Central Kalimantan	2. Bengkulu	2. West Sumatra
3. East Kalimantan	3. Jambi	3. Riau
4. South-east Sulawesi	4. South Sumatra	4. Jakarta
5. West Nusa Tenggara	5. Lampung	5. Central Java
6. Maluku	6. West Java	6. Bali
7. Islands around Java and Sumatra, except Nias Island	7. Yogyakarta	7. West Kalimantan
	8. East Java	8. North Sulawesi
	9. Central Sulawesi	9. South Sulawesi
	10. East Nusa Tenggara	10. East Timor
	11. Irian Jaya	

been widely used to control the disease in the infected areas.

In order to prevent the spread of CSF infection to free areas, the importation of pig and pig products from infected and suspected areas are prohibited and importation of CSF vaccine is not allowed. Serological investigations using sampling methods should be implemented in free areas to monitor the possibility of disease introduction. In suspected areas, movement of pigs and pig products from infected areas is also prohibited. However, additional measures of intensive serological investigation have to be undertaken to detect the introduction of the disease as early as possible.

In the infected areas, the control strategies adopted are:

1. control movement:
   - pig and pig products entering infected areas should be subjected to quarantine regulation;
   - pigs from other infected areas should be vaccinated at the place of origin;
   - pigs from free and suspected areas should be vaccinated in the animal quarantine at the place of destination;
2. routine vaccination with a coverage of at least 60% of the pig population, with criteria as follows:
   - using only officially recommended vaccines;
   - the supply of vaccine for a commercial pig producer is self-supporting and vaccination is carried out by a practice veterinarian under the responsibility of a government veterinarian;
   - the supply of vaccine for small pig farms is subsidised by the Government and vaccination is carried out by the government veterinarian at the animal health post.

Conclusion

1. Control measures to prevent further spread of CSF within Indonesia are particularly important. Those areas currently free of disease should be protected by strict measures to control the movement of pigs and pork products from areas known to have CSF.
2. Plans to eradicate CSF from areas or islands in Indonesia in the near future should be effectively established. Eradication of the disease on individual islands (such as Bali) seems extremely expensive and is likely to fail due to the reintroduction of disease from neighbouring areas if control of the movement of pigs and pork products is not well undertaken and laboratory support is lacking.

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Developments and Advances in the Prevention and Control of CSF in Yunnan Province

Li Chundi[1] and Zhang Yinguo[2]

Abstract

Yunnan Province is in the southwestern border area of China, bounded by three countries (Burma, Laos and Vietnam) and has a 4000 kilometre boundary. The administrative divisions are divided into eight national autonomous prefectures, five districts, three municipalities and 126 counties. The population of the Yunnan province is 410 million, of 26 nationalities. The total number of pigs is 420 million. The system of prevention and control of animal diseases has been gradually improving since 1980. Laws and decrees, technical standards and a network of prevention and control of animal disease epidemics have been enacted or established in Yunnan Province. We have effectively controlled the occurrence or outbreak of animal epidemics, and, especially in the prevention and control of CSF, have achieved remarkable success. The results of a continuous sampling survey for 10 years indicate that the mortality of pigs caused by CSF has dropped from 4.2% to 0.4%, and the number of dead pigs from 1.2 million in 1980 to 160 000 in 1998. This paper briefly introduces the animal veterinary organisations, personnel and developments and advances in the diagnosis, monitoring and control of CSF in Yunnan Province.

According to 'National Regulations of Epidemic Prevention in Livestock and Poultry', the government has enacted and issued 'Yunnan Provincial Enforcement Detailed Regulations of Epidemic Prevention in Livestock and Poultry', 'Measures and Techniques of Prevention and Control of CSF in Yunnan Province' (1985), 'Yunnan Provincial Supervisory Regulations of Animal Quarantine and Animal Health or Veterinary' (1996) and 'Laws of Animal Epidemic Prevention, The People's Republic of China' (1997). 'Administrative Measures of Planned Vaccination of Live Pigs in Yunnan Province' will be enacted and issued soon.

These laws and regulations standardise the system of epidemic prevention in livestock and poultry in our province and also fully reflect governmental action in the area of epidemic prevention and control.

[1] Yunnan General Veterinary Station
[2] Yunnan Tropical and Subtropical Animal Virus Diseases Laboratory

Animal Veterinary Organisations and Personnel

In our province we have already established five levels of animal veterinary organisations, including province, prefecture, county, township and village (Figure 1). The total personnel or staff of these organisations is more than 25 000 (Table 1). There are 13 000 village veterinary surgeons, 7745 personnel in veterinary stations in townships, 4910 personnel in veterinary stations of prefectures and counties and 26 in provincial veterinary stations among them. Each veterinary personnel at different levels has obtained an appropriate educational background and taken part in training courses for veterinary techniques. The titles for technical personnel are research fellow, senior veterinarian, veterinarian, assistant veterinarian, technical staff and village veterinary surgeons, respectively. The levels of techniques are distinct and the disposition of personnel is very reasonable.

System of Vaccines Transport and Storage

In order to guarantee the quality and immunising effect of vaccines, we have successfully established

Ministry of Agriculture, P.R. China

Administrative system	**Technical system**
Bureau of Animal Husbandry and Veterinary, Ministry of Agriculture	National General Station of Veterinary
↓	↓
Bureau of Animal Husbandry, Yunnan Province	Yunnan General Station of Veterinary
↓	↓
Bureau of Animal Husbandry, District or Prefecture	Veterinary Station of District or Prefecture
↓	↓
Bureau of Animal Husbandry, County	Veterinary Station of County
↓	↓
Bureau of Animal Husbandry, Township	Veterinary Station of Township
↓	↓
Administrative Organ of Village	Veterinary Surgeon of Village
↓	↓

Figure 1. Diagrammatic sketch of veterinary organisations in Yunnan Province.

Table 1. Disposition of animal veterinary organisations and personnel.

Name of organisation	No. of organisations	No. of staff
National General Station of Veterinary	1	35
Yunnan General Station of Veterinary	1	36
Veterinary Station of District or Prefecture	16	540
Veterinary Station of County	125	4370
Veterinary Station of Township	1570	7745
Veterinary Surgeon of Village		13 000
Total		25 756

the five-level (province, prefecture, county, township and village) cold-chain system for vaccine storage.

Network for Animal Disease Diagnosis and Monitoring

We have established a three-level (province, prefecture, county) network for animal disease diagnosis and monitoring, equipped as necessary (Figure 2). The equipment used meets advanced world standards. In CSF diagnosis and monitoring, an ELISA reader and a fluorescent microscope are available to the whole province. In the prefectures and some counties, the CSF antibody can be monitored and CSFV diagnosed.

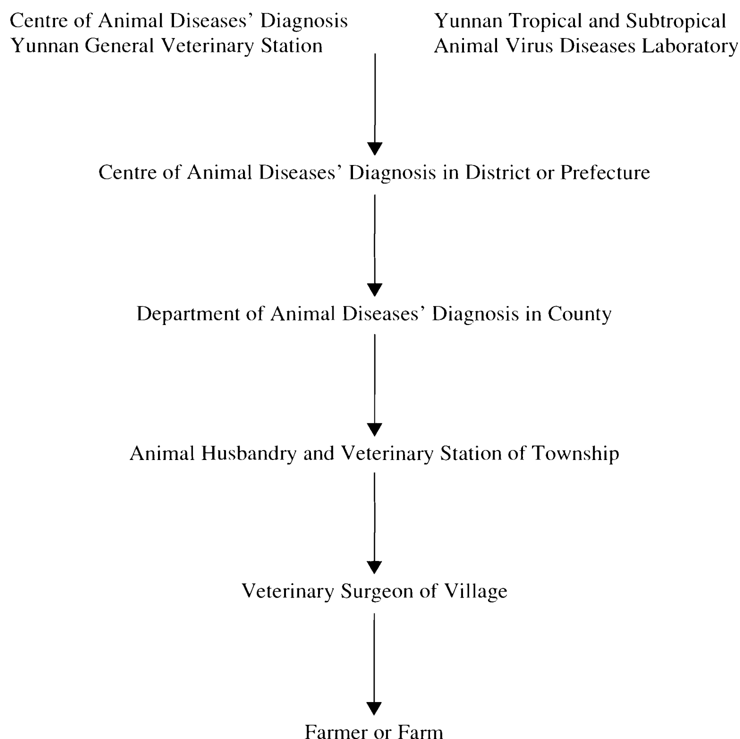

Figure 2. Diagrammatic sketch of network for the diagnosis, monitoring and control of animal diseases in Yunnan Province.

Vaccination and Prevention

The techniques of vaccination and prevention of CSF have gone through stages and gradually become successful now in Yunnan Province (Figure 3). CSF has been effectively controlled in the whole province.

Vaccinated live pigs in whole epidemic area (include contiguous area)

Limited by labour power and material resources, the main measure taken to prevent and control CSF in Yunnan Province was to vaccinate live pigs in the whole epidemic area (including the contiguous area) in the 1950s and early 1960s. As the immune (or vaccinated) density was very low, CSF breaks out frequently and causes huge economic loss.

Vaccinated whole live pigs in spring and autumn (twice per year)

The main measure was to make a concentrated effort to vaccinate all live pigs in spring and autumn (twice per year) to prevent and control CSF in the late 1960s and early 1980s. The main flaw of this measure was that many new susceptible pigs, imported into farms beyond the vaccinated period, could not be vaccinated in time. So the prevention and control of CSF was not effective in the whole province. The mortality of pigs caused by epidemic diseases was 13%, among that 40% by CSF. The number of dead pigs was more than 1 million in 1983 (Table 2).

Table 2. Vaccinated animal numbers and CSF mortality 1980–1998.

Year	Breeding numbers	Mortality	Immune rate (%)
1980	32 000 000	1 200 000	
1983	32 000 000	1 000 000	
1989	33 000 000	580 000	75.0
1990	33 000 000	470 000	79.0
1991	34 000 000	380 000	80.2
1992	36 000 000	350 000	80.2
1993	36 000 000	330 000	82.0
1994	37 000 000	340 000	83.0
1995	39 000 000	280 000	85.0
1996	41 000 000	240 000	90.0
1997	42 000 000	220 000	95.0
1998	42 000 000	160 000	95.0

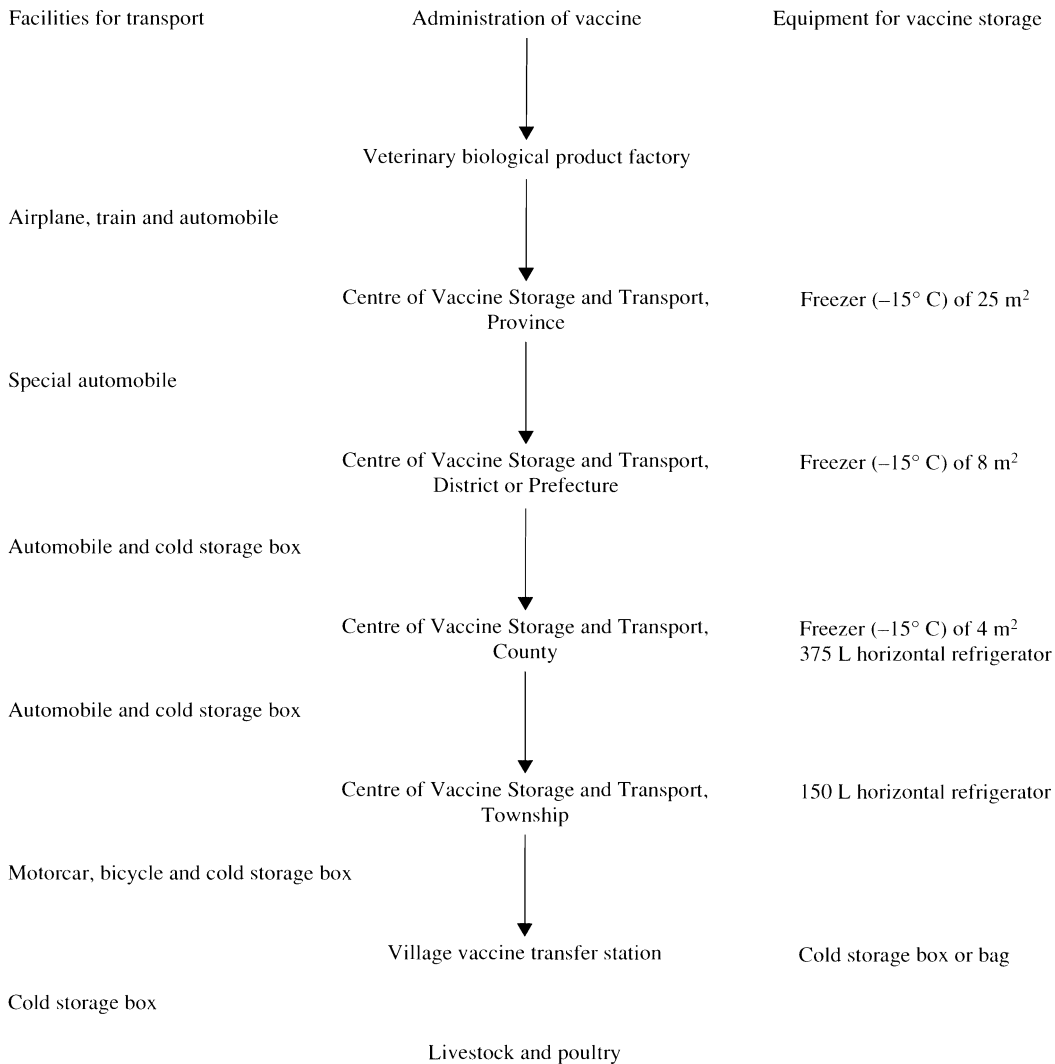

Figure 3. Diagrammatic sketch of equipment for cold chain vaccine storage system and administration.

Vaccination Program for CSF

In light of serious infections of CSF in Yunnan Province, and in order to control this epidemic disease to ensure the development of pig production, the government has enacted 'Vaccination Program for CSF in Yunnan Province' and applied the program in some counties for three years. The results demonstrate that the program was feasible and effective in the prevention and control of CSF. At the end of 1985, we issued 'Enforcement Regulations of Vaccination Program for CSF in Yunnan Province' and have applied and disseminated it in the whole province.

The main points of the program include:

- The used vaccine was lapinised hog cholera attenuated vaccine. Breeding pigs were vaccinated once per year and piglets at 60 days old (large piglets, tested by rabbit neutralisation assay, was more than 16 times, the piglets can resist the challenge of virulent virus, at 8 times they can resist natural infections, and less than four times had no protection. The half-life of antibody titre is 10 (3 days. The titre of maternal source antibody in 60-day-old piglets dropped to zero or less than eight times in 90% piglets).

- We vaccinated the pigs according to month, two months, or seasons.
- CSF has been effectively controlled by the enforcement of this vaccination program.
- The mortality of pigs caused by epidemic diseases dropped from 13% (1983) to 6% (1993), among that the mortality caused by CSF dropped from 40% to 20%. The number of dead pigs was 330 000 per year.

Planned Vaccination for CSF

In order to decrease the costs of CSF vaccine, we have used cell culture (bovine testicular cell) vaccine instead of lapinised attenuated vaccine, since 1990. Because the properties of these two kinds of vaccine are different, the vaccination program based on lapinised attenuated vaccine was not available.

The cases of CSF increased. The mortality of pigs caused by CSF was 23% among the mortality caused by epidemic diseases. So we firstly designed the test to screen the effective vaccinal dose of cell culture vaccine. The results indicate that using one dose (labelled in the direction by vaccine factory) of vaccine to inject piglets, if the piglets had no maternal source antibody of CSFV, the immune duration was nearly 10 months.

In the current situation, most maternal pigs have been vaccinated. In order to eliminate the interference of maternal source antibody, we used 3–4 times the dose to vaccinate piglets. The immune duration was 8–10 months. According to the results, we have gradually applied planned vaccination.

The main points of the planned vaccination include:

- Samples from pigs and piglets were collected to monitor the titre of antibody or maternal source antibody of CSFV (Table 3).

Table 3. Results of monitoring immune antibody.

Location	No. of samples	Methods	No. positives	Positive rate (%)
Kunming	510	ELISA	435	85.3
Qujing	455	ELISA	366	80.4
Yuxi	330	ELISA	268	81.2
Honghe	522	ELISA	434	83.1
Dali	250	ELISA	198	79.2
Lincang	211	ELISA	144	68.2
Lijiang	397	ELISA	323	81.4
Total	2675		2168	81.0

- According to the results we vaccinated animals in time. If possible we vaccinated the 0-day-old piglets and ensured the rate of immune density was more than 90%.
- CSF has been effectively controlled by enforcing the planned vaccination.

According to results of the sampling survey in 1998, the mortality of pigs caused by epidemic diseases dropped from 6% (1993) to 4% (1998), among that, the mortality caused by CSF dropped from 20% to 10%. The number of dead pigs was 160 000 per year.

How To Deal with the Outbreak of CSF

According to many years' local experience, if there is an outbreak of CSF in a village (with high pig density) or pig farm and there is already a definite diagnosis, in critical situations we used large doses (15–20 times normal dose) of lapinised attenuated vaccine or cell culture vaccine to vaccinate the animal as soon as possible. Using this measure, we can rapidly control the disease and decrease the economic costs. Generally, in three days after vaccination the disease could be stabilised.

According to the history of prevention and control of CSF in Yunnan Province, lapinised hog cholera attenuated vaccine and cell culture vaccine, studied and prepared by Chinese scientists, has been applied and disseminated for many years, and is very effective (Table 4).

We have made great efforts for nearly 20 years and controlled CSF in Yunnan Province. The mortality of pigs caused by CSF dropped from 45% in the early 1980s to 10% in 1998 among the mortality of pigs caused by epidemic diseases. The number of dead pigs per year dropped from 1.2 million in 1980 to 160 000 in 1998 (Table 2). The achievement of prevention and control of CSF was remarkable.

We have collected the serum from 2675 vaccinated pigs from six prefectures (Kunming, Qujing, Yuxi, Honghe, Dali, Lincang and Lijiang) to monitor the antibody of CSFV in the pigs' group in 1998. The results showed more than 81% pigs were up to standard (Table 3). The results tallied with the cases of CSF, obviously decreased in the whole province, and also indicated that the veterinary personnel working in the whole province have done excellent and reliable work.

To sum up, in order to prevent and control CSF effectively in a border and multinational province where pigs have been raised dispersedly, we should establish a good troop for epidemic prevention, screen safe and effective vaccine, enhance national consciousness of epidemic prevention, prevent and control the epidemic by laws and regulations, make a good job of monitoring the epidemic situation, draw up a plan for epidemic prevention in a critical situation, and pay more attention to the basic research of veterinary medicine. Only in this way can CSF be controlled effectively and eradicated finally.

Table 4. Developments and advances in the prevention and control of CSF by vaccine.

Year	Type of vaccine	Measures of vaccination	Effect
1960	Tissue vaccine (spleen and lymphoid tissue, prepared extemporarily)	Vaccinated live pigs in whole epidemic area (including contiguous area)	Outbreak frequent
1970	Tissue vaccine (spleen and lymphoid tissue)	Vaccinated whole live pigs in spring and autumn (twice yearly)	Outbreak frequent
1985	Lapinised Hog Cholera attenuated vaccine	Vaccination program for CSF	Outbreak occurrence
1996	Cell culture (bovine testicular cell) vaccine	Planned vaccination for CSF	Cases occurrence

Enactment of Laws and Regulations

1985 National Regulations of Epidemic Prevention in Livestock and Poultry.

1985 Yunnan Provincial Enforcement Detailed Regulations of Epidemic Prevention in Livestock and Poultry.

1985 Measures and Techniques of Prevention and Control of CSF in Yunnan Province.

1996 Yunnan Provincial Supervisory Regulations of Animal Quarantine and Animal Health or Veterinary.

1997 Laws of Animal Epidemic Prevention, The People's Republic of China.

1999 Administrative Measures of Planned Vaccination of Live Pigs in Yunnan Province.

Classical Swine Fever in Lao PDR

Sounthone Vongthilath[1] and Stuart Blacksell[2]

Abstract

Pig disease is the major problem for pig farmers in Lao PDR. Classical swine fever (CSF) is endemic in Lao PDR with many outbreaks reported annually. It accounts for a large number of pig deaths in all pig-raising systems. Pig-raising is popular throughout Lao although the northern Lao people tend to keep a higher number of pigs because of traditional customs. In Northern Lao, it has been reported there is an average of 3.7 pigs per family, in Central Lao, 1.4 pigs per family, and in Southern Lao 2.3 pigs per family. There are three pig-raising systems in Lao PDR: smallholder, small family business and semi-intensive.

DUE TO THE recent economic crisis in this region, investment in the livestock production sector has decreased marginally during the past few years although the demand for meat and fish is gradually increasing in line with the population demands of Lao Peoples' Democratic Republic (Lao PDR).

The problem faced by the Department of Livestock and Fisheries (DLF) of Lao PDR is primarily to facilitate food security to the provincial areas in addition to increasing the supply of good quality meat and fish to the major towns of the country.

Pork production is approximately 5 kg/person/year and accounts for 25% of total meat production. Regarding the export market, 3% of pork production is sent to other countries and pork production is increasing at a rate of 3% per year.

The estimated livestock populations in Lao PDR of the previous 10 years are summarised in Table 1.

Pig Breeds and Husbandry Techniques

Four native pig breeds are recognised in Lao PDR. In a survey undertaken by the DLF to determine the phenotypic characteristics of pigs in Lao PDR and provide recommended groupings, four distinct groups, *Moo Chid*, *Moo Laat*, *Moo Daeng* and *Moo Nonghaet* were recognised. Of the imported breeds, Landrace and Duroc are popular although raised to a much lesser extent than native breeds. Pig feed in Lao PDR is usually rice bran, corn, cassava, alcohol production waste, edible grasses or weeds and waste food. Commercial pig feeds are generally only used in urban and peri-urban areas of Vientiane City.

Approximately 64% of families in Lao PDR raise pigs for sale or consumption. Pig-raising is generally performed by smallholder farmers and is popular as a form of supplementary income for rice farmers. Pig-raising is popular throughout Lao although the northern Lao people tend to keep a higher number of pigs because of traditional customs. In Northern Lao, it has been reported there is an average of 3.7 pigs per family, in Central Lao, 1.4 pigs per family, and in Southern Lao 2.3 pigs per family. There are three pig-raising systems in Lao PDR: smallholder, small family business and semi-intensive.

Table 1. Livestock in Lao PDR 1988–98.

Year	Buffalo	Cattle	Goat/Sheep	Pigs
1988	1 040 730	764 100	89 200	1 267 880
1989	1 026 160	816 530	105 160	1 349 980
1990	1 071 750	841 900	139 410	1 392 100
1991	1 103 910	892 390	154 720	1 433 160
1992	1 130 720	992 980	103 890	1 560 920
1993	1 134 200	1 019 840	127 550	1 624 670
1994	1 168 230	1 081 010	141 600	1 673 390
1995	1 191 410	1 145 870	152 930	1 723 590
1996	1 211 700	1 186 000	159 000	1 772 000
1997	1 223 800	1 227 500	165 000	1 813 000
1998	1 092 740	1 126 600	122 170	1 432 140

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The smallholder system employs 1–3 native pigs in a village situation. The pigs may roam freely in the village or be penned, and are given poor quality feed or forage in the village. This method of production accounts for 96% of all Lao pig production. The small family business system employs 3–6 native or imported breed pigs generally in a village or backyard situation. The pigs are penned and are given improved feed. This method of production accounts for 3% of all Lao pig production. Semi-intensive pig farming systems employ large numbers of imported breed pigs in a farm situation and are fed commercial feed that may be mixed with local improved feeds. This method of production accounts for 1% of all Lao pig production.

Constraints to Production

Pig disease is the major problem for pig farmers in Lao PDR. Classical swine fever (CSF) and most probably many other viral diseases account for a large number of pig deaths in all pig-raising systems. Bacterial diseases such as *Salmonella cholerasuis* and *Erysipelothrix rhusiopathiae* (the aetiological agent of swine erysipelas) also have a large impact, as well as parasitism in village pigs, such as *Ascaris suum,* resulting in retarded growth of the pigs.

The lack of quality vaccine, vaccine cold chains and veterinary or para-veterinary staff to apply the vaccines is also a problem. Problems in delivery and processing of quality feeds is also another constraint.

Figure 1. Incidence of CSF infections diagnosed in Lao PDR during 1998.

Figure 2. Incidence of CSF infections diagnosed in Lao PDR to June 1999.

Commercial pig feed is expensive by local standards and is therefore employed only in larger production systems. Improved feeds such as cassava, corn and rice bran are used by some farmers but availability, expense of production and processing time are a problem.

Marketing and management can be difficult, depending on the location, as travel to market to sell the pigs may be expensive as well as difficult, or impossible, due to poor or impassable roads. In such cases, a middle-man trader may visit the village and offer sub-standard prices, further compounding the marketing problems for small-scale pig producers.

Local or native pig breeds are not genetically improved and therefore do not provide as good a feed-conversion ratio as imported breeds.

Current CSF Situation

CSF is endemic in Lao PDR with many outbreaks reported annually. The level of incidence is influenced to a large extent by the demands of local pork requirements where pigs are transported by vendors or middle-men. Illegal international animal trade may also contribute to the spread of CSF as the country is an established thoroughfare to major markets in neighbouring countries. Additionally, as a large percentage of pig production in Lao PDR is derived from village smallholders where farmers keep animals in a free-ranging fashion, this factor may have an influence in some sporadic outbreaks of CSF.

The routine diagnosis at the ACIAR project laboratory in Vientiane is performed using the CSF

antigen capture ELISA (CSF AC-ELISA). Samples are submitted to the laboratory via a provincial and district sample submission network. In 1998, the project laboratory received 257 samples. Fifty were CSFV antigen positive representing 35 outbreaks (19.5% of the total samples submitted) (Figure 1). In 1999, 87 samples were received with 19 positive samples from 11 outbreaks (21.8%) (Figure 2).

External Assistance

The Lao Government has quite limited national budget resources for the financial support of CSF control activities. Nevertheless, in the previous year the Lao Government has received strong support from donor agencies in the form of bi-lateral or regional veterinary services projects. The Australian Centre for International Agricultural Research (ACIAR), the European Union project 'Strengthening livestock services' have provided support which has enabled Lao PDR to implement a CSF research program to meet the needs of the country.

The following summarises the work of selected projects working on CSF in Lao PDR over the previous 12 months.

ACIAR project

ACIAR project 9438 is a three-year project providing laboratory facilities for CSF diagnosis for both antigen detection and serology. A resident Australian scientist provides training and support to local staff in the implementation of the diagnostic techniques. Furthermore, serological surveys to determine the prevalence of CSF antibodies throughout the country have been undertaken. During 1998–99, the following were accomplished:

Infrastructure and institutional strengthening

- Commissioning and opening a refurbished laboratory for CSF and FMD Diagnosis;
- Training Lao staff in the diagnosis of CSF using ELISA techniques;
- Establishment of a provincial sample submission network to facilitate the collection and transport of samples to the project for diagnosis;
- 20 staff from four provinces trained in surveillance and data analysis prior to CSF survey work.

Serological surveys

- Using active surveillance techniques, approximately 2000 sera were collected from four provinces;
- More than 2000 sera were collected from abattoirs in eight provinces.

European Union project

Project ALA/96/19, 'Strengthening of livestock services and extension activities', is a six-year project. The main objectives are to strengthen veterinary services and the extension network at all levels for an efficient and sustainable delivery of animal health and production services, and thereby to reduce disease incidence, improve management practice and increase livestock productivity. This project has no explicit focus on CSF but different components can support the Government of Lao PDR in CSF control activities. The project has started only recently and is expected to become fully operational in 1999.

Conclusion

CSF is the most important pig disease in Lao PDR. Control strategies for it face constraints in technical and human resource areas. The ACIAR project has provided valuable resources and training in CSF diagnosis and control. Strong support via external assistance is required to continue research into CSF in Lao PDR.

Pig Production and Classical Swine Fever in Vietnam

Nguyen Tien Dzung[1]

Abstract

Vietnam is a Southeast Asian country bordered by China to the north, PR Laos and Kampuchea to the west, and the South China Sea to the east and south. It has 78 million inhabitants in an area about 320 000 km^2. The climate is tropical, hot and humid and north of the 16th parallel, it is cold in winter (November–April). Livestock consists mainly of pigs (18 million), cattle and buffalo (6 million) and poultry (180 million). This paper outlines pig production in Vietnam and then details the situation of classical swine fever (CFS), still the most important disease in pig production.

MORE THAN 80% of pig production in Vietnam occurs in the household or smallholder sector of the population. Typically, each household owns 2–3 pigs. At the same time, commercial or intensive pig-raising with small herds (10–20 pigs) is occurring in this sector, especially in the deltas of the Mekong and the Red rivers, where the focus is mainly on breeding. Large pig farms (hundreds to thousands of sows) also occur in the south and to a lesser degree in the north.

Native pig breeds are the I, the Mong cai, the Baxuyen and the H'mong. They are found now only in the household sector and in lower numbers than European pigs. The latter have the advantage of being lean and fast-growing, and are becoming preferred in pig husbandry.

Feed in the traditional household sector consists of kitchen waste, paddy brands, vegetables and some grains. The pigs are often unleashed to supplement-feed in the backyards. The use of industrially-processed feed is growing and many foreign companies dominate the market.

Classical Swine Fever

Clinical signs

The disease was first diagnosed in Vietnam in 1924. Since then it has been considered the most devastating disease in pig production. The course is normally acute and sub-acute. The clinical signs of the disease are the same as in 1924. In the 1990s, the chronic form of the disease became generalised. It is characterised by stunting and constipation in growing pigs and reproduction trouble in breeders. Virus carrier state has been repeatedly reported.

Epidemiology

The prevalence of CSF is not well recorded. Statistics data exist but are difficult to interpret. The seasonal character of CSF occurrences was established, more than 80% of outbreaks occurring in the cold season. The condition was linked to the massive slaughtering of pigs for the Tet (the Lunar New Year) festivities in that season and, consequently, the movement of new stock piglets to replace slaughtered ones. Nowadays, as life improves, pork meat demand extends all year, and so pigs are slaughtered all year. In addition, vaccination occurs more often. The seasonal character is no longer valid and the disease has become endemic. The spreading of the disease is strongly linked with the circulation of pigs and pork meat.

Diagnosis

CSF is diagnosed mainly by clinical signs. At the extreme, every diseased pig that does not respond to antibiotic treatment is considered as being infected with CSF. At autopsy, the lesions are, in most cases, sufficient to establish CSF diagnosis, but such tests are not often applied to living pigs as no compensation policy exists that allows the veterinarian to do so.

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Two laboratories (one in Hanoi, another in Ho Chi Minh City) are now capable of CSF diagnosis. The techniques used there are immunofluorescence and ELISA. The number of samples received for CSF diagnosis is not as high as in European countries, because the test is costly and pig owners are not willing to pay the charges.

ELISA has helped to detect virus carrier sows. The prevalence of CSFV carrier pigs was reported as high as 15% on some farms.

Prevention and control

Vaccination against CSF has been practised since 1960 in Vietnam. At that time in the north, the vaccine was produced using the C-strain, and in the south, the Japanese GPE$^{(-)}$ strain. Now all home vaccine is produced with the C-strain in the freeze-dried form. Vaccines are sufficiently produced, but some foreign vaccines are also authorised for import. The vaccination campaigns are executed twice per year under the responsibility of the Provincial Veterinary Service. Complementary vaccination is realised by the practitioners. The pig owners pay the vaccine and the service cost, except in the mountainous regions where the State pays the vaccination cost.

Results of vaccination are assessed and reported by the Provincial Veterinary Service only as the proportion of pigs being vaccinated. The figure varies greatly from one to another location. The vaccination efficacy remains obscure. CSF is a communicable disease and vaccination is compulsory under the State Ordinance on Veterinary Activities issued in 1993. But vaccination is still far from being satisfactorily implemented. Difficulties encountered in vaccination campaigns are:

- lack of vaccine-keeping facilities (the cold chain);
- pig-catching (in many regions, pigs are raised unconfined); and
- lack of acceptance by the pig owners.

These conditions are more pronounced in the regions where the economy still bears the character of auto-sufficiency. Hopefully, the situation is changing through the policy adopted by the Vietnamese State, i.e. to convert its economy into a market economy.

Other prevention measures adopted for fighting CSF are the inspection of animal movement and pork meat. Only animals previously vaccinated against CSF are allowed to be transported.

Measures to be applied in a CSF outbreak are also established and are similar to those adopted elsewhere. Another weak point in the control of CSF is the lack of certain diagnosis at the early stage of the outbreak. Being afraid of restrictions on animal movement imposed by the control measures, pig owners often do not inform the authorities of the presence of CSF.

Classical Swine Fever in Hong Kong

T.M. Ellis, L.D. Sims and K.C. Dyrting[1]

Abstract

Classical swine fever (CSF) is considered to be endemic in Hong Kong and outbreaks of acute CSF have occurred in any month of the year during the past 12 years. It is a notifiable disease. Vaccination with live attenuated vaccine is widely used in pig herds. However, outbreaks of disease have apparently occurred in vaccinated herds. In the past two years, few outbreaks of typical acute CSF have been confirmed. The status of chronic and lower virulence cases of CSF within pig herds is uncertain. Recently, diagnosis has been based on clinical signs and gross and microscopic pathological findings. Introduction of testing for virus antigen or genome detection is planned to confirm CSF diagnosis, to determine the prevalence of lower virulence or chronic disease and to investigate cases of apparent vaccine breakdown.

THE PIG-RAISING industry within Hong Kong consists of approximately 330 farms with a total herd of approximately 400 000 pigs. Farm size varies considerably from 20–30 sows to more than 1000 sows. The pigs are mostly run indoors on solid or slatted floors. Farms are required to prevent untreated waste from entering watercourses and most have installed and operate waste treatment facilities involving anaerobic and aerobic fermentation systems on site. Pigs are usually fed imported formulated rations.

Disease in pigs with clinical signs and pathology typical of classical swine fever has been seen at the Castle Peak Veterinary Laboratory of the Agriculture and Fisheries Department since the laboratory was established in the early 1970s (D. Higgins, pers. comm.). Early diagnosis was based on clinical and pathological findings with no confirmatory antigen or virus detection tests conducted.

In the late 1980s, antigen detection by immunoperoxidase staining of CSF antigen in cryostat sections of tonsils was used to confirm the diagnosis of CSF in pigs with clinical signs and pathology typical of acute or chronic CSF (K.S. Lo, pers. comm.). Immunological reagents from CVDL Lelystad were used. Subsequently, diagnosis was based on clinical findings and gross and microscopic pathology from 1992 until this year, when a PCR procedure for CSF based on primers selected by Dr F. Leung, Zoology Department, University of Hong Kong, was introduced.

Recent CSF Incidence Data

The number of CSF outbreaks reported and diagnosed from pig-raising farms in Hong Kong since 1987 is summarised in Table 1. Details of the number of pigs affected and the number of deaths in the outbreaks are shown as well as the month(s) during which outbreaks occurred for that year. The procedures used for diagnosis of CSF in these outbreaks are also summarised.

Control of CSF in Hong Kong

The disease is at present accepted as being endemic in Hong Kong and, as such, no specific restrictions are placed on farms where disease is diagnosed. Advice is given on farm control including decontamination and management procedures to lessen the spread. The main control method recommended is vaccination.

Five CSF vaccines are registered for use in Hong Kong including Pest-Vac (Fort Dodge), Suvac (Sanofi), Pestiffa (Rhone Merieux), Kinavac (Syva Laboratories), and a lapinised hog cholera vaccine from Taiwan. The registered vaccines are all live attenuated vaccines based on the lapinised China strain of CSF. Vaccines are generally used following

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Table 1. Classical swine fever cases in Hong Kong since 1987.

Year	Months	No. outbreaks	No. pigs affected	No. deaths	Method of diagnosis
1987–88	Jan, Mar, Jun, Jul, Aug	9	501	206	Clin/Path, Ag detect by immunoperoxidase staining
1988–89	Jan, Mar	3	1000	930	Clin/Path, Ag detect by immunoperoxidase staining
1989–90	Apr, May, Jul, Aug, Sep, Oct, Nov, Feb, Mar	21	2826	928	Clin/Path, Ag detect by immunoperoxidase staining
1990–91	Apr, May, Jun, Sep, Nov, Feb	10	1362	569	Clin/Path, Ag detect by immunoperoxidase staining
1991–92	Apr, Jun, Nov, Dec, Jan, Feb	11	438	315	Clin/Path, Ag detect by immunoperoxidase staining
1992–93	Apr, May, Jul, Nov, Dec, Mar	9	1155	729	Clin/Path, Ag detect by immunoperoxidase staining
1993–94	Apr, May, Jun, Jul, Aug, Nov, Dec, Jan	17	2117	607	Clin/Path
1994–95	Apr, Nov, Jan, Mar	5	5325	128	Clin/Path
1995–96	Dec, Feb	2	900	120	Clin/Path
1996–97	Jul, Sep, Oct, Dec, Jan	5	655	280	Clin/Path
1997–98		0	0	0	Clin/Path
1998–99	Feb	1	150	100	Clin/Path, PCR

the manufacturers' recommendations with respect to age of vaccination and requirement for secondary or booster vaccinations.

However, an unusual practice has been reported locally where piglets are vaccinated just after birth before colostrum has been ingested. The effect on the immature lymphoid system of these piglets, even by the attenuated lapinised China strain, is not known at this time, and it is interesting to speculate what effect this may have on infections with other porcine viruses or bacterial infections.

Discussions with veterinary wholesalers supplying vaccines indicate that approximately 725 000 doses of the registered CSF vaccines are used in Hong Kong annually.

Future Activity

Introduction of antigen detection and antibody ELISAs for CSF, establishing tissue culture facilities with immunofluorescent staining for CSF-infected cells and further utilisation of the PCR test are all planned to improve diagnostic capability for CSF.

These procedures will be used to confirm CSF diagnosis and further investigate cases where CSF vaccination does not seem to have prevented the disease. Additionally, the effect of day-old piglet vaccination on persistence and efficacy of the CSF vaccines will be investigated and the industry will be advised of any adverse effects of this practice. The impact of other viral agents known or suspected to occur in Hong Kong on the response to CSF vaccination or infection with field strains of CSF virus will also be examined.

Acknowledgment

The assistance of Mr Pang Tak Hing in collecting information about CSF vaccine usage in Hong Kong and providing statistical information on CSF case occurrence since 1987 is gratefully acknowledged.

EMERGING VIRAL INFECTIONS AND FLAVIVIRUS RESEARCH

Nipah Virus—Considerations for Regional Preparedness

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Abstract

The discovery of Nipah virus in Malaysia has created a significant regional problem for countries in Southeast Asia, to develop plans for an adequate response to this major new emerging zoonotic disease. Biosafety of personnel conducting field and laboratory investigations of suspect Nipah virus infections is a major priority. Since pigs have functioned as a significant amplifying host, there is a need for consensus on the risks of the trade in pork and live pigs. Horses, dogs and cats have been shown susceptible to infection and so consideration has been given to the international movement of these other animals. It is recommended that where on-going surveillance indicates freedom from active infections, the pig industry may be considered safe to continue operations. A wildlife reservoir has been postulated as the source of the virus, and Pteropid and other species of bats implicated serologically. Studies are needed to confirm the wildlife species involved in the natural ecology of the Nipah virus, and to allow assessment of the risk factors that may lead to further 'jumps' of Nipah infection from wildlife to domestic animals and to people.

THE OUTBREAK of zoonotic Nipah virus disease in pigs and people in Malaysia in 1998 and 1999 introduced a virus of greater importance and impact than most newly emerging viral diseases. Large numbers of farmers died, more than 105. The disease was controlled by 'stamping out' of known and suspected infected pig herds. Over 1.1 million pigs were killed.

The emergence of such a zoonotic disease causing the deaths of so many people so quickly is quite alarming. To deal effectively with this new threat may require changes in the management of veterinary and public health services and the pig industry. There is now an awareness that unknown threats may arise at any time.

Although no human cases resulted from eating pork, nonetheless, sales of pork dropped dramatically. Consumer confidence in pork may recover only slowly. In the control of the disease, half the pigs in peninsular Malaysia were destroyed, also substantially disrupting the pork industry and its export trade. Hence, the outbreak had enormous economic consequences.

Specific issues include whether Nipah virus has been eradicated from domestic animals and what needs to be done to monitor the situation. Has Nipah virus spread to other countries through the movement of infected pigs? Wildlife have been implicated as a potential source of the virus. Can new infections occur in Malaysia or elsewhere as a result of a new 'jump' from the virus's wildlife reservoir? Veterinary

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authorities need to consider how to detect any new outbreaks in a timely manner, and to ensure that their spread is limited.

This paper and a companion paper (Daniels et al. 2000) provide information regarding the diagnosis of Nipah virus infections, and suggest strategies for managing a pig industry in the 'post-Nipah' era.

The Nipah Virus Outbreak in Malaysia

An epidemic from a single source

In 1999, Nipah disease occurred at several locations in Malaysia and spread to Singapore. However the first cases of human Nipah encephalitis had occurred in the Ipoh district of Malaysia in early 1997. Testing of convalescent sera of these people showed antibodies to the new virus and immunohistochemistry showed Nipah viral antigen in brains from some deceased patients. Retrospective investigations in the Ipoh area showed that the movement of disease from farm to farm could be traced to trading in pigs prior to people becoming sick. Large movements of pigs from Ipoh to other areas further south were associated with the later stages of the outbreak. Abattoir workers in Singapore became sick after processing pigs from Malaysia. Only people working in the pig industry became affected, mostly people actually handling pigs or closely exposed to sick pigs. Since the virus was spread from place to place by the movement of infected pigs, control was based on controlling the infection in pigs.

A new virus isolated

Initially, Japanese encephalitis (JE) was suspected because of this association of human cases with pigs, but JE control measures were not successful in halting the outbreak. In March, 1999, Dr Chua Kaw Bing and Professor Lam Sai Kit at the University of Malaya isolated an unknown virus from human patients (Chua et al. 1999). Rota et al. (1999) showed the new virus was related to but different from Hendra virus, a zoonotic paramyxovirus that had been associated with a small number of deaths of horses and people in Australia. The new virus in Malaysia was called Nipah virus, after the home village of the patient.

A wildlife reservoir

Although retrospective studies indicated that the Nipah epidemic had a point source in the Ipoh district, the actual source of the virus and the means by which pigs became infected are not precisely known. Comprehensive studies of animals on farms and of wildlife have revealed that a substantial proportion of Malaysian fruit bats of the genus *Pteropus* had neutralising antibodies to Nipah virus. By analogy, Pteropid fruit bats have previously been strongly implicated as the reservoir host of the related Hendra virus in Australia (Young et al. 1996; Halpin et al. 1996; Williamson et al. 1998). In Malaysia, occasional bats of other genera in the Pteropid family have also shown antibodies. Until further evidence is available it is reasonable to consider that fruit bats may be the wildlife reservoir of Nipah virus.

Nipah and Hendra viruses

Both Nipah and Hendra viruses are newly discovered viruses in the family Paramyxoviridae, and are more closely related to each other than to viruses of other genera in the family. Other recently discovered members of the Paramyxoviridae in pigs, Menangle virus in Australia (Philbey et al. 1998) and La Piedad, Michoacan virus (LPMV or blue eyed pig disease virus) in Mexico (Moreno-Lopez et al. 1986), are not closely related to Nipah virus. These other two viruses are in the *Rubulavirus* genus (Berg et al. 1991; Westernberg et al. 1999), while Nipah and Hendra viruses are best classified in a proposed new genus in the Paramyxoviridae family, and are more closely related to the genus *Morbillivirus* than to the genus *Rubulavirus*.

Molecular analysis of Nipah and Hendra viruses (Wang et al. 1998; Yu et al. 1998; Rota et al. 1999) showed that Nipah virus is different from Hendra virus at the nucleotide and amino acid levels, and should be classified as a new virus in the same group as Hendra.

However, there are serological cross reactions between Nipah and Hendra viruses, which allowed diagnostic tests for Hendra virus to be used successfully in the initial investigations of the Nipah virus outbreak. Hendra virus antigens in ELISA tests detect Nipah virus antibodies, and anti-Hendra virus antibodies detect Nipah virus antigens in immunohistochemistry tests. Currently, ELISA tests for Nipah antibodies in domestic animals use reagents prepared from Nipah virus.

Nipah Viral Disease in Animals

Pigs

Pigs may be thought of as the amplifying host for Nipah virus. The infection in pigs is frequently asymptomatic.

Alternatively, pigs may show an acute febrile illness with temperatures of 40 °C or greater accompanied by signs of respiratory and/or neurological disease. The respiratory signs ranged from open

mouth breathing or increased or forced respiration to harsh non-productive cough, which lead to descriptions such as barking cough syndrome or 'one-mile' cough. The neurologic signs observed included head pressing, agitation/biting at bars, tetanic spasms, trembling and muscle fasciculation. Mortality in pigs was low, but has not been accurately quantitated. Sows and boars sometimes died without showing other clinical signs, or with a bloody nasal discharge. Abortion was an early sign in some sow herds. Nipah virus infection spread rapidly among pigs on an affected farm, suggesting a highly contagious disease.

Experimental infection of pigs at AAHL reproduced the respiratory and neurological diseases described on farms. Animals could be infected by the oral route, infections were frequently asymptomatic, and virus was isolated from the throat and nasal passages of both diseased pigs and those not showing clinical signs. Rapid infection of in-contact pigs confirmed the infection to be contagious (Middleton et al. 1999).

The pathology of Nipah disease in pigs was different from that in people, cats and dogs. The primary lesion was a pneumonia with bronchiolitis, alveolitis and vasculitis. Viral antigen was present in vascular endothelium, in respiratory epithelium at all levels including the trachea, and in airway exudates. Syncytial cells were present in respiratory epithelium and vascular endothelium. In pigs, lesions of vasculitis were not as marked elsewhere in the body as they were in other species. Lesions of encephalitis have not been seen in pigs: the CNS disease in this species is a primarily non-suppurative meningitis.

Other species

A sick dog in Malaysia showed respiratory distress and was semi-comatose. The lungs were consolidated and the kidneys were inflamed. Nipah viral infection was diagnosed by immuno-histochemistry, with viral antigen in the kidney, among other organs. Importantly, serological studies of dogs in and around infected areas showed no evidence of transmission of Nipah virus infection from dog to dog.

Of two experimentally infected cats, one showed fever, respiratory distress and became moribund while the other developed fever and respiratory disease and recovered (Middleton et al. 1999). Both cats excreted Nipah virus in their urine.

All horses registered with racing authorities in Malaysia and Singapore, as well as a large number of other recreational horses, were screened serologically—more than 3000 horses. The only place where reactors were detected was a polo club adjacent to the infected pig farms in the Ipoh district. A small number of horses became infected, either on pig farms or on the roads in proximity to vehicles transporting pigs. Again, there was no evidence of spread of Nipah virus infection among the horses on this property.

Serological investigations of other animals on pig farms, including goats, rats and other rodents has shown no evidence of spread of Nipah virus infection among these species.

Working Safely with Nipah Virus

Nipah virus is obviously dangerous, and is best worked with in Biosafety Level 4 (BSL4) laboratories, including the use of encapsulated air suits (Figure 1). Procedures that increase the amount of virus in the environment, such as virus isolation, antigen production, tests which involve growth of the virus in cell culture and the experimental infection of animals should not be attempted except under BSL4 conditions of containment.

In countries where Nipah virus infections may be suspected, preliminary investigations can be made using the following principles. These will allow investigation of disease on farms, collection of specimens from suspected infected animals and processing of samples for approved laboratory examinations with minimum risk of infection to veterinary staff.

Basic principles for such investigations are that:

- personnel be adequately clothed and equipped to maximise their safety, and
- procedures that involve multiplication or propagation of the virus either in vivo or in vitro should not be attempted.

In a suspect Nipah virus investigation, all animals sampled and all sera should be considered infectious, and therefore dangerous, until they have been inactivated and/or tested negative. The most dangerous procedures are the clinical and post mortem examinations, blood collection, separation of the serum and preparation of the serum for testing. Specific recommendations for working safely on the farm and in the laboratory are presented in Appendices 1 and 2.

Protective clothing is important and the expense is not prohibitive. A face mask incorporating a HEPA filter, that filters virus particles, is available quite cheaply from the 3M company, for a few dollars. These are suitable for most farm and laboratory work. Staff conducting necropsies should have a higher level of protection, positive air pressure respirators that deliver HEPA filtered air to a breathing hood that covers the entire face (Figure 2). This equipment is more expensive, costing several hundred dollars. However, it is readily available from the 3M company. Central veterinary laboratories in the

Figure 1. Scientists at AAHL working with Hendra virus under Biosafety Level 4 conditions, wearing fully encapsulated suits with independent air supply.

Southeast Asian region should consider being equipped and having a team trained to conduct field examinations safely.

Laboratory Diagnosis of Nipah Virus Infections

Since Nipah virus should be worked with only in BSL4 laboratories, indirect ELISA tests using inactivated antigens have been developed for various species. However, it is still necessary to ensure that the specimens being tested do not contain live Nipah virus. Sera are heat inactivated and detergent treated prior to testing to kill any infectious virus present. With ELISA tests, false positives may occur. Reactor sera may be sent to a BSL4 laboratory for confirmation by serum neutralisation test (SNT), since the SNT is currently accepted as the 'gold standard' test for Nipah virus serology. The ELISA is a useful screening test in surveillance programs.

Serology may identify a farm where further investigations are needed. Such farms and animals should be treated as infected until proven safe. The procedures for conducting on farm investigations and post mortem examinations have been outlined in Appendix 1.

Formalin fixed tissues are safe, and so immunohistochemistry for the demonstration of Nipah viral antigen using an immunoperoxidase detection system is a most useful means of confirming active infection in animals that can be performed in most central veterinary laboratories. Nipah virus antigen has been detected in a range of organs from a number of species, during natural or experimental infections. Animals in the acute phase of infection should be selected for sampling, and a number of animals should be tested rather than single animals, especially if the investigation is in pigs.

A slightly less safe alternative for the detection of Nipah virus in infected animal tissues is the reverse transcriptase polymerase chain reaction (RT-PCR), since the samples will usually be unfixed tissues. Nipah virus specific primers have been designed. However, PCR is a difficult technology to manage because of the possibility of false positive reactions from contamination.

Virus isolation is the least safe method for demonstration of the Nipah agent, and the procedure entails

Figure 2. Scientists conducting field investigations of Nipah virus disease in Malaysia, wearing goggles and a face mask incorporating a HEPA filter (a) or a positive air pressure respirator, in which external air is taken in via a pump incorporating a HEPA filter and delivered by flexible hose to the head enclosed in a perspex and nylon full face mask.

a high level of risk for laboratory staff. Virus isolation is not recommended to play a major part in Nipah virus investigations.

Surveillance and Disease Control

Serological surveillance

In Malaysia, after Nipah ELISA tests were transferred to the national veterinary laboratory, all pig farms were tested twice within a 90-day period. Farms testing positive according to predetermined epidemiological criteria were culled. An ongoing surveillance program will continually monitor the national pig herd to give confidence that no new Nipah virus infections are occuring.

Since infections of pigs may be asymptomatic, serological surveillance is recommended for other countries that wish to confirm freedom from Nipah virus infection. The ELISA test is recommended as the screening test, but false positives will be detected. Before Nipah virus infection is diagnosed, ELISA reactors should be confirmed by SNT.

On-farm surveillance

The case description for Nipah disease in pigs has been outlined above. Nipah virus disease should be declared a notifiable disease and suspicious signs of illness in pigs reported to the veterinary services for further investigation.

Such reporting by farmers is a minimum requirement for a safe pig industry. Otherwise any new outbreaks of Nipah virus infection in swine will again be detected only by the deaths of farmers and farm workers from Nipah encephalitis.

Of course, the veterinary profession can do better than this. Nipah virus disease causes subtle changes in the disease pattern on farms. Recognition of such changes requires continuous recording of morbidity and mortality data, and evaluation of these records. Herd health monitoring would give timely early warning of new Nipah infections in pig herds.

Animal production systems around the world are moving from small-holder systems to intensive systems. Herd health monitoring should be seen as an essential component of the management of intensive

systems. Although there are computer programs to assist with herd health monitoring, a pencil and notebook can be just as useful. The essential activities are firstly to record data such as births, deaths, abortions and important signs of disease, and secondly to have these records examined or analysed by a trained person. Better control of disease in the herd is possible, and so the farmer benefits financially. Managers of large pig farms in the 'post-Nipah' era have a responsibility to their workers and the public to monitor the health of the enterprises to ensure that undiagnosed disease problems are not causing risk to themselves and others.

Biosecurity of farms

Prevention and control of infectious diseases in the modern intensive farm depends on two practices—surveillance for diseases or herd health monitoring, as noted above, and biosecurity, or the effective voluntary quarantining of the farm from the rest of the industry.

In Malaysia, Nipah virus was transmitted from farm to farm by the sale and purchase of infected pigs, via the normal trade in breeding stock and later by the sale of cheap pigs as farmers quit the industry. The epidemic could not have occurred if farmers had a strict policy of keeping their farm self contained. It must be appreciated that the introduction of animals of unknown health status carries the risk of introducing disease, and that there are no easy profits from between farm trading of animals.

Governments can legislate for biosecurity of farming enterprises, but the responsibility rests with individual farmers. Farmers must be educated by the veterinary profession to understand the risks of accepting other people's unwanted pigs. Large investments need to be protected, not only from Nipah but also from the many other infectious diseases of the pig industry. A Code of Practice, developed by people in the industry, has been suggested as a means of developing consensus on good farming practices, and voluntary compliance with those objectives.

The problem of a wildlife reservoir of Nipah virus

An added complication in the case of Nipah virus is the management of the risk of reinfection of pigs from wildlife, from fruit bats. At this point, until the involvement of bats is confirmed and the natural history of the virus in the bats is more completely understood, it seems prudent that fruit bats be considered the probable initial source of the Nipah virus. What has happened once can happen again. Hence pig farms should not be located in areas to which bats are attracted, such as orchard districts. Fruit trees should not be grown on pig farms.

Trade and the Movement of Animals

Nipah encephalitis of people crossed an international boundary through the movement of infected pigs for slaughter in Singapore. Governments in Southeast Asia need a formal decision-making framework for the management of trade in pigs and pork that poses no risk of movement of Nipah infections.

Consumption of pork has not resulted in the spread of Nipah viral infections, whereas the handling of live or recently killed infected pigs has resulted in human disease. Issues to be considered include the asymptomatic infection of pigs, the (unknown) period of viraemia and whether there is persistence of the virus in recovered animals. Criteria are needed whereby farms, regions or countries may be considered free of Nipah virus infection. Where there is a high probability of infection occurring, clinical or serological surveillance may be requested as evidence of freedom. In countries that have never reported Nipah disease, acceptance of the status quo may be adequate.

The international movement of companion and performance animals has also been disrupted to some extent by the Nipah virus outbreak. A number of countries require serological testing of horses, dogs and cats prior to their importation. OIE guidelines would be useful for the management of the trade in such animals. Where national or regional freedom could be declared and recognised, requirements for testing could become unnecessary. Again, a full description of the ecology of the Nipah virus in its wildlife reservoir would be helpful in these considerations.

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Appendix 1

Recommended Procedures for Working Safely on Farms where Nipah Virus Infection is Suspected

1. Where inspection or sampling of a farm for Nipah virus is conducted, appropriate protective clothing and safe work procedures should be adopted.
2. On arrival at the farm, designate an area which includes the departmental vehicles as a 'clean' area. Ensure that any potential infection is not introduced from the animal pens back into the clean area. Mark the boundary of the clean area so it is easily identified, and place buckets of disinfectant at the boundary of the clean area and the potentially infected farm area.
3. Before leaving the clean area, put on appropriate protective clothing:
   - long sleeve overalls;
   - rubber boots;
   - gloves, taping these to the sleeves of the overalls, preferably two sets of gloves;
   - eye protection (goggles, safety glasses or safety mask);
   - nose and mouth protection (a face mask that will filter virus particles).

   If necropsies of affected animals are to be done, staff conducting the necropsy should wear positive air pressure respirators (PAPRs) and always wear double gloves.
4. Before moving into the infected area, organise all equipment to minimise the number of times staff will have to return to the clean area from the infected area.
5. Enter the infected area (animal pens) and conduct a visual examination of the disease situation. Note the health status of all animals, the distribution of any sick or dead animals, the location of any classes of animals to be sampled, and suitable locations to either establish a sampling coordination area or to conduct post mortem examinations.
6. If pigs are to be sampled for serum, establish a sampling coordination area where tubes can be labelled and recorded. If necropsies are to be conducted, select a site where contamination of other animals can be minimised and which can be cleaned and sterilised after the job is done.
7. Within the infected area, carry a spray bottle of viricidal disinfectant so that hands and equipment can be progressively washed and sterilised throughout the course of operations, to prevent

the build up of contamination on people and equipment.

8. When operations have been completed, collect all rubbish into appropriate containers. Place all needles or disposable scalpel blades into a 'sharps' container. Place necropsied carcasses in a body bag ready for burial or burning. Spray the outside of the bag with disinfectant.
9. Wash all visible contamination (blood, faeces) from equipment, boots, hands and clothing. Proceed to the boundary of the clean and infected areas.
10. The outside of sampling containers (blood tubes, tissue jars) should be clean and sprayed with disinfectant. The containers should be tied in a plastic bag then placed in a transport container (ideally a plastic or metal container, but at least a plastic bag) and the outside of this extra container also sprayed with disinfectant.
11. Spray all clothing, and wash boots in the buckets of disinfectant. Wash all equipment in the disinfectant before taking it to the vehicles. If waterproof overalls are to be reused, ensure that these have been completely sprayed with disinfectant.
12. After everything has been disinfected, move to the vehicles, store samples and equipment and remove protective clothing. If cloth overalls have been used, wet these in disinfectant and store in leakproof plastic bags. If disinfected waterproof overalls are to be reused, store these in clean plastic bags.
13. Spray face masks and safety glasses again with viricidal disinfectant, and store for reuse.
14. Discard items such as gloves and any other rubbish into biohazard or other strong plastic bags and tie the bag. (It is good practice to leave removal of the inner pair of gloves to the last step in the undressing procedure.) At the lab, burn or autoclave the bag.
15. If it is necessary to return to the vehicles during the course of operations in the infected area, ensure disinfection of boots, gloves etc at the boundary before moving from the infected to the clean area.

Care of equipment

16. The Racal positive air pressure respirators (PAPRs) supplied by the 3M company comprise a battery operated motor and air filter in a plastic case worn on the back, a head mask with perspex face shield and a flexible air hose linking the two. All exterior surfaces should be sprayed with disinfectant after operations. All components will be reused and should be kept clean and decontaminated.
17. The batteries are recharged between use, ideally with complete discharging first. 3M supply a unit for this purpose. When not in use batteries should be discharged and recharged at regular intervals to prolong battery life and to ensure equipment is always ready for immediate use.
18. Filter cartridges in units need not be changed too frequently, say at intervals of a month if in heavy use. Protect the filter cartridge by placing a dust filter on top of the cartridge inside the lid of the unit.
19. The cheaper face masks may also be reused. They should be kept clean and sprayed lightly with disinfectant after use, to remove surface contamination.

Appendix 2

Recommended procedures for working safely in the laboratory with samples where Nipah virus infection is suspected

1. Where testing for Nipah virus is requested, samples may be contaminated with Nipah virus, and appropriate protective clothing and safe work procedures should be adopted.

Receipt of blood samples

2. Blood samples should arrive bagged and in an outer container, and the tubes already disinfected at the time of collection. Even so, staff opening containers or receiving blood tubes should be appropriately dressed:
   - long sleeve laboratory gown, that does not open at the front;
   - shoes that offer protection to the feet;
   - gloves that pull over the sleeves of the gown;
   - eye protection (goggles or safety glasses);
   - nose and mouth protection (face mask that will filter virus particles).

3. Conduct all operations in a Class II Biohazard cabinet, where possible:
   - open the outer container wearing full protective clothing described above;
   - spray the bag containing the tubes with disinfectant;
   - place the bag of tubes in the biohazard cabinet and open it carefully, checking for broken or leaking tubes;
   - spray tubes thoroughly with disinfectant, wipe dry, and place in rack for transport to the centrifuge;
   - record tube numbers and prepare labelled serum tubes for receipt of separated serum.
4. Centrifuge the blood collection tubes to clear the serum in the normal way, in a closed laboratory centrifuge. After spinning, allow the centrifuge to sit 5 minutes before opening. When opening the centrifuge, be sure to wear full protective clothing including mask and eye wear. Centrifuges can generate aerosols.
5. In the biohazard cabinet and wearing full protective clothing, open each tube and use a disposable pipette to transfer serum to a labelled tube. Dispose of pipettes and blood tubes in a biohazard plastic bag contained within the cabinet.
6. Whenever withdrawing tubes, waste disposal bags or the hands from the cabinet, disinfect with a disinfectant spray first.

Recording and storing

7. The testing laboratory may receive blood tubes for serum separation, or may receive sera already separated by a regional laboratory.
8. Sera are labelled with a testing number and stored at 4 °C to await processing.

Serum processing

9. Sera are next aliquotted into inactivation buffer in masterplates as outlined in the ELISA protocol supplied with the reagents. This should be done in a separate room from the blood separation procedure, and this room not used for any other purpose. The only people to work in this room should be trained operators, wearing full protective gear and working in a certified biohazard cabinet. Sera in inactivation buffer are next heat inactivated, after which the samples are considered non-infectious.
10. The remaining serum in the tubes is stored in a –20 °C freezer that is not used for any other purpose and that is kept secure. These sera are considered still infectious until tested negative, and the infectious status of the freezer is clearly indicated on the freezer.

Flavivirus Research at the Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand

Timothy P. Endy[1]

Abstract

The Armed Forces Research Institute of Medical Sciences (AFRIMS) in Bangkok, Thailand was established in 1960. The Department of Virology has a long tradition of flavivirus research that started with studies on dengue haemorrhagic fever (DHF). AFRIMS has contributed to flavivirus research with observations on the epidemiology of dengue and Japanese encephalitis (JE) and the development of key assays to test for these viral pathogens. Notable achievements were the development of the plaque reduction neutralisation assay and the IgM/IgG enzyme immuno-assay for JE and dengue as well as the JE vaccine trial in Thai children. The Department of Virology, AFRIMS, continues this strong tradition of flavivirus research initiated in the 1960s. Today, scientific protocols are being performed with both Thai and international collaborators on the immunology of DHF, the pathogenesis of asymptomatic dengue disease, the long-term circulation of dengue serotypes in Thailand and vaccine development of a tetravalent dengue vaccine. Japanese encephalitis is an additional important focus of our flavivirus research. Active research protocols are ongoing on the epidemiology and clinical manifestations of JE, the occurrence of natural JE infection and immunologic boosting in children vaccinated with the killed JE vaccine and the development of new second generation JE vaccines. The Department maintains a high level of expertise and is a resource on flavivirus diagnostics, the epidemiology and immunology of flavivirus diseases in Southeast Asia, and flavivirus vaccine development.

THE UNITED States Army Medical Component, Armed Forces Research Institute of Medical Sciences (USAMC–AFRIMS) has a long and distinguished history of research in tropical infectious diseases. Established in 1958 as part of the Southeast Asian Treaty Organization (SEATO), USAMC–AFRIMS has been instrumental in documenting and reporting the emergence of dengue and dengue haemorrhagic fever, Japanese encephalitis, chikungunya, hepatitis A, hepatitis E, cholera, antibiotic-resistant enteric organisms, drug-resistant malaria, drug-resistant scrub typhus and HIV virus (subtype E). Its current extensive network of field sites throughout Southeast and Southwest Asia continues to provide essential information on these diseases as well as potential emerging new infectious diseases.

Epidemiology of Dengue in Thailand

Dengue virus is the causative agent of dengue fever (DF) and dengue haemorrhagic fever (DHF) and has emerged in the last 50 years as one of the most important global health problems of the 20th century. In Thailand, the reported number of cases of dengue disease has increased since 1958 to more than 50 000 cases of DHF and DF per year, with periodic outbreaks of more than 100 000 cases. Little is known about the long-term circulation of dengue serotypes and their association with epidemics and severe dengue disease in a country hyperendemic for dengue.

A longitudinal diagnostic study of children with suspected dengue at the Queen Sirikit National Institute of Child Health (QSNICH, formally known as the Bangkok Children's Hospital) was conducted in Bangkok, Thailand from 1973 to 1998 (Nisalek et al. in prep.). During this period of observation 17 277 children were admitted with suspected severe dengue disease, of whom 14 680 were diagnosed

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with acute dengue infection: 2276 serologically consistent with primary dengue and 12 206 with secondary dengue. Dengue virus was isolated from 4881 patients and identified as den-1 in 1241 children (25% of all isolates), den-2 in 1575 (32%), den-3 in 1597 (33%) and den-4 in 468 (10%).

The predominant isolated dengue virus serotype by year was: den-2 from 1973 to 1986; den-3 in 1987; den-2 in 1988 and 1989; den-1 in 1990 to 1992; den-4 in 1993 and 1994; and den-3 from 1995 to 1998. No association was observed between specific dengue serotypes and severe dengue years.

In one country hyperendemic for dengue, at least three dengue serotypes can be isolated in any given year. One predominant serotype emerges and re-emerges as the cause of the yearly epidemic over time without specific dengue serotypes associated with large epidemics.

The Current State of Dengue Diagnosis

A rapid immunochromatographic test for IgM and IgG antibodies to dengue viruses was evaluated. The rapid test demonstrated 100% sensitivity in the serologic detection of dengue virus infection and was able to distinguish between primary and secondary dengue virus infections through the detection of IgM and/or IgG antibody. The specificity of the test for non-flavivirus infections was 88% (3 of 26 positive), while for JE virus infections the specificity of the test was only 50% (10 of 20). The rapid test demonstrated a good correlation with reference laboratory assays and may be useful for the rapid diagnosis of dengue virus infection (Vaughn et al. 1998).

Understanding the Immune Response in Dengue Disease

Fever, headache, eye pain and severe muscle and joint pain classically characterise the acute illness caused by dengue virus infection and described as dengue fever. Shock and haemorrhage associated with dengue infection, dengue haemorrhagic fever (DHF), was first noted in Manila in 1953 and recognized in Bangkok, Thailand as a clinical entity in 1958 (Gubler 1997). In 1958, there were 2158 reported cases of DHF in Thailand with 300 reported deaths.

Since 1958, Thailand has experienced a dramatic rise in the annual reported cases of dengue and is now considered hyperendemic for dengue with periodic large outbreaks of disease occurring every three to five years. The reported annual incidence of DHF increased from 9 per 100 000 in 1958 to 189 per 100 000 in 1998.

The largest reported outbreak of DHF in Thailand occurred in 1987 with 174 285 reported cases of DHF with an incidence rate of 325 per 100 000 (Nisulak et al. 1999). Plasma leakage is the hallmark of DHF though little is known about the pathophysiology of severe dengue disease.

Our group has ongoing studies that are demonstrating several important viral and host determinants of disease severity in dengue haemorrhagic fever. The association of viral factors such as certain dengue specific serotypes with more severe dengue disease and the demonstration that peak virus titre correlates with clinical severity have been recently demonstrated in our studies on dengue (Nisulak et al, 1999, Vaughn et al. 1999). T lymphocyte activation, increased cytokine levels and apoptosis also correlate with early immune activation in acute dengue illness and is related to the development of plasma leakage and disease severity (Green et al.).

Conclusion

The Department of Virology, AFRIMS maintains a high level of expertise in the field of arbovirology and flaviviruses. It is a resource for information on regional viral disease threats, diagnostic assays, product development, vaccine field site development, and phase III vaccine trials.

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Serological Responses to Japanese Encephalitis in Thai Swine

Ananda Nisalak[1]

Abstract

Studies of Japanese encephalitis virus (JEV) in Thailand have contributed to our understanding of the transmission cycle of JEV between *Culex* mosquitoes, swine and humans. Studies performed in the Chiangmai Valley, Northern Thailand in 1970 revealed that JEV infections in sentinel and indigenous swine occur every month in Thailand except during January and February. Most infections occur in a 2–3 month period, from May to July. The role of vector density and JEV transmission was studied in three suburban communities in Bangkok from January to June 1987. All three communities demonstrated comparable rates of transmission of JEV to sentinel swine despite one community having a fraction of the mosquito density of the other communities. Peak seroconversion rates in swine preceded the peak mosquito density. In 1985, a field study of JEV demonstrated the isolation of virus in mosquito pools at the same time that infections were noted in sentinel swine. Of 64 seronegative swine placed in five villages, 58 seroconverted and JEV was isolated in four swine. Serologic studies of swine in 1993 demonstrated that JE-specific IgM is detected first followed by haemagglutination inhibition and IgG antibody. Both virologic and serologic data demonstrated that 35%, 80% and 100% of sentinel swine became infected by study days 8, 17 and 57 respectively. Understanding this transmission cycle and the serologic response of swine to JEV infection as sentinels for human disease will further our understanding of the pathogenesis of this disease.

JAPANESE encephalitis (JE) virus is a mosquito-borne flavivirus first isolated in Japan in 1935 from the brain tissue of a fatal encephalitis case. Early epidemiological studies demonstrated the seasonal occurrence of this disease in Japan and suggested that an insect vector may be transmitting the virus. In 1938, the virus was isolated from *Culex tritaeniorhynchus* mosquitoes and subsequently shown to be its principal vector. Since the first clinical descriptions of this disease, much has been learned about the epidemiology of JE. High JE seroprevalence rates has been well documented among pigs, horses, and in the bird population in Japan and several other countries. Other vertebrates including cattle, sheep, dogs, and monkeys have also been found to have appreciable JE seroprevalence rates. Pigs are considered to be the primary amplifying host of JEV. The peak seasonal occurrence of JE varies geographically. In Thailand, epidemic peaks occur between late June and early August or September. In China, Japan, and Korea, epidemic peaks tend to be in August.

Studies at AFRIMS

In 1969, an epidemic of Japanese encephalitis occurred in the Chiangmai Valley and other areas of northern Thailand. Several epidemiological studies of JEV in Chiangmai Valley were conducted by AFRIMS from 1970 to 1971. These studies examined the relationship of rainfall, *Culex* mosquito density, human encephalitis cases and JEV infections of sentinel pigs. The studies demonstrated that JEV transmission to pigs and humans is highly endemic in Northern Thailand and occurs throughout the year. Peak incidence of cases occurs during the rainy season and correlates with the vector density and infection rate in swine.

Studies by AFRIMS from 1985 to 1987 explored the relationship of JE and vector density during an

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urban outbreak of this disease. In 1985, an unexpected outbreak of Japanese encephalitis occurred in Bangkok. An epidemiological survey was initiated in three suburban areas. *Culex tritaeniorhynchus* and *Cx Gelidus* comprised 71–96% of all mosquitoes collected by CO_2-baited CDC traps at the three sites. JEV was isolated in both species and the minimal infection rate (MIR) was comparable in both species.

The proportion of sentinel pigs that had JE antibodies increased proportionately and correlated with vector abundance at each site. Vector abundance was highest during the monsoon (May–October), moderate during the transition season (March–April and November–December), and lowest during the dry (January–February) seasons.

Mosquitoes collected during the monsoon yielded the greatest amount of JE isolates. Swine seroconversions were greater during the monsoon and transition seasons than in dry seasons. Indices of JE transmission activity (vector abundance, pig seroconversions, and MIRs) increased proportionately with rainfall.

In 1985, AFRIMS conducted a field trial study using an inactivated Japanese encephalitis vaccine (Biken) in 65 224 school children in Kamphangphet province, Northern Thailand. Studies of the transmission of JEV were included in the vaccine trial. Two sentinel pigs, seronegative for JEV, were placed in each village throughout the province every two weeks during the vaccine trial. Mosquitoes were collected at each site and swine tested for seroconversion to JEV every two weeks.

Results of the survey demonstrated that transmission of JEV was widespread. JEV was isolated from mosquito pools (the first sample was collected in the last week of May), and new anti-JE antibody was detected in a serum sample collected from a sentinel pig on 24 May, 1985. Of 54 seronegative pigs placed in five villages, 48 seroconverted. JEV was recovered from the blood of four pigs.

In 1993, studies conducted at AFRIMS examined swine as a potential animal model for JEV vaccine testing. Antibody analysis after acute JEV infection demonstrated that JE-specific IgM is detected first followed by haemagglutination and IgG antibody. Both virologic and serologic data demonstrated that 35%, 80% and 100% of sentinel swine became infected by study days 8, 17 and 57 respectively.

Conclusion

Pigs are important in the transmission cycle of JEV. Virtually all-domestic swine that became infected develop viraemia capable of infecting mosquitoes. Our studies with sentinel swine demonstrated that they became infected within one week of entering an endemic region with infection occurring throughout most of the year. Serologic data demonstrated that anti-JEV IgM is detected first followed by haemagglutination-inhibition and IgG antibodies.

Genetic Variations in Chinese Field Strains of Hog Cholera Virus

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Abstract

RT-PCR was employed to amplify the portion of the E2 gene encoding major immunogenic sites at the N terminal of the hog cholera virus E2 glycoprotein. This fragment was amplified directly from 23 field strains from hog cholera (HC) tissue samples, which had been responsible for serial HC outbreaks in nine geographically distinct provinces in China. Computer-based phylogenetic relationships among these strains and reference strains were obtained by analysing nucleotide sequence data. This resulted in classification of the 23 strains into two major groups. Nineteen belong to Group 2 and were further subdivided into Subgroups 1, 2 and 3. The remaining four strains, together with the Chinese reference Shimen and attenuated vaccine C strains, belong to Group I. These findings reveal, surprisingly, that HCV field strains prevalent in China in recent years are genetically divergent from the Shimen and vaccine C strains, indicating a different origin for this virus in China.

HOG CHOLERA (HC), caused by the hog cholera virus, a pestivirus belonging to the Flaviviridae family, is a highly contagious domestic animal disease worldwide, having important economic ramifications. The disease first occurred in the 1920s in China, where now there are two standard HCV strains, a virulent Shimen strain and an attenuated vaccine strain. The latter, appearing in 1957, was derived from serial passages of virulent Shimen strain through rabbits, and so is now known as hog cholera lapinised virus (HCLV). Since that time, HCLV has been used in China as a unique vaccine strain to prevent HC in pigs (Yin et al. 1997).

After spreading gradually worldwide, HCLV became known as 'C' strain. Because of a nationwide immunisation policy of twice-yearly inoculations of pigs, in spring and autumn, hog cholera is well controlled in China, with large-scale outbreaks rarely seen. However, sporadic onsets can be found each year.

It is noteworthy that a mild and atypical form of hog cholera, having a long duration, atypical clinical and pathological symptoms, and relatively low morbidity and mortality, has been observed often since the late 1970s, and that a certain proportion of vaccinated pigs contract it. The reason for this remains unknown, but is assumed to be due either to insufficient immunisation caused by non-rationalised vaccination procedures, or to a genetically variant, less virulent HCV.

Although serologic investigation has not shown different serotypes, studies using monoclonal antibody typing (Lowings et al. 1996), gene sequence analysis (Lowings et al. 1994, 1996; Vilcek et al. 1996) and restriction enzyme mapping (Lowings et al. 1996) have shown distinct HCV genetic groups, thus furthering understanding of HCV molecular epidemiology and evolution. However, this kind of work has not been carried out in China, a geographically huge country, where exist factors likely to influence virus variation and evolution. Such factors include a complex ecology, the coexistence of various animal husbandry methods, a vaccination-based disease eradication policy, and highly mobile commercial herds.

In order to understand HCV's genetic history in China, we have analysed E2 gene variation in

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23 HCV field strains by nucleotide sequencing and phylogenetic analysis. The 23 strains cover 23 sites in nine provinces, almost one-third of the country. Sequence data obtained from this fraction of the country reveal the existence of at least two distinct HCV groups in China. Most of the strains prevalent in recent years are clustered in Group 2 and are surprisingly divergent from the classical Shimen and vaccine HCLV strains, both of which belong to Group 1. Thus, a different origin of HCV in China has been proposed. This investigation has yielded results similar to those of previous studies (Hofmann et al. 1994; Lowings et al. 1996; Vilcek et al. 1996).

Materials and Methods

Viruses

All 23 HCV field strains used were from spleen or lymph node tissue samples from pigs that died from clinically-diagnosed HC. Each of the 23 samples was collected from a different site in nine Chinese provinces; thus, each strain was taken from a single site. The area involved in the investigation comprises about one-third of China.

RT-PCR and sequencing

One set of degenerate primers were chemically synthesised based on a previously published study (Lowings et al. 1996). Sense primer is 5'TC(GA)(AT) CAACCAA (TC)GAGATAGGG3' corresponding to Alfort position 2467-2487. Antisense primer is 5'CA CAG(CT)CC(AG)AA(TC)CC(AG)AAGTCATC 3' corresponding to Alfort position 2738-2716 (Meyers et al. 1989).

Total RNA, prepared directly from collected tissue samples, using TRIzol reagent according to the manufacturer's (Gibco/BRL) instructions, was used to amplify a region encoding predominant neutralising epitopes at the E2 N terminus by previously established RT-PCR protocol (Li et al. 1998). Utilising ABI PRISM 377 DNA Sequencer, PCR products were gel-purified and automatically sequenced without cloning.

Phylogenetic analysis

DNAsis computer software (HITACHI Software Co. Ltd) was used to construct a phylogenetic tree of HCV strains. In addition to the 23 nucleotide sequences and their derived amino acid sequences obtained in this study, six reference sequences representing two groups and four subgroups were retrieved from the GenBank database: Shimen (Accession No. U72047, Li et al. 1998) and HCLV or C (Accession No. 72048, Li et al. 1998), Alfort (Accession No. J04358, Meyers et al. 1989), Brescia (Accession No. M31768, Moormann et al. 1990), C1W (Accession No. L36164, Lowings et al. 1994) and ALD (Accession No. D49532, Ishikawa et al. 1995).

Results and Discussion

Sequence-based phylogenetic analysis is a powerful tool widely used for molecular epidemiology and viral evolution. The current study shows that the region encoding predominant neutralising epitopes on the E2 gene 5' is not highly variable, but variable enough (van Rijn 1993) to distinguish HCV groups. Analysis of this region is highly consistent with the results for the 5' NCR region, NS5B region, and even monoclonal antibody typing (Lowings et al. 1996).

Utilising sequence data analysis, extensive study of phylogenetic relationships among HCV isolates from more than 200 samples revealed HCV's worldwide molecular epidemiology history (Hofmann et al. 1994; Geerts et al. 1995; Lowings et al. 1996, 1999; Stadejek et al. 1996; Vilcek et al. 1996; Vilcek and Paton 1998; Harasawa and Giangaspero 1999; Widjojoatmodjo et al. 1999). However, there exists no relevant information for China.

China has veterinary world importance, not only because it is geographically huge, but also for the following reasons. HC has been endemic in China for more than half a century; outbreaks were dominated by typical and acute infection of pigs at all ages with high morbidity and mortality before the 1970s, then featured atypical and chronic infection and sporadic onset with relative low morbidity and mortality since the 1980s.

In recent years, infection of piglets has been seen much more frequently than of young and adult pigs. In addition, China has pigs numbering 475 million, the largest in the world. This, coupled with China's vaccination-based control policy and the high mobility of commercial herds, maximises opportunities for HCV transmission.

In order to understand the background of HCV molecular epidemiology in China, and to make this kind of analysis comparable to a previous study (Lowings et al. 1996), the exact same primer set was used to sequence the partial E2 gene of field strains collected in China so far. Nucleotide sequences of 23 Chinese field strains, along with six reference sequences, were aligned and compared to analyse variation and determine the phylogenetic relationship among the strains (see Figure 1). It was determined that the 23 field strains could be divided into two major groups with a nucleotide homology of less than 80%. Most, 19 of the 23, were genetically

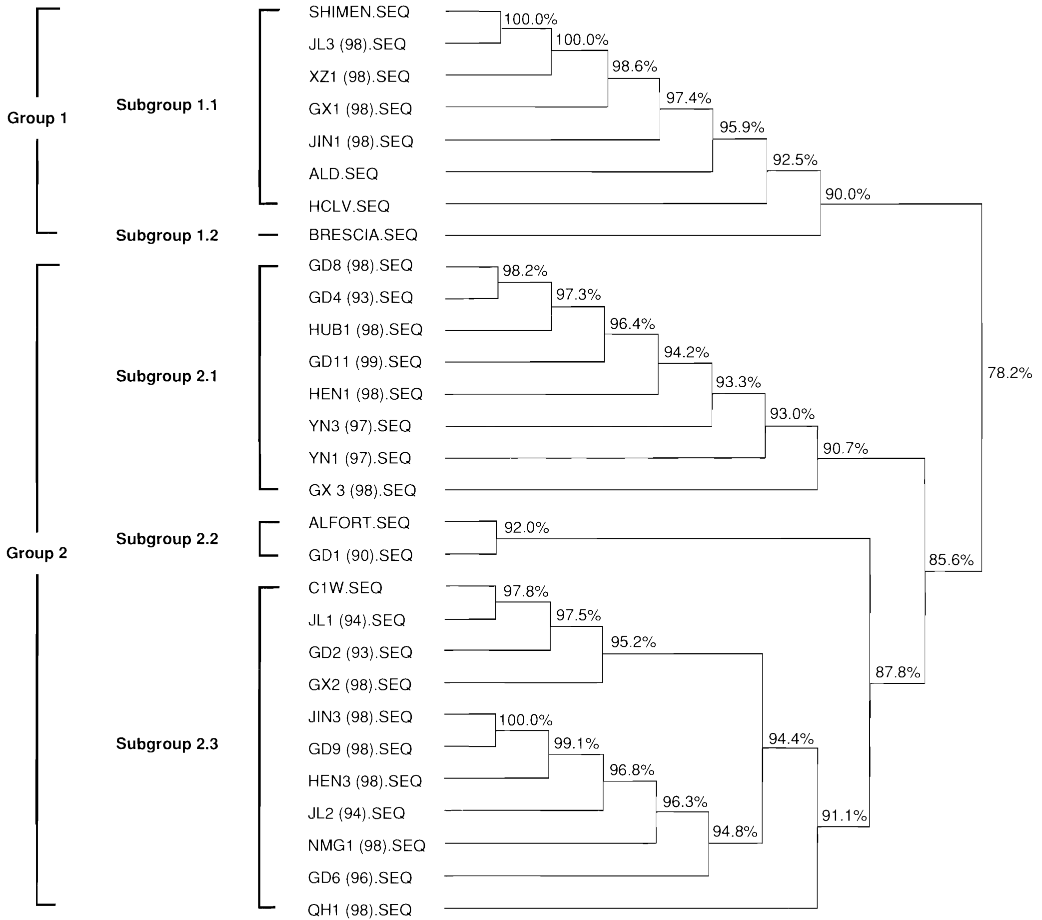

Figure 1. Key to field-strain designation: Capital letters indicate the provincial location of the virus strain, i.e. JL: JiLin; XZ: XiZang; GX: GuangXi; GD: GuangDong; HUB: HuBei; HEN: HeNan; YN: YunNan; NMG: NeiMongGu; and QH: QingHai. The numbers following the letters indicate the order in which the strains were collected. Parenthesised numbers indicate the year that the virus strain was collected. Reference strains are identified by their original names from previous publications. Percentages represent the homology between two lineages.

divergent from the classical Shimen strain (isolated in the 1950s and now a Chinese reference strain), their variation, compared with Shimen and HCLV, existing in the E2 gene. The 19 strains, together with Alfort and C1W (an Italian field strain isolated in 1985), belong to Group 2 and could be further divided into three subgroups. The remaining four strains, together with Brescia, ALD, HCLV and Shimen, belong to Group 1. This would indicate that our classification is comparable to those published previously (Lowings et al. 1996; Stadejek et al. 1996; Vilcek et al. 1996).

The phylogenetic tree shows that all seven strains from GuangDong province (GD strains), belong to Group 2 and could be further divided into three subgroups, indicating HCV variety in the province. Located at extreme south of China, GuangDong is one of the most economically developed of the Chinese provinces. It has had heavy pig trading, with most of its pig populations having been introduced from overseas, as well as from inner Chinese provinces. The high pig herd mobility would suggest cross-transmission of divergent HCV strains in that area. In addition, an evolutionary relationship might

be deduced from the fact those seven GD strains have spanned 10 years, from 1990 to 1999, implying a possible relationship with Alfort, since the oldest strain, GD1, was closest to it. This finding would explain how the European HCV strain was transmitted to China. HCV variety was also found in neighbouring GuangXi Province (GX strains), where two groups of HCV exist: strain GX1 belongs to Group 1, whereas strains GX 2and 3 belong to Group 2.

Of six references, four virulent strains, Alfort, Brescia, C1W and ALD, were previously clustered into four subgroups (Lowings et al. 1996) and were taken in this study as lineage references. These four reference sequences confirmed the reliability of our grouping of 23 Chinese field strains into two major groups and four subgroups. Moreover, three of the reference strains, though not Brescia, revealed close counterparts to Chinese strains in their lineage, indicating evolutionary origins for Chinese HCVs other than the one from which Shimen derived.

This is a first report on HCV molecular epidemiology in China. Although the data obtained so far are limited, thorough investigation is under way to determine nationwide HCV variation.

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DNA-mediated Protection Against Hog Cholera Virus

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Abstract

Four DNA fragments encoding hog cholera virus (HCV) E2 glycoprotein with different function motifs were obtained by PCR amplification. Four corresponding eukaryotic expression plasmids were constructed and designated as: (a) pcDST containing the entire E2 gene insertion with the signal sequence at 5' end and the transmembrane one at 3' end; (b) pcDSW containing the E2 gene insertion with the signal, but not the transmembrane sequence; (c) pcWT containing the E2 gene insertion with transmembrane, but not the signal sequence; and (d) pcDWW containing the E2 gene insertion without both the signal and the transmembrane sequences. All four plasmids have been readily transfected in BHK cells with pcDST and pcDSW capable of secreting E2 antigen. Plasmids pcDST and pcDSW were shown to induce humoral immune response against HCV in mice when administered intramuscularly, but no immune responses were detected with either pcDWT or pcDWW. The antibody level elicited by pcDSW was higher than that induced by pcDST. The results showed the different function motif of E2 gene exerted a significant influence on DNA-mediated immune response. When the pcDSW was used to immunise rabbits and pigs, both rabbits and pigs were shown to be protected from the virulent challenge of HCV (hog cholera lapinised virus for rabbits and Shimen strain for pigs).

Hog Cholera (HC), characterised by symptoms of haemorrhagic fever and immune depression, is a contagious swine disease usually leading to substantial economic losses. The pathogen is HC virus (HCV), a member of pestivirus genus, Flaviviridae family, and also known as classical swine fever virus (CSFV). The hog cholera lapinised virus (HCLV) vaccine, developed in China in the 1950s, has played a key role in the control of the pandemic of hog cholera. However, an atypical form of HC and immunisation failure has emerged in China since the 1970s, especially in recent years. Infection of piglets has been seen much more frequently than in young and adult pigs. In some countries such as Germany and The Netherlands where HCV had been eradicated, the epidemic of HC emerged again. It is important to develop a safer and more efficacious vaccine with a new type of HC strain in control of hog cholera.

DNA vaccine can be developed as an alternative to the traditional vaccines. Vaccination of DNA constructs encoding prominent immunogens of viruses has been shown to induce antiviral immunity in various animal models. For example, HIV (MacGregor et al. 1998; Ugen et al. 1998; Boyer et al. 1999), HBV (Yuen et al. 1999) and malaria (Wang et al. 1998) in humans; BHV1 (Hurk et al. 1997; Lewis et al. 1999) and BRSV (Schrijver et al. 1997, 1998) in bovine and FMDV (Huang et al. 1999; Ward et al. 1997), PRV (Gerdts et al. 1999; Haagmans et al. 1998), PRRSV (Pirzadeh and Dea 1998) and *E. coli* (Turnes et al. 1999) in swine. Although some host animal trials have been shown to be promising in protection against the viral challenge, much more remains to be done to enhance the efficacy of the naked DNA vaccination. The objective of this study was to determine the HCV-specific immune responses elicited from the DNA constructs and the animal protection from a virulent challenge.

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Materials and Methods

Construction of eukaryotic expression plasmids of E2 gene of HCV

There is a signal peptide preceding the mature E1 protein and three transmembrane domains at its C terminus. Four E2 gene fragments were amplified by PCR using plasmid pHCE2 as template (Li 1998) to contain various combinations with or without the signal sequence and the sequences of the transmembrane domains. The primers were chemically synthesised as shown in Table 1.

The underlined sequences of the primers are corresponding to the E2 gene sequences. The remaining sequences are for restriction enzyme sites for cloning purposes. The CCACCATG sequence has also been adeed to enhance the translation level of E2 gene acording to the Kozak rules (for forward primers). Forward primer Ps contains the signal sequence and Pws not. Reverse primer Pt contains the transmembrane sequence and Pwt not. Random combination of forward and reverse primers would lead to the PCR amplification of four different E2 gene fragments. The PCR procedure was as follows: 95• 60S, 60• 70S, 72• 60S, 30 cycles. In order to construct HCV DNA vaccine plasmid, each of the four amplified E2 gene fragments was digested with Bam HI and Eco RI and then cloned into the same-enzyme digested plasmic $pcDNA_3$ (Invitrogen). The in-frame sequence of E2 gene fragment in each plasmid was verified by auto-sequencing using ABI PRISM™ 377 DNA sequencer.

Bacterial transformation and plasmid preparation

Transformation of *E. coli* and the DNA plasmid preparation were done according to the method as described (Wicks et al. 1995). The concentration and purity of plasmid DNA were assayed by measuring the absorbency at 260 nm and 280 nm with Gene-Quant (Pharmacia).

Evaluation of eukaryotic E2 expression plasmids in vitro

The expression efficiency of the E2 gene of the four plasmids was analysed by in vitro transfection of BHK-21 cells followed by direct ELISA detection of the cells and culture supernatants. Transfection was performed with the LIPOFECTIN reagent (Life Technologies) according to the manufacturer's instructions with the following modification. Briefly, the mixture of DNA and LIPOFECTIN Reagent was placed on 40• 60% confluent monolayer of BHK-21 cells for 12 hours. The suspension was replaced by Gibco RPMI 1640 containing 10% newborn calf serum and 40 μg/mL of G_{418}. The cells were incubated at 37• for 10 days to let the transfected cells be confluent and the untransfected control cells be dead. Then the G_{418} resistance cells were passaged one more time by repeating the above procedure. Forty-eight hours later, the G_{418} resistance cells and the culture supernatant were harvested for assaying the E2 expression by direct ELISA. The pig anti-HCV antibody was prepared as described by Tsai Chun Lin (1969). Peroxidase-conjugated anti-HCV antibody was made by the method similar to that described by Wilson et al. (1978).

Immunisation of mice

Female 18• 20 g KM mice were purchased from the Laboratory Animals Center of Changchun Institute of Biological Products. Prior to the vaccination, the mice were anaesthetised by i.p. injection of 0.2 mL saline containing ketamine and xlyazine (100• g/g and 10• g/g body weight, respectively). The DNA vaccine was prepared by diluting the purified DNA preps to 1μg/μl in PBS. Approximately 100• g of the DNA vaccine plasmid were given to each mouse with 50 ug DNA injected to the tibias anterior (TA) area of each hind leg. A total of six mice was inoculated with each of the plasmid preparations. Mice inoculated with the same amount of blank vector $pcDNA_3$ were used as control. The mice were immunised at weeks 0, 2 and 4 and the sera were collected by tail bleeds at weeks 3, 4, 5, 6, and 7.

Rabbit immunisation and challenge

Six New Zealand white rabbits (about 2 kg) were purchased from the Laboratory Animal Center of the university. They were divided into three groups (pcDST, pcDSW and $pcDNA_3$ as control). Two rabbits were given for each group. The rabbits were injected intramuscularly at the TA areas of two legs with 0.5 mg per leg of the vaccine DNA preparation and intradermally (i.d.) at 10 skin sites on the back

Table 1. The primer sequences used to amplify the different E2 gene fragments.

Name	Primers	Primers location in the E2 gene
Ps	5'•CGGGATCCGCCACCATGGTATTAAGAGGACAGGTCGTGC•3'	-63•-42nt
Pws	5'•CGGGATCCGCCACCATGGGCCGGCTAGCCTGCAAG•3'	-3• 15nt
Pt	5'•CGGAATTCCTACTGTAGACCAGCGGCGAGCTGTTC•3'	1102• 1125nt
Pwt	5'•CGGAATTCCTAGTCAAACCAGTACTGATACTCGCC•3'	966• 984nt

(20 μg per site). The inoculation was repeated three times at two-week intervals. All immunised rabbits were challenged with a dose of 10 MID HCLV two weeks after the last immunisation.

Immunisation of pigs

Eight pigs at two months of age were purchased from a HCV-free farm on the outskirts of Changchun City. The pigs and their sows had never been vaccinated with HCV vaccine and their sera were tested to be HC-negative by indirect ELISA (Yu et al. 1999). The immunisation protocol was the same as described for the rabbits. Six pigs were immunised with pcDSW and two with $pcDNA_3$ as control.

Antibody assays

Anti-HCV antibodies were detected by an indirect ELISA using the recombinant E2 as antigen according to the method described previously (Yu et al. 1999). Briefly, each well of Nunc immunosorb plate was coated with 1.2• g recombinant E2 antigen in carbonate buffer saline (CBS, 0.05M pH9.6) for overnight at 4• and then blocked for 1 hour at 37• with 3% gelatin in PBS. Serum samples were diluted in PBS at 1• 200 prior to the addition to the appropriate wells. After 2 hours of incubation at 37• the plates were washed three times with PBS/0.05% Tween-20 and then added with anti-mouse (or pig) peroxidase-conjugate (Sigma). After repeating the above incubation and washes, the plates were added with 0.045% H_2O_2 and OPD (0.4 mg/mL) in phosphate-citrate buffer (0.1 M citric acid, 0.2 M sodium phosphate dibasic). Allow the reaction for 30 minutes and add 2 M H_2SO_4 to stop the reaction. Absorbency at 490 nm was read on a DG3022A ELISA-Reader.

Results

HCV E2 expression in vitro cells

In order to determine whether the signal sequence and the transmembrane domain of E2 gene of HCV exert any influence on the immune response of DNA vaccine, four different plasmids, pcDST, pcDSW, pcDWT and pcDWW, were constructed and then used to transfect BHK-21 cells. Each plasmid-transfected cell and their culture supernatant were assayed respectively by ELISA for HCV E2 expression. The results showed that E2 products could be detected from the cells transfected with plasmids pcDST and pcDSW, but not with pcDWW and pcDWT. Moreover, E2 expression was detected in the culture pellets of cells transfected by both pcDST and pcDSW and in the culture supernatant of pcDSW-transfected cells as well, but not in the supernatant of pcDST-transfected cells based on the ELISA assay (data shown in Table 1). These results suggested that E2 glycoprotein could be anchored on the cell membrane by the expression of transmembrane domain of E2 gene and could only be secreted into the culture supernatant as the transmembrane domain was deleted.

Comparison of mouse immune responses induced by four different plasmids

Four plasmids were used to immunise the four mouse groups with six mice in each group. The collected sera were diluted 200-fold and their antibody responses against HCV were evaluated by an indirect-ELISA using the recombinant E2 as antigen (Yu et al 1999). Serum conversion was judged by the ratio P/N• 2.0.

The results showed that the mice immunised with pcDST and pcDSW exhibited serum conversion at week 4 (i.e. 2 weeks after the second immunisation). The antibody responses were increased gradually to week 7, the end of bleed. The specific antibody level induced by pcDSW was slightly higher than that induced by pcDST (Table 3). However, immune responses were not detected for either pcDWT or pcDWW (data not shown).

Protection of rabbits against HCLV challenge

Plasmids pcDST and pcDSW were used to vaccinate rabbits. All immunised rabbits were challenged with a dose of 10 MID HCLV two weeks after the last immunisation. The body temperatures of the rabbits were measured twice a day for 3 days before challenge and for 2 days after challenge, and then 4 times a day for 2 weeks 2 days after challenge.

After challenge, $pcDNA_3$ controls were deemed not to yield immunity against HCLV infection since a significant increase of body temperature over 1• was observed at 84–96 hours after challenge and lasted for about 36 hours. In pcDST group a mild increase of body temperature over 0.5• was observed lasting a short time about 12 hours. However, no body temperature increase was observed in pcDSW

Table 2. The expression of HCV DNA vaccine plasmids in BHK-21 (direct-ELISA, OD_{490}).

Groups	pcDST	pcDSW	pcDWT	pcDWW	$pcDNA_3$ control
Supernatants	0.070	0.656	0.025	0.037	0.014
Transfected cell pellets	0.521	0.510	0.213	0.175	0.200

Table 3. The antiHCV IgG levels induced by pcDST and pcDSW (indirect-ELISA).

Groups	OD_{490}				
	3w	4w	5w	6w	7w
pcDSW	0.086±0.021*	0.139±0.023	0.167±0.032	0.240±0.020	0.283±0.032
pcDST	0.073±0.021	0.135±0.016	0.163±0.025	0.212±0.015	0.267±0.038
$pcDNA_3$	0.061±0.032	0.060±0.018	0.069±0.020	0.058±0.030	0.070±0.021

*the mean value from 6 immunised mice.

Table 4. Antibody responses against HCV in pigs immunised with DNA vaccine (indirect ELISA).

Groups	OD_{490}					
	Days after immunisation				Days after challenge	
	0	15	30	45	4	8
pcDNA3	0.12±0.01	0.14±0.01	0.13±0.01	0.13±0.02	0.13±0.01	0.14±0.01
pcDSW	0.12±0.01	0.13±0.02	0.22±0.05	0.30±0.03	0.40±0.04	0.45±0.03

group which, thereby, was deemed to yield an efficient immunity against HCLV infection. These results probably suggest that pcDSW was likely to provoke stronger immune response than pcDST, although both were effective in eliciting the HCV immune response in rabbits.

Protection of pigs against HCV challenge

Plasmid, pcDSW seems to be the most effective construct in eliciting an immunity against HCV based on the mouse and rabbit studies and so was chosen to be used to immunise pigs. Two weeks after last inoculation all six pcDSW-immunised pigs completely resisted the challenge with a lethal dose of HCV Shimen strain as determined by the observation of clinical signs, visible pathological inspection and antigen detection of HCV. Two of them just showed a slight fever without HC symptoms and pathological lesions and recovered next day. The two $pcDNA_3$ control pigs suffered severe HC after challenge, which showed typically acute HC symptoms and pathological lesions. One died 10 days later and another was killed at moribund stage. The titre of HCV-specific antibodies elicited by DNA vaccine increased with the time and the recalling reaction of immune response after challenge was observed for immunised pigs but not for controls (as shown in Table 4).

Discussion

In this study, four different functional HCV E2 fragments were amplified by PCR and subcloned into eukaryotic expression vector $pcDNA_3$ for the construction of the four E2 expression plasmids. Since E2 gene is one partial sequence of a large open reading frame and does not contain the initiative code, the sequence CCACCATG, which is compatible with Kozak's rule, was added to the 5' end of E2 to enhance the efficiency of translation initiation.

A signal peptide precedes the N terminus of mature E2 protein and three transmembrane domains locate at its C terminus (Rumenape et al. 1993). In order to understand whether these two function motifs exert any influence on the immune response level, four different E2 gene expression plasmids with or without the signal sequence and the transmembrane sequences were constructed. Immunisation of mice with these plasmids showed that pcDST and pcDSW (both contain signal sequence proceeding E2) could elicit an immune response whereas pcDWT and pcDWW (both do not contain signal sequence) could not. The result was different from that obtained by Haddad et al. (1997). The reason may be that E2 is a glycoprotein and cannot be processed to be a mature protein without a signal sequence.

Zijl et al. (1991) has found that the presence of the transmembrane domains at the C terminus of E2 is required to obrain complete protection mediated by the recombinant pseudorabies virus (PRV) expressing HCV E2. However, it might be different for the HCV DNA vaccine. The mouse serology experiment in this study showed that the level of immune response elicited by the E2 DNA plasmid without transmembrane domains was higher than that with transmembrane domains when administered intramuscularly.

The reason is unclear. It can be speculated that muscles are the major vaccine injection sites and there are few AP cells (APC) in muscles. Muscle cells do not possess the function of antigen presentation (AP). Therefore, if the antigen expressed by muscle cell with the transmembrane domain, it would be associated with the membrane and thus the antigen would not be transported properly. When the transmembrane domain is omitted, the E2 antigen could be secreted out of the cell and delivered elsewhere through tissue liquids, lymph liquids and even blood and thus the antigen message could be presented properly to the immune system.

It is further evidence that pcDSW could completely protect immunised rabbits from the HCLV challenge but pcDST only provided limited protection. All six pigs immunised with pcDSW were protected from the challenge of virulent HCV Shimen strain. The results showed pcDSW could induce a strong immune response.

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