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MANUAL ON THE DIAGNOSIS OF NIPAH VIRUS INFECTION IN ANIMALS

Food and Agriculture Organization
of the United Nations
Regional Office for Asia and the Pacific

Animal Production and Health Commission
for Asia and the Pacific (APHCA)

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Dedicated to
the memory of those who died
in the Malaysian Nipah virus outbreak.

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FOREWORD

The outbreak of Nipah virus in Malaysia in 1999 created history in the category of new emerging diseases. It caused major losses, both in animal and human lives and to trade, and created a significant set back to the swine sector of the animal industry in Malaysia. The control and eradication of Nipah virus was an example in international cooperation with prompt participation and inputs from many countries.

While retrospective epidemiological investigations now indicate that the disease may have caused mortality in pigs and humans at least one year earlier, the discovery and identification of the infective agent in March 1999 was the turning point in controlling the major outbreak which began in late 1998. The Government of Malaysia acted very boldly in eliminating the carrier animals at the infected foci in a number of locations across the country. The culling of infected pigs has successfully stopped the infection of humans in its tracks, after 257 people were registered as infected with this virus. The removal of pigs during the outbreak period, and subsequent mopping up and surveillance operations affected 1 006 farmers and 1.2 million pigs. The effect on the loss of human lives and the economies of these swine farmers as well as others in related activities will remain for a long time.

The outbreak of a new disease and the discovery of a new infective agent like Nipah virus has created new experiences and expertise. The identification of species of fruit bats as the probable natural host of Nipah virus and the related Hendra virus raises the possibility that these or other novel paramyxoviruses may be more prevalent than we think, given the occurrence of the fruit bat species in many countries in the region. This manual is the result of the experience of many scientists and experts who had been involved in the initial control programme and the subsequent scientific investigations on this

disease and the infective agent. It should serve as an effective guide to other scientists, diagnosticians, laboratory personnel, field operatives and others who are interested in this subject.

Nipah virus is a zoonotic agent that has caused death in animals and humans. In Malaysia, the effective carrier was pigs, and transfer to humans was through direct contact with infected pigs. Although the virus characteristics do not allow the disease to spread from human to human and become pandemic, the safety procedures in handling infected animals cannot be over-emphasized.

In mid-2001, the Office International des Epizooties (OIE) declared the Malaysian pig population officially free of Nipah virus infection. I would like to take the opportunity to thank all the scientists and personnel from all agencies and countries involved in the control of the Nipah virus disease, without whose efforts the disease could not have been effectively controlled in such a short time. My sincere congratulations are directed to Hume Field and the team of scientists who have successfully put together the materials in this manual.

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SUMMARY

The emergence of Nipah virus poses a threat to animal and public health, as well as to commerce and trade. Preliminary research has established that species of bats (genus *Pteropus*) are a natural host of the virus, however the occurrence of the virus across the distribution of other pteropid species is unknown. The overlapping distribution of these species (and so the consequent opportunity for contact) across much of the range of the genus makes the wider occurrence of Nipah or a related virus probable. The presence of Hendra virus in Australian pteropid species illustrates this.

The serious zoonotic nature of Nipah virus makes the development of and adherence to safe working practices a prerequisite to any investigation or research. It also dictates that the most appropriate initial detection methodologies for Nipah virus are those that don't involve live virus, namely ELISA serology and immunohistochemistry. When using these tests however, it needs to be remembered that (like most laboratory tests) they have imperfect sensitivity and specificity, and false positive and false negative results will occur. These issues can be addressed by using appropriate sampling methodologies, by implementing quality assurance measures for testing, by determining criteria for test interpretation prior to testing, and by maintaining collaborative relationships with international reference laboratories.

Advance planning for emergency management of disease outbreaks is the first step in effective outbreak control, and requires legislative, management, and operational preparedness. The conduct of the outbreak investigation and control in Malaysia, the subsequent surveillance for further infection, and finally measures to demonstrate freedom from infection illustrate this. In addition, experience in Malaysia has shown that the successful management of the pig industry into the future requires a partnership approach

between government agencies, industry representatives, and individual farmers. This approach provides a blueprint for the ongoing management of pig industries for freedom from Nipah virus.

The Malaysian Nipah virus experience, tragically costly in human, animal, and economic terms, has provided a spectrum of valuable information. To ignore the opportunity to learn from the experience is to ignore an opportunity to prepare for future eventualities, and squanders the lives and livelihoods lost to Nipah virus.

CHAPTER ONE

THE EMERGENCE OF NIPAH VIRUS

Introduction

Diseases that are rapidly increasing in incidence or distribution are said to be 'emerging'. The definition encompasses not only diseases associated with previously unknown (or novel) agents, but also those known diseases that are 're-emerging' spatially or temporally. What triggers disease emergence? Modern epidemiological principles contend that disease is multi-factorial - that in addition to the presence of the infectious agent, additional factors are generally necessary for infection and disease to occur. Such factors may relate to the agent, to the host, or to the environment. Putative contributing factors to disease emergence include ecological changes, changes in human demographics and behaviour, increased international travel and commerce, advances in technology and industry, microbial adaptation or change, and breakdown of public health measures (Morse 1995).

Many emerging infections are zoonoses. The introduction of a "new" zoonotic infection into a human or domestic animal population can follow the incursion of humans (accompanied by their domestic animals, livestock, and crops) into previously remote natural habitats

where unknown disease agents exist in harmony with wild reservoir hosts. Upon contact with new and naive species, an agent may 'jump species' and establish in a new species which has no natural immunity or evolved resistance (unlike the natural host which may have evolved with the agent over time). The maintenance of monocultures of genetically similar or identical individuals may further promote susceptibility to infection. Further, artificially maintained high population densities may facilitate the rapid spread of pathogens throughout livestock populations. Zoonotic infections may be passed directly to humans from the natural reservoir, or they may be transmitted to humans via an intermediate, amplifying host.

The emergence of Nipah virus in Malaysia

Approximately 1.1 million pigs were culled to contain a major outbreak of disease in pigs and humans in Peninsular Malaysia between September 1998 and May 1999. Of 257 reported and attributed human cases in Malaysia, 105 were fatal. The disease in pigs was highly contagious, and characterized by acute fever with respiratory involvement and sometimes nervous signs in all age classes. Sows and boars sometimes died peracutely (Nor *et al.* 2000). The predominant clinical syndrome in humans was encephalitic rather than respiratory, with clinical signs including fever, headache, myalgia, drowsiness, and disorientation sometimes proceeding to coma within 48 hours (Chua *et al.* 1999; Goh *et al.* 2000). In total, at

least 115 people died as a result of the outbreak. In addition to the 105 fatal cases in Malaysia, two farm workers who returned home to Indonesia (Dr Mohd Taha Arif, Ministry of Health, Kuala Lumpur: personal communication) and one abattoir worker in Singapore (Paton *et al.* 1999) died. In Malaysia, numerous others infected during the outbreak died subsequently, and many of the surviving encephalitis cases suffer nervous sequel. The majority of human cases were employed in the pig industry and had a history of direct contact with live pigs (Parashar *et al.* 2000).

Preliminary characterization of an isolate from a human case at the Centers for Disease Control and Prevention (CDC) in Fort Collins and Atlanta, USA, showed the primary causative agent in the outbreak to be a previously undescribed virus of the family *Paramyxoviridae* (CDC 1999), (Chua *et al.* 1999). This and later investigations showed the new virus, named Nipah virus, to be more closely related to Hendra virus than to other paramyxoviruses (Chua *et al.* 2000; Harcourt *et al.* 2000; Wang *et al.* 2000). Hendra virus is a recently emerged and zoonotic virus first described in horses and humans in Australia in 1994 (Murray *et al.* 1995). Nipah virus has subsequently been isolated from pigs and dogs on infected pig farms (Chua *et al.* 2000), and experimental infections of pigs and cats have confirmed the susceptibility of these species to infection and disease (Middleton *et al.* 2001).

Epidemiological evidence suggested that during the outbreak, the primary means of spread between farms and between regions was the movement of pigs. The primary mode of transmission on pig farms was believed to be via the oro-nasal route. The epidemic is believed to have started in the northern Malaysian State of Perak, from where 'fire sales' (panic selling in the face of a disease outbreak) dispersed pigs across the country. Secondary modes of transmission between farms within localized farming communities may have included roaming infected dogs and cats, and sharing of boar semen (although at present, virus has not been identified in porcine semen). Lorries transporting pigs may also have introduced the virus onto farms.

The early epidemiology of the disease in Perak, and the spillover mechanism that first introduced the infection to pigs remains undetermined. However, retrospective investigations suggest that Nipah virus has been responsible for sporadic disease in pigs in Peninsular Malaysia since late 1996, but was not recognized as a new syndrome because the clinical signs were not markedly different from those of several endemic pig diseases, and because morbidity and mortality were not remarkable (Aziz *et al.* 1999; Bunning *et al.* 2000).

The economic and social impacts of the outbreak

The outbreak had a devastating impact on the pig industry in Malaysia. Most of the 257 human encephalitis cases and the 105 fatalities were pig industry people, and their loss is keenly felt by all associated with the industry. Major economic costs have been incurred in controlling the outbreak, in lost domestic and export markets, and in allied businesses.

The government paid US$35 million in compensation for the 1.1 million pigs destroyed at an average price of US$32 per pig. An estimated cost of US$136 million was spent in the control programme from the Department of Veterinary Services. Tax revenue estimated at US$105 million was lost from the pig industry. Approximately 618 homes and 111 shops, as well as schools and banks, were evacuated in bringing the outbreak under control, causing great financial loss to the families and business involved. In addition, the pig industry in Malaysia also provided employment to farm workers and primary supporting services like drug and vaccine sales, feed and transport. It was estimated that 36 000 people from this group had suffered from the loss of employment due to closure of farms (Nor & Ong 2000b).

Prior to the outbreak, Malaysia had a standing pig population of 2.4 million. During the stamping out operation an estimated 901 228

pigs from 896 farms were destroyed in the infected areas between 28 February to 26 April 1999. A further 50 farms were culled under the national testing and surveillance programme. In total, approximately 1.1 million pigs were destroyed which cost about US$97 million, assuming that the average price per pig was US$95. Also, prior to the epidemic, Malaysia had been exporting pigs to Singapore and Hong Kong. The loss of this export trade meant a loss of about US$120 million in 1999, assuming average price per pig of US$120. In addition, local pork consumption during the peak of the outbreak dropped by 80 percent and farmers supplying this market suffered financial loss estimated to be about US$124 million during the outbreak period alone.

The episode caused a drastic change in the direction of the future of the pig industry in Malaysia. Pig farming is now allowed only in "identified pig farming areas", with farmers in other areas encouraged to undertake other agricultural and livestock activities.

The putative natural host

Fruit bats of the genus *Pteropus* have been identified as a natural reservoir host of Nipah virus (Johara *et al.* 2001). Surveillance of wildlife species for evidence of the origin of the virus was an integral part of the outbreak investigation, and when laboratory evidence indicated that Nipah and Hendra viruses were closely related,

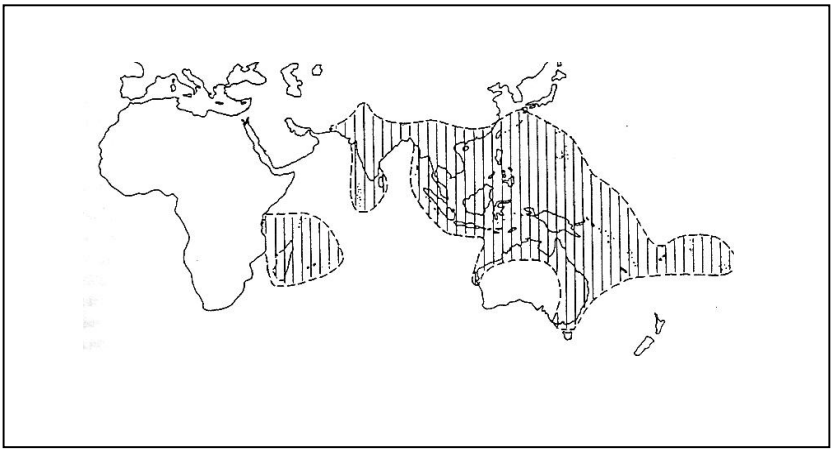
Malaysian bat species were targeted for surveillance. In common with most countries in the region, Malaysia has a great diversity of bat species. Of 324 bats from 14 species surveyed, neutralizing antibodies to Nipah virus were found in 21 bats from five species (four species of fruit bats, including two flying fox species, and one insectivorous species), although only two flying fox species showed a substantial seroprevalence. Cross neutralization of Nipah antigen by antibodies to Hendra virus was excluded as the cause of the reactivity. Subsequently, Nipah virus was isolated from the urine of a free living colony of *Pteropus hyomelanus* in Malaysia (Chua *et al.* 2001). Experimental infections of an Australian species, *Pteropus poliocephalus*, showed that this species supported a permissive cycle of infection with a human isolate of Nipah virus (Deborah Middleton *et al.*, Australian Animal Health Laboratory, Geelong, Australia: unpublished data).

Flying foxes occur across South-east Asia. The world distribution of flying foxes (genus *Pteropus*) extends from the west Indian Ocean islands of Mauritius, Madagascar and Comoro, along the sub-Himalayan region of Pakistan and India, through South-east Asia, the Philippines, Indonesia, New Guinea, the South-west Pacific Islands (to the Cook Islands), and Australia (Figure 1). There are about 60 species of flying foxes in total. Flying foxes range in body weight from 300 g to over 1 kg, and in wingspan from 600 mm to 1.7 m.

They are the largest bats in the world, do not echolocate but navigate at night by eyesight and their keen sense of smell. Females usually have only one young a year, after a six-month pregnancy. The young are independent after about three months. All flying fox species eat fruits, flowers or pollen, and roost communally in trees (Hall & Richards 2000). Flying foxes are nomadic, known to travel over considerable distances. Radiotracking studies in eastern Australia have shown individuals to undertake periodic movements of up to 600 km (Eby 1991). Where the distributions of different species overlap, roosts are shared. Thus the potential exists for interaction between flying fox populations across the region.

In the course of investigating the origins of Nipah virus, ubiquitous peridomestic species were also extensively surveyed. The uniformly negative serology results from surveyed peridomestic rodents, insectivores, and birds in Malaysia (Asiah *et al.*, unpublished data) indicate that these animals did not play a role as secondary reservoirs for Nipah virus. While evidence suggests that dogs readily acquired infection following close association with infected pigs, targeted surveillance indicated that Nipah virus did not spread horizontally within dog populations.

Figure 1: World distribution of flying foxes (genus *Pteropus*) *(adapted from* Hall & Richards 2000*)*



CHAPTER TWO

WORKING SAFELY WITH NIPAH VIRUS

Nipah virus is classified internationally at the highest biosecurity level - BSL4 - and as such warrants the highest level of care in the field and laboratory. What precautions are necessary during investigations on farms where Nipah virus infection may be suspected? How should diagnostic specimens be handled in the laboratory where Nipah virus infections are suspected but not confirmed? How should sera from suspected outbreaks be handled? In addition to the discussion herein, it is recommended that a recent review of the principles of working safely during investigations of dangerous zoonotic agents (Abraham *et al.* 2001) be read.

Risk assessment in field investigations – general principles

A necessary prelude to any investigation of possible zoonotic disease is an assessment of possible risk to those involved in the investigation. The following approaches (from the CSIRO Australian Animal Health Laboratory's Standard Operating Procedures for the Field Investigation of Animal Disease) are suggested:

- Review the situation prior to commencement of any examination of live or dead animals. Consider differential diagnoses based on the species involved, clinical syndromes, previous diagnostic

tests and epidemiological features of the disease including whether people are already known to be affected.

- Inquire whether the area has a history of particular zoonoses.
- Note the presence of any assistants, farm workers or other people at the investigation site, and their likely proximity to potential sources of infection.
- Note the location of the investigation site in relation to any environmental features which may increase the spread of the infection as a result of the investigation (such as proximity to watercourses, dams, public thoroughfares and other farming establishments).
- Review what appropriate precautions may have already been taken by yourself and others. For example, restricting public access, or vaccination of personnel where a vaccine exists.
- Special precautions should be taken for personnel who are pregnant, immunocompromised or inexperienced.
- Communicate clearly any concerns or advised precautions to assistants and other people at the investigation site. Manage the investigation site in accordance with a duty of care.
- Avoid contact with secretions, excretions and body fluids of potentially infected animals while conducting clinical examinations or collecting specimens. Wear suitable protective clothing, including examination gloves.

- Keep the use of sharps to a minimum and be sure to dispose of scalpel blades and needles in an appropriately designed "sharps" container.
- Apply insect repellent (such as DEET) in areas/situations where insect vectors are seen as a potential hazard. Apply to any exposed parts of the body and protective clothing.
- During examination and sampling of live animals, ensure adequate restraint to reduce the risk of accidental infection of personnel.
- Wash hands and equipment after examinations or specimen collection. Disinfect protective clothing, refuse and biological waste, or otherwise dispose of safely.

Safety procedures on Nipah-infected or suspected farms

It is emphasized that precautions needed for working safely on farms extend beyond issues of personal protective equipment. Thought must also be given to appropriate work procedures to ensure that the activities of the investigation do not spread the infection, or increase the risk of exposure, to other locations. The following approaches are suggested (Daniels *et al.* 2000):

- On arrival at the farm, designate a "clean" area (which commonly includes the farmhouse, offices and the departmental vehicles), and operate from that area using procedures to ensure that any potential infection is not introduced from the animal

pens back to that area. Place buckets of disinfectant at the boundary of the clean area and the potentially infected farm area. Use viricidal disinfectants such as sodium-hyperchlorite, Betadine, Dettol, Lysol, Virkon or Savlon. Ensure the boundary is easily identified by all staff on site.

- Within the clean area, put on appropriate protective clothing: long sleeve overalls, rubber boots, gloves (preferably two sets, taped to the overall sleeve cuffs), eye protection (goggles, safety glasses or safety mask), and nose and mouth protection (a face mask that will filter virus particles). People conducting necropsies on affected animals should preferably wear positive air pressure respirators (e.g. 3M Racal ^{TM}) and double glove with puncture resistant gloves (Figure 2).
- Before moving into the infected area, organize all equipment to minimize the number of times staff will have to return to the clean area from the infected area. If it is necessary to return to the vehicles during the course of operations, ensure disinfection of boots, gloves, etc. at the boundary before moving from the infected to the clean areas.

Figure 2: Protective equipment worn by those performing necropsies *(note long sleeve overalls, double puncture-resistant gloves taped to overalls, and positive air pressure respirators)*

- 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. If pigs are to be sampled for serum, establish a work area where tubes can be labelled and recorded. The use of collection tubes which facilitate clotting avoids the

need for centrifugation (and the associated possibility of aerosols being created). If necropsies are to be conducted, select a site where contamination of other animals can be minimized and which can be cleaned and sterilized after the job is done.

- Within the infected area, carry a spray bottle of disinfectant so that hands and equipment can be progressively washed and sterilized throughout the course of operations, to prevent the build up of contamination on people and equipment.
- When operations have been completed collect all rubbish into appropriate containers. Place all needles or disposable scalpel blades into a "sharps" container. Assist the farm owner to dispose of necropsied carcasses, by placing in a body bag ready for burial or burning. Spray the outside of the bag with disinfectant.
- Wash all visible contamination (blood, faeces) from equipment, boots, hands and clothing. Proceed to the boundary of the clean and infected areas and sterilise all clothing, aprons, equipment and samples. 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.
- The outside of sampling containers (blood tubes, tissue jars) should be cleaned 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.

- When everything has been disinfected return to the vehicles, store samples and equipment and remove protective clothing. If cloth overalls have been used, wet these in disinfectant and store in leak-proof plastic bags. If disinfected waterproof overalls are to be reused, store these in clean plastic bags.
- Spray face masks and safety glasses again, and store for reuse.
- Discard items such as gloves and any other rubbish into biohazard or other strong plastic bags and tie the bag. At the laboratory, burn or autoclave the bag.
- Change into clean clothes before leaving the premises. (It is good practice to leave removal of the inner pair of gloves to the last step in the undressing procedure.) Wash all clothing at least daily and do not use the same clothing between farms.
- Wash vehicles, including tires and wheels, with disinfectant before leaving the farm.

Care of equipment

- 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.

- The batteries are re-charged 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.
- 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.

Safety procedures in the laboratory with Nipah-infected or suspected samples

Where Nipah virus infection is suspected as a possible differential diagnosis, appropriate protective clothing and safe work procedures should be adopted (Daniels *et al.* 2000).

Receipt of Blood Samples

- Blood samples should arrive ideally in an inner plastic or metal container, or at least bagged, and in a leak-proof outer container (see Appendix 7), and the tubes already disinfected at the time of collection. Even so, staff opening containers or receiving blood

tubes should be appropriately dressed with a 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), and nose and mouth protection (face mask that will filter virus particles).

- Conduct all operations in a Class II Biohazard cabinet where possible, and:
  - open the outer container wearing full protective clothing described above;
  - spray the inner container containing the tubes with disinfectant;
  - place the inner container 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; and
  - record tube numbers and prepare labelled serum tubes for receipt of separated serum.
- Should centrifugation of the blood collection tubes be necessary to clear the serum, use a closed laboratory centrifuge. (Centrifugation can create aerosols - using collection tubes which facilitate clotting and thereby avoiding the need for centrifuging should be considered.) 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.

- 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.
- Whenever withdrawing tubes, waste disposal bags or the hands from the cabinet, disinfect with a disinfectant spray first.
- Allocate, label and record a testing (accession) number for the sera.
- Store sera at 4^{o}C to await processing.

Serum processing

- Where sera from herds or animals suspected of Nipah virus infection are processed, sera are aliquoted 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 is 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. After the heat inactivation step, the samples are considered non-infectious. (Sera may be treated by heat inactivation at 56^{o}C for 30 minutes following a 1:5 dilution in PBS buffer containing 0.5 percent Tween20 and 0.5 percent

Triton-X100 prior to testing. Alternatively, irradiation is an option (Daniels *et al.* 2001b).)

- The remaining serum is stored in a –20^{o}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 this is clearly indicated on the freezer.

Use standard operating procedures (SOPs) that utilize a step-wise diagnostic approach, with more dangerous procedures being undertaken only if the results of less dangerous screening tests indicate a need. Built into the SOP should be sampling strategies (for suspect outbreak and surveillance) to ensure that an adequate number of sera are collected, an adequate number of animals necropsied, and an appropriate range of tissues collected.

Laboratories should consider carefully what can be done safely with their facilities, and develop standard operating procedures that are written down, approved by senior management, and in which staff are regularly trained and retrained. Relevant recommendations for SOPs have been developed by several authors (Daniels *et al.* 2000; Nor & Ong 2000). A comprehensive discussion of diagnostic tests is presented in Chapter 4.

It is timely to suggest that veterinarians adopt a basic universal precaution approach to handling all animals and samples submitted to laboratories to minimize the risk of zoonotic disease. A basic requirement of such an approach is the prevention of exposure of the skin and mucous membranes to the body fluids of sick or potentially infected animals. Hence internal examinations and necropsies should not be conducted without gloves and other protective measures such as appropriate clothing and footwear. Depending on the circumstances, personal judgement should be exercised regarding the need for protection of the mucous membranes of the face and the need for respiratory protection.

CHAPTER THREE

REACHING A PRESUMPTIVE DIAGNOSIS ON-FARM

There are no pathognomonic features ascribable to Nipah virus disease in pigs. Differential diagnoses should include the following:

- Classical swine fever
- Porcine reproductive and respiratory syndrome
- Aujeszky's disease (Pseudorabies)
- Swine enzootic pneumonia due to *Mycoplasma hyopneumoniae*
- Porcine pleuropneumonia due to *Actinobacillus pleuropneumonia*
- Pasteurellosis

While not pathognomonic, disease in sows may support a presumptive diagnosis of Nipah virus. Severe respiratory symptoms, neurological symptoms, or increased mortalities in sows are not common features of other diseases.

The clinical disease in pigs

Nipah virus will infect pigs of all ages. Clinical observations in the Malaysian outbreak suggested a different clinical picture in different classes of animals. For example, sows were noted to present primarily a neurologic syndrome and porkers a respiratory syndrome.

It may also be that observed clinical signs reflected another variable such as husbandry. For example, the housing of sows and boars in stalls which precluded substantial exercise may have masked respiratory involvement.

Clinical observations in weaners and porkers:

Affected weaners and porkers showed acute febrile illness with respiratory signs ranging from rapid and laboured breathing to harsh non-productive coughing. In severe cases, there was blood-tinged mucous discharge from the nostrils. In less severe cases, open mouth breathing was a feature. Neurological signs were also observed, and included trembling, twitching, muscular spasms, rear leg weakness and varying degree of lameness or spastic paresis.

Clinical observations in sows and boars:

Affected sows and boars were found dead overnight, or exhibiting acute febrile illness with laboured breathing (panting), increased salivation and serous, mucopurulent or blood-tinged nasal discharge. Neurological signs including agitation and head pressing, tetanus-like spasms and seizures (Figure 3), nystagmus, champing of mouth, and apparent pharyngeal muscle paralysis were observed. Abortions were reported in affected sows.

Clinical observations in suckling pigs:

Mortality of suckling pigs was estimated to be 40 percent. The relative contribution of the effects of infection in suckling pigs and sow inability to nurse is unknown. Healthy but confirmed seropositive sows were observed to nurse healthy piglets. Most of the infected piglets showed symptoms of open mouth breathing, leg weakness with muscle tremors and neurologic twitches.

Clinical disease in pigs can be very subtle and a large proportion of pigs in a farm may not exhibit any clinical signs. The incubation period is estimated to be 7 to 14 days. Transmission studies in pigs in Australia at the CSIRO Australian Animal Health Laboratory established that pigs could be infected orally and by parenteral inoculation. It was observed that infection could spread quickly to the in-contact pigs. Neutralizing antibodies were detectable 10-14 days post-infection.

Figure 3: Acute onset neurological signs including seizures, tetanic-like spasms and jaw champing are seen in sows

Other susceptible domestic species

Some farmers reported deaths in dogs at the same time as in pigs during the Malaysian outbreak. Two such animals exhibiting a distemper-like syndrome were examined. Nipah virus was isolated from the tissues of one and Nipah virus antigen demonstrated in both by immunoperoxidase staining of tissue sections (Chua *et al.* 2000). Serological surveys of dogs in infected areas showed that up to 50 percent of clinically normal dogs had anti-Nipah virus antibodies by ELISA. Some farmers reported cats to be clinically affected also.

The susceptibility of cats was confirmed by experimental infections (Middleton *et al.* 2001).

Over 3 000 horses in Malaysia were subjected to serological examination (by serum neutralization test). Two of these horses were found to have neutralizing antibodies to Nipah virus. Immunohistochemistry on formalin-fixed tissues from a third horse with a history of neurological symptoms showed Nipah virus infection. All three horses were from a single property surrounded by infected pig farms.

A survey of ubiquitous peridomestic small mammals including rodent and bird species on and around infected pig farms found no evidence of infection.

Necropsy findings in pigs

Necropsies should be conducted of recently dead and acutely diseased pigs. Animals chosen should be representative of the affected ages and types, and should include a number of animals to increase the sensitivity of the sampling procedure.

The post-mortem findings due to Nipah virus infection in pigs are relatively non-specific. The lung and the meninges were the key organs affected. The majority of the cases showed mild to severe

lung lesions with varying degrees of consolidation, emphysema and petechial-to-ecchymotic haemorrhages, and blood-tinged exudates in the airways. On cut surface, the interlobular septa were distended. The meninges showed generalized congestion and oedema. Other visceral organs were apparently normal.

The epidemiological pattern of the disease

Clinical disease consistent with case descriptions across all classes of pigs, and a history of introduction of new pigs constitutes a suspicious epidemiological pattern. An increased incidence of sow illness and death should be treated with particular suspicion. Simultaneous reports of unexplained illness or deaths in dogs or cats should strengthen consideration of Nipah virus infection, as should concurrent human disease characterized by early signs of encephalitis (Chua *et al.* 1999) on a suspected farm. In human cases, the observed incubation period ranges from 4 to 18 days with the first symptom being a severe headache. Farm workers have been reported to develop illness after pigs have recovered.

Nipah virus in Malaysia was spread from farm to farm by the movement of infected pigs. The extensive testing and surveillance programme which followed the outbreak control programme showed that farms that did not receive pigs generally remained uninfected, even when an adjacent farm was infected. Thus, a check for any

violation of farm biosecurity should be conducted where Nipah virus is suspected. Also, farms which took prompt action to cull populations of grower pigs from suspected sources also avoided infection with Nipah virus, where growers and breeders were housed separately.

As Nipah virus infection has now been eradicated from the Malaysian pig herd, it is most probable that any new outbreaks will reflect another spillover of the virus from the wildlife reservoir. Thus any future investigation might consider whether contact between the affected pigs and fruit bats may have occurred, although this scenario would only be relevant for the first premises to be infected. Factors to consider would be the system of housing of the pigs, and the presence of fruit or flowering trees that could attract foraging bats to the vicinity of the farm.

CHAPTER FOUR

CONFIRMATORY LABORATORY DIAGNOSIS

Procedures for the laboratory diagnosis of Nipah virus infections include serology, histopathology, immunohistochemistry, electron microscopy, polymerase chain reaction (PCR), and virus isolation. The recommended initial screening tests are ELISA serology and immunohistochemistry, neither of which amplify infectious virus, and so are safer tests in the laboratory.

Serological tests

In determining a sampling strategy it should be remembered that Nipah virus infection is highly contagious in pigs. By the time a farm is suspected to be infected, it is likely that a substantial proportion of pigs will have antibodies. As a guide, if it is expected that more than 20 percent of the pigs may have already seroconverted, 15 serum samples from each age group (adult, grower and weaner) will give a 95 percent probability of detecting seropositive animals (Daniels *et al.* 2001b).

ELISA

ELISA serology can be conducted safely and quickly without access to PC4 facilities, and can be a most useful diagnostic tool. Where

laboratories are establishing an ELISA capability, it is recommended that as well as standardizing against positive and negative controls, the test should be validated against a reference panel of at least 500 sera representative of those to be routinely tested. Testing of a random sample of the 500 sera by serum neutralization test in a PC4 facility will give assurance that the sera are indeed negative for antibodies to Nipah virus. It also allows for an estimate of ELISA test specificity relative to the SNT to be calculated (Daniels *et al.* 2001b).

The current ELISA configuration developed by CSIRO Australian Animal Health Laboratory has a blocking step to minimize non-specific reactions. The negative control antigen is prepared in Vero cells in an identical manner to the virus-infected cell lysates, and used in a pre-absorption step and as a mock antigen in parallel with viral antigen on the test plates. In this way any high levels of non-specific binding are removed or identified. Recombinant Nipah virus G and M protein antigens, generated using baculovirus expression systems, have been used experimentally but have not yet been adopted routinely (Daniels *et al.* 2001b).

ELISA serology can also be a useful surveillance tool. It is emphasized that surveillance programmes need to be designed carefully, based on epidemiological principles, and in the knowledge that the ELISA screening test does not have 100 percent specificity.

Thus, there will be false positives. The response to such ELISA reactors must be planned with the relevant veterinary and public health authorities in advance. To the pig producers false positives in the ELISA create much anxiety, while to the public health authorities the possibility of false negatives is a concern. The sensitivity of the testing procedure can be addressed through careful epidemiological design of the sampling strategy (Daniels *et al.* 2001a; Daniels *et al.* 2001b).

Serum neutralization tests

The serum neutralization test (SNT) is the accepted reference serological test, but because Nipah virus is a BSL4 level agent, biosafety considerations require that this work be carried out in a PC4 facility. In developing diagnostic and surveillance capabilities for Nipah virus, a partnership with an international reference laboratory with PC4 capabilities is strongly recommended (Daniels *et al.* 2001b).

Specimens for submission for serology:

Serum should be removed from the clotted blood samples within 24 hours to avoid haemolysis. For air transport to a laboratory (for example, an overseas reference laboratory), the serum samples should be packed by a trained person in accordance with International Air Transport Association (IATA) packing instruction

602 (see Appendix 6). The recipient country will require a valid import permit, so prior consultation with the reference laboratory is necessary.

Histopathology

The pathogenesis of Nipah virus infection involves primarily vascular endothelium in all species. In pigs, the respiratory epithelium is also involved. Although formation of syncytia is a feature of Nipah virus histopathology (Hooper *et al.* 2001), these structures are not identifiable in all cases, so histopathological changes are not pathognomonic. While histopathology is a useful diagnostic tool, it should be noted that specificity may be lacking where diseases causing lung and/or brain pathology (Aujeszky's disease, Swine fever, Enzootic pneumonia) coexist with (or precede) Nipah virus infections.

In pigs, most of the principal histopathological lesions of Nipah virus infection were observed in the lung tissues. Generalized vasculitis with fibrinoid necrosis, haemorrhages, and infiltration of mononuclear cell sometimes associated with thrombosis were observed notably in the lungs, kidneys, and lymphoid tissues. There was moderate to severe interstitial pneumonia with widespread haemorrhages in the interlobular septa. Lesions seen in the bronchi and bronchioles were those of hyperplasia of the columnar

epithelium, peribronchiolar and peribronchial infiltration of lymphocytes, exudation to the lumen of live and dead cells and other debris and single cell necrosis of columnar cells. Numerous neutrophils were seen within the alveoli and within bronchioles and bronchi. Syncytial cell formations were seen in the endothelial cells of the blood vessels of the lung and within the alveolar spaces. In the brain, some degree of meningitis, characterized by oedema and infiltration of lymphocytes, plasma cells and macrophages, as well as vasculitis characterized by swollen vessel walls containing some macrophages were observed.

Immunohistology has shown a high concentration of the viral antigens in the endothelium of the blood vessels, particularly in the lungs. Evidence of viral antigens has also been seen in cellular debris in the lumen of the upper respiratory tract (Hooper *et al.* 2001).

Immunohistochemistry

Immunohistochemistry (IHC) is highly recommended for initial Nipah virus diagnosis. It is one of the safest of tests as it is performed on formalin-fixed tissues. Since the primary pathology occurs in the vascular endothelium, viral antigen can be detected in a range of tissues (see Appendix 4). Thus it is important that laboratory submissions include a wide range of tissues, and not just lungs. Nipah virus antigen has been detected in porcine meninges (but not

brain tissue), lungs, trachea and kidneys. In pregnant animals the uterus, placenta and foetal tissues should be submitted (Daniels *et al.* 2001a; Daniels *et al.* 2001b).

Immunohistochemistry, as with other laboratory tests, will not have perfect sensitivity and specificity. Imperfect sensitivity can be compensated by sampling an adequate number of animals at necropsy, perhaps over a period of a few days if disease is progressing on the farm. Importantly, an adequate range of tissues should be sampled from each animal. Laboratories using IHC should practise the test, keeping records of their observations. On some occasions, there will be apparent reactions that are difficult to interpret, and the specificity of the test in any laboratory will be greatly improved if the operators are familiar with the conditions and artefacts that are normally seen in their region. Consultation and sharing of specimens with colleagues in other laboratories and countries is recommended for mutual self-help. This is one of the key points in development of a laboratory quality assurance system for IHC (Daniels *et al.* 2001b).

Specimens for submission for histopathology and Immuno-histochemistry:

A wide range of (10 percent) formalin-fixed tissues packed (for air transport) in minimal formalin and in accordance with IATA packing

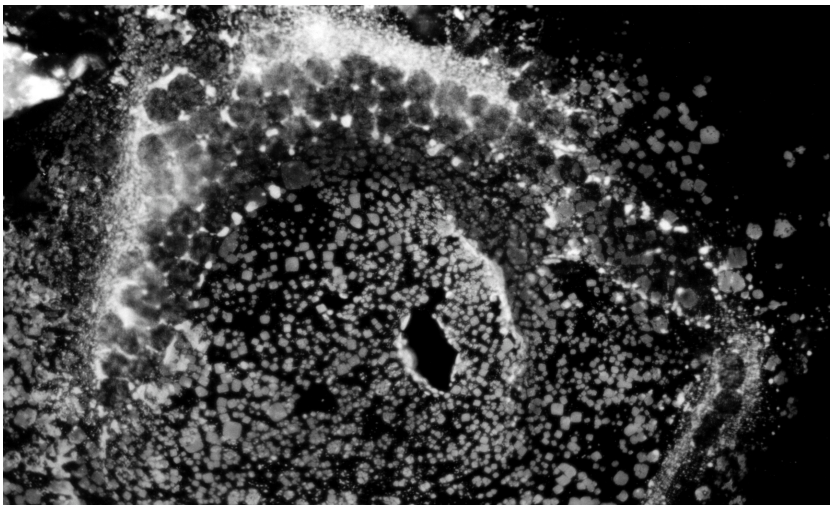
instruction 650 (see Appendix 7). Multiple lung and airway samples are recommended.

Virus isolation

Ideally, to confirm any new Nipah virus outbreak, virus should be isolated. Because Nipah virus is a BSL4 level agent, biosafety considerations require that this work be carried out only in a PC4 facility. Nipah virus grows well in Vero cells, with development of characteristic syncytia with the nuclei arranged around the periphery of the multi-nucleated cell (Figure 4). This arrangement differs from most syncytia seen in cell cultures with the closely related Hendra virus (Hyatt *et al.* 2001). Brain, lungs, kidneys and spleen should be cultured (see Appendix 4). CPE usually develops within 3 days, but two 5-day passages are recommended before discontinuing the attempt (Daniels *et al.* 2001a). Identification of virus isolates may be attempted by immunostaining of fixed, infected cells, neutralization with specific antisera, PCR of culture supernatants, and electron microscopy. Suspected new isolates should be sent to an international reference laboratory for molecular characterization (Daniels *et al.* 2001b). Teamwork among the international scientific community is strongly recommended in the handling of emerging diseases such as Nipah virus.

Figure 4: Nipah virus syncytium with nuclei characteristically forming a ring around the periphery of the multinucleated cell

(in this case with the cytoplasm stained to demonstrate Nipah virus antigens)



Specimens for submission for virus isolation:

A wide range of fresh tissues (lungs, spleen, kidneys, tonsil, central nervous system) packed (for air transport) by a trained person in accordance with IATA packing instruction 602 (see Appendix 6).

Electron microscopy

Negative contrast EM and immuno-electron microscopy are useful to rapidly obtain information on the structure and antigenic activity of

viruses in cell culture. Details of both techniques, and their application to the detection and analysis of Nipah virus (and Hendra virus) infections are described by Hyatt *et al.* (Hyatt *et al.* 2001).

PCR

Diagnostic assays for Nipah (and Hendra) virus are in routine use by the CSIRO Australian Animal Health Laboratory (based on the M and N genes) and the US Centers for Disease Control (based on the N gene). While a valuable tool, the methodology warrants strict attention to internal quality assurance to avoid spurious results (Daniels *et al.* 2001a).

CHAPTER FIVE

CONTROL AND ERADICATION

Advance planning

Outbreak control operations require a high level of organization across the spectrum of legislative, managerial, logistical, technical, and procedural activities. Thus, advance planning for the emergency management of disease outbreaks is the first step in effective outbreak control. The Australian AUSVETPLAN provides a useful model for such planning (Daniels 2001), encompassing plans for:

- management activities (control centres, high level coordination, information management, laboratory preparedness);
- control procedures (destruction and disposal of animals, valuation and compensation, decontamination of premises);
- various livestock enterprises; and
- various known diseases.

Any plan for Nipah virus preparedness should comprehensively address:

- laboratory preparedness issues, such as biosafety, scientific skills, quality assurance, epidemiology, technology transfer (see Chapters 2, 3 and 4);
- diagnostic methodologies (see Chapters 3 and 4); and

– control and eradication techniques (this chapter), pig industry issues, such as farm biosecurity and herd health monitoring (see Chapter 6).

The remainder of this chapter outlines the stamping-out approaches adopted in the Malaysian outbreak (Nor & Ong 2000; Ong *et al.* 2000; Mangkat 2001).

The organization of the control operation

Importantly, the Department of Veterinary Services (DVS) of Malaysia had legislation in place that enabled Nipah virus to be declared as a new notifiable disease, and facilitated the control and prevention of spread of the disease by empowering the DVS to declare disease control and eradication areas. The Director of the Department of Veterinary Services in each state was thus able to prohibit the keeping, movement, sale, or slaughter of pigs, and to order the examination and destruction of infected or suspect infected animals, and the closure and destruction of premises.

A taskforce of relevant Ministers, Deputy Ministers, and Secretaries General was set up by the Cabinet. Their role was to provide policy direction, to coordinate the functions of the various Ministries and Departments involved, and to closely monitor progress. Major decisions such as the depopulation of infected zones, the demolition of pig farms, the evacuation of villagers, and the payment of

compensation were made by the Cabinet Taskforce. In each affected state, special committees chaired by the Chief Minister or State Secretary were established. These committees coordinated all control operations in the state, monitored the outbreak situation, and reported to Cabinet Taskforce. In districts where major culling operations were conducted, district committees were set up to provide logistic support.

In addition, technical committees were established to carry specific activities – to act as Secretariat for the Cabinet Taskforce, to coordinate and monitor field and laboratory studies on disease investigations, to provide technical input to the culling operation, the payment of compensation, surveillance and logistics support, and public awareness and education programmes. The DVS set up a 24-hour operation room to coordinate and supervise the control operation with the state veterinary authorities, to convey DVS and Cabinet Taskforce directives to the state veterinary authorities, to provide and monitor the budget and logistic requirements of the state veterinary authorities, and to facilitate the flow of data and information to operations room and Cabinet Taskforce, and to act as a resource centre for other agencies, the media and interest groups.

Movement controls on pigs

After Nipah was declared as a disease under the legislation, all movement of pigs or pig meat (local, intrastate and interstate) was banned with immediate effect by cancelling all previously issued permits. Media releases and public notices were used to advise of the restrictions. The ban was enforced by increased DVS and police patrols on roads from infected areas. The movement ban was later amended to allow the movement of pigs outside declared zones to Government abattoirs, with each consignment transported under permit and escorted by DVS officers.

Mass culling of active disease farms

Infected zones of 2 km radius and buffer zones of 10 km radius were imposed around infected premises. All pigs within the buffer zone were culled over a 2-month period (a total of 901 228 pigs from 896 farms). The Department of Veterinary Services, the Department of Transport, the Army, other related government agencies and non-government organizations were involved in the culling operation. Prior to culling, farm owners were served with a notice of culling. Farmers and residents were evacuated, and the area sealed with police roadblocks. All personnel involved in the culling operation were reminded to put on personal protective equipment before entering infected areas.

The pigs were culled by shooting, and disposed of by burying in deep pits within the infected area, either on-farm or off-farm (see Figure 5). Chlorinated lime and detergents were used to disinfect premises and burial sites (see Figure 6). Evidence of infection of dogs in one outbreak area prompted a decision to shoot all stray dogs in infected areas. At the same time, peri-domestic animals and dog studies were conducted to determine possible transmission of virus through these animals.

Financial assistance

The Malaysian Government approved establishments of two funds: the Humanitarian Fund - to relieve hardship caused by the loss of family members, and the Nipah Trust Fund - to provide financial assistance for the pigs culled. A committee headed by the Secretary General of Ministry of Agriculture operated the trust funds, with the day to day management entrusted to the DVS Director General.

A national testing and surveillance programme

As the culling programme neared completion, a national testing and surveillance programme was implemented to determine the Nipah status of all pig farms in Peninsular Malaysia. The programme resulted in the culling of a further 50 seropositive farms (Ong *et al.* 2000), and enabled a claim of freedom from Nipah virus infection in the swine population of Peninsular Malaysia.

A national abattoir monitoring and testing programme

The third phase of the Malaysian Nipah virus control and eradication programme involved ongoing monitoring of pigs sent to abattoirs. The programme incorporated a trace-back system based on ear-notch identification to allow pigs to be traced back to farms of origin. The porker class of pig was targeted for screening, as the presence of antibodies in pigs of this age denoted infection on the farm of origin within the last four months. The programme aimed to demonstrate that Nipah virus was not circulating on pig farms, and thus to restore public confidence in pork consumption.

Figure 5: Culling and disposal by shooting and burying

Figure 6: Disinfection of burial sites using chlorinated lime

CHAPTER SIX

MANAGING PIG INDUSTRIES FOR FREEDOM FROM NIPAH VIRUS INFECTION

There are three broad areas that require attention in managing pig industries for freedom from Nipah virus infection (Daniels 2000; Daniels *et al.* 2001b):

- actions from the pig industry;
- actions from governments; and
- definition and management of the risk posed by the wildlife reservoir.

Actions from the pig industry

It is recommended that the pig industry in at-risk countries adopts a code of practice to preclude the possibility of an outbreak of such major proportions as was seen in Malaysia. This would involve the implementation of simple good management practices which are advantageous to producers even in the absence of threats such as Nipah virus. Firstly, where animals are intensively farmed there is a need for herd health monitoring, through record keeping and analysis, to identify any change in the health of the herd on each farm. Early recognition of syndromes consistent with the clinical case description (see Chapter 3) followed by laboratory testing (see

Chapter 4) will be the most efficient means of containing any potential outbreaks. Full implementation of this approach implies a strong involvement of veterinarians (probably employed as farm consultants) who have skills in epidemiology for the management and analysis of animal health records. Commitment to this style of management is of broader benefit to the farmers, as it enables the control of the whole range of disease and production issues.

Secondly, the principles of farm-gate biosecurity need to be strictly and widely applied. In Malaysia, Nipah virus spread from farm to farm through the trading of pigs (as have many other diseases of pigs previously). Where it is necessary to purchase new breeding stock, the methods for the introduction of these animals to the herd must be clearly defined. This step may include serological testing and/or quarantine.

There is little economic sense in risking the whole farm for the sake of small financial benefit from trading a few pigs, and a realignment of commercial practices is needed to better manage the risks from introductions. As with herd health monitoring, strict on-farm biosecurity allows better control of a range of porcine diseases, to the financial benefit of farmers and the long-term protection of their investment.

Actions from governments

The Nipah virus outbreak in Malaysia resulted in trade and policy responses from governments internationally. In the absence of agreed OIE guidelines, various bilateral restrictions regarding trade in pigs and pork, and other animal movements were developed by Malaysia's neighbours and trading partners. After the outbreak was controlled, a period of serological surveillance was necessary to demonstration of freedom from Nipah virus (see Chapter 5). The design and management of such programmes require veterinarians who are trained and proficient in epidemiological procedures. Cost effective sampling strategies applicable to local circumstances need to be designed, and acceptance of the suitability of the programme negotiated with the client bodies, be they trading partners, public health authorities or the OIE. A laboratory testing capability has to be established and maintained, and the interpretation of test results has to be undertaken using epidemiological principles and knowledge of test performance. Ideally, a partnership with an international reference laboratory will allow follow up testing on any samples giving results of concern.

The outbreak in Malaysia highlighted a number of areas of veterinary expertise essential for an efficient response capability: epidemiology, laboratories and equipment, and diagnostic testing and quality control.

Epidemiology is the discipline within veterinary science on which the rational scientific management of the health of animal populations is based. Within the context of Nipah virus, epidemiologists are needed in both the private and public sectors to:

- interpret and respond to the information collected in herd health programmes;
- design and manage surveillance programmes;
- conduct outbreak investigations;
- advise on the drafting of legislation for the management of the domestic industry; and
- advise on appropriate restrictions on the movement of animals and animal products.

While many countries have allocated resources to the training of epidemiologists from time to time, these people are frequently transferred or promoted, giving rise to difficulties in maintaining a critical mass of trained people everywhere they are needed. An answer in the medium term is to ensure the study of epidemiology to a high standard in undergraduate degrees, and to provide further training opportunities which address the needs of animal health authorities and the intensive animal industries.

The provision of laboratory services for Nipah virus diagnosis is a complex issue, given that work propagating the virus should be done under the PC4 conditions. Such facilities are expensive to maintain, and it may not be cost effective to have a PC4 lab in each country. Systems of bilateral and regional collaboration should be negotiated.

Diagnostic tests need to be both sensitive and specific (Daniels *et al.* 2001a), and a new generation of such tests requires ongoing research and development effort. In addition, the application of quality assurance (QA) programmes is of great value in ensuring that tests are performing within predetermined limits of acceptability and minimizing spurious reactions. Regional QA programmes based on collaboration among a number of laboratories have a role in the future for all laboratory testing.

Another area to be addressed is management and regulation of the pig industry. If a code of practice is required of industry, there may need to be a framework of legislation within which to work. Experience in Malaysia has shown that successful management of the pig industry for freedom from Nipah virus requires a partnership approach between government agencies, the industry representatives and the individual farmers. The expectations that each group has of the others can be defined through consultation and communication. Ultimately the government must legislate and the industry must

adopt methods of operation that will protect individual farms from infection, and prevent any spreading of new infection.

Managing the risk from the wildlife reservoir

Preliminary research has established that species of bats (genus *Pteropus*) are a natural host of the virus (Chua *et al.* 2001; Johara *et al.* 2001); however the geographic range of the virus in the Malaysian species, and the presence or absence of infection in related species outside Malaysia is unknown. A regional and collaborative approach is needed to map the distribution of the virus in bat populations, the incidence of infection in bats, risk factors for infection in bats, and risk factors for spillover to pigs. The pathogenesis of the virus in bats also remains to be described, particularly the mode of transmission, to enable industry to develop cost effective risk management measures. While spillover from the natural host may be a very rare event, it is worth noting that there have now been three separate outbreaks of Hendra virus disease in horses in Australia, indicating a "jump" from the wildlife reservoir on three separate occasions (Field *et al.* 2000; Hooper *et al.* 2000). Thus, wherever there is an intensive pig industry in tropical countries with similar fruit bat fauna to that in Malaysia there is a possibility of a new Nipah virus outbreak. Careful and informed management of the industry by both the private sector and government offers the best protection for public health and industry prosperity.

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APPENDIX 1

STANDARD OPERATING PROCEDURES (SOPS) FOR WORKING ON SUSPECTED NIPAH VIRUS INFECTED PREMISES

It is recommended that investigating teams take a laminated photocopy of these pages into the field for reference.

General SOPs for working on suspected Nipah virus infected premises:

On the infected farm, establish a base that may be considered "uninfected" for the purposes of operator safety from which to conduct the investigation.

1. Mark the boundary of the clean area so it is easily identified.

2. Place buckets of disinfectant at the boundary of the clean area.

3. Before entering the infected area, put on appropriate protective clothing.

4. Organize all equipment to minimize returns 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 the post mortem examinations.

6. In the infected area, collect all rubbish into appropriate containers.

7. Place all needles or disposable scalpel blades into a “sharps” container.

8. Carry a spray bottle of viricidal disinfectant so that hands and equipment can be progressively washed and sterilized throughout the course of operations.

9. Before leaving the infected area, wash all visible contamination (blood, faeces, etc.) from equipment, boots, hands and clothing.

10. Disinfect boots and gloves in the buckets of disinfectant at the boundary every time before returning to the clean area.

11. Clean and spray with disinfectant the outside of sampling containers (blood tubes, tissue jars).

12. Secure the containers in a plastic bag then place them in a transport container (ideally a plastic or metal container, but at least a plastic bag).

13. Spray the outside of this extra container with disinfectant.

14. Spray all clothing, and wash all equipment in the disinfectant before taking them to the vehicles.

15. If waterproof overalls are to be reused, ensure that these have been completely sprayed with disinfectant.

16. After everything has been disinfected move to the vehicles, store samples and equipment and remove protective clothing.

17. Wet any cloth overalls in disinfectant and store in leak-proof plastic bags.

18. If disinfected waterproof overalls are to be reused, store these in clean plastic bags.

19. Spray face masks and safety glasses again with viricidal disinfectant, and store for reuse.

20. 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 laboratory, burn or autoclave the bag.

APPENDIX 2

STANDARD OPERATING PROCEDURES (SOPs) FOR PERFORMING NECROPSIES AND COLLECTING SERUM SAMPLES ON NIPAH VIRUS INFECTED PREMISES

It is recommended that investigating teams take a laminated photocopy of these pages into the field for reference.

Specific SOPs for performing necropsies:

1. Appropriate clothing:
   - Long sleeve overalls
   - Rubber boots
   - Gloves, taping these to the sleeves of the overalls, preferably two sets of gloves
   - A plastic apron that can be disinfected or discarded
   - A face shield or similar eye and mucous membrane protection
   - A mask with a HEPA filter or a positive air pressure respirator (PAPR) is mandatory where Nipah virus infection is known to occur, and recommended where the disease is suspected

2. In the infected area, select a site where contamination of other animals can be minimized and which can be cleaned and sterilized after the job is done.

3. Plan carcass disposal in advance. To what extent will the necropsy increase the level of contamination at the premises? What protocol for disinfection should be followed? Will the carcass be burnt or buried?

4. Instruct personnel not involved in the post mortem examination (for example the farmers) to remain away from the work site.

5. Procedures that create aerosols should be done in such a way as to minimize the dispersal of aerosol particles.

6. Place all needles or disposable scalpel blades into a "sharps" container.

7. Place necropsied carcasses in a body bag ready for burial or burning. Spray the outside of the bag with disinfectant.

Specific SOPs for collecting blood samples:

1. Appropriate clothing:
   - Long sleeve overalls
   - Rubber boots
   - Gloves, taping these to the sleeves of the overalls, preferably two sets of gloves
   - A plastic apron that can be disinfected or discarded
   - A face shield or similar eye and mucous membrane protection
   - A mask with a HEPA filter or a positive air pressure respirator (PAPR) is mandatory where Nipah virus infection is known to occur, and recommended where the disease is suspected.

2. In the infected area, establish a sampling coordination station where tubes can be labelled, recorded and prepared for removal from the infected area.

3. Place all needles or broken blood tubes into a "sharps" container.

4. Pack and unpack samples as described above.

APPENDIX 3

CHECKLIST OF EQUIPMENT AND SUPPLIES FOR NIPAH VIRUS FIELD INVESTIGATIONS

1. Protective clothing:
   - overalls (long sleeved disposable or non-disposable)
   - plastic aprons (minimum 3)
   - gumboots
   - goggles or face shields (minimum 3)
   - disposable breathing masks, incorporating a HEPA filter (one for each person)
   - positive air pressure respirators (PAPRs) (minimum 3)
   - waterproof disposable gloves
   - rough textured rubber kitchen gloves (minimum 3)
   - wide packing tape

2. Necropsy kit
   - tranquilizers appropriate for each species to be examined
   - specialized restraint equipment for each species (for example, pig snares)
   - euthanasia solution
   - knives – skinning and boning
   - steel
   - scalpel handle and blades
   - "sharps" container
   - scissors – several pairs, various sizes and types
   - large rat-toothed forceps
   - butcher's saw, tomahawk, bone cutters
   - ruler
   - string
   - plastic sheeting
   - plastic waste disposal bags
   - 2 buckets – able to accommodate a gum boot

- plastic scrubbing brush
- towels
- detergent, soap
- disinfectant (for example, Virkon diluted at 20 g/litre)

3. Specimen collection
    - supply of 10 percent formalin
    - plain blood tubes for serum collection and blood tubes with anticoagulant
    - disposable needles and syringes, “sharps” container
    - venoject holder and needles if venoject tubes are to be used
    - sample containers for tissues – various sizes including one for fixing brain
    - labels and permanent markers for sample ID
    - sterile swabs, transport media and sample tubes for attempted virus isolation
    - selection of sealable plastic bags in various sizes
    - appropriate boxes for use as outer containers for transport of samples to the lab
    - biosecure consignment boxes to comply with IATA packing instructions 602 or 650; if samples are to be consigned, plus all necessary documentation for consignment of specimens such as relevant import permits and dangerous goods forms

APPENDIX 4

A GUIDE TO SAMPLING TISSUES FOR NIPAH VIRUS DIAGNOSIS: SAMPLES FROM NATURAL AND EXPERIMENTAL CASES IN WHICH NIPAH VIRUS HAS BEEN IDENTIFIED

Sample	Virus isolation	Immunohistochemistry	PCR
Nasal swabs	Pig Human		
Throat swabs	Pig Human Cat		
Urine	Human Cat		
Serum/blood	Pig Cat		
CSF	Human		Human
CNS (including meninges)	Cat	Pig Human Dog Cat Horse	Human Dog Cat
Lung	Pig Cat	Pig Human Dog	

Sample	Virus isolation	Immunohistochemistry	PCR
Spleen	Pig Cat	Human Dog	
Lymph node	Cat		
Kidney	Cat	Pig Human Dog	Dog
Other organs	Pig tonsil Human Unspecified tissues Cat tonsil	Human heart Human unspec. tissues Pig trachea Dog heart Dog adrenal	Human unspec. tissues Dog liver

Note: Adapted from Daniels *et al.*2001

APPENDIX 5

LABORATORIES WITH PC4 FACILITIES AND NIPAH VIRUS RESEARCH PROGRAMMES

1 CSIRO AUSTRALIAN ANIMAL HEALTH LABORATORY

Contact details:

Project Leader, Diagnosis and Epidemiology
CSIRO Australian Animal Health Laboratory
Private Bag 24 (5 Portarlington Road)
Geelong 3220
Australia
Phone: +61 3 5227 5000
Fax: +61 3 5227 5555
E-mail: Peter.Daniels@csiro.au
Website:
http://www.csiro.au/index.asp?type=faq&id=Australian%20Animal%20Health% 20Laboratory

2 CENTERS FOR DISEASE CONTROL AND PREVENTION

Contact details:

Special Pathogens Branch
Mail Stop G14, DVRD/NCID
Centers for Disease Control and Prevention
1600 Clifton Rd Atlanta, GA 30333
USA
Phone: +1-40 4 639 1115
Fax: +1-40 4 639 1118
E-mail: TKsiazek@cdc.gov or PRollin@cdc.gov
Website: http://www.cdc.gov/ncidod/dvrd/spb/index.htm

3 LABORATOIRE P4 JEAN MERIUEX

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APPENDIX 6

PACKING INFECTIOUS SUBSTANCES – INTERNATIONAL AIR TRANSPORT ASSOCIATION (IATA) PACKAGING INSTRUCTION 602

The following is taken from the IATA Dangerous Goods Regulations (42nd edition, CD-rom version 42.01).
Please refer to the IATA internet site (http://www.iata.org) for further information.

(STATE VARIATIONS: AUG-03, CAG-04, USG-13, VUG-02
OPERATOR VARIATIONS: AF-04, AS-02/08, CI-01, CO-07/08, CS-07, FX-09, SW-01, US-08)
The General Packing Requirements of Subsection 5.0.2 must also be met.

General Requirements
Shippers of infectious substances must comply with these Regulations and must ensure that shipments are prepared in such a manner that they arrive at their destination in good condition and that they present no hazard to persons or animals during shipment. The packaging must include:
(a) inner packagings comprising:
- a watertight primary receptacle(s);

- a watertight secondary packaging;
- other than for large body parts and whole organs which require special packaging, an absorbent material which must be placed between the primary receptacle(s) and the secondary packaging. Absorbent material is not required for solid substances.

Multiple primary receptacles placed in a single secondary packaging must be wrapped individually or for infectious substances transported in liquid nitrogen, separated and supported to ensure that contact between them is prevented.

The absorbing material, for example cotton wool, must be sufficient to absorb the entire contents of all primary receptacles.

(b) an outer packaging of sufficient strength meeting the design type tests found in Subsection 6.5 and bearing the Specification Markings as required by 6.0.6 for shipments of infectious substances other than those containing large body parts and whole organs which require special packaging. Also infectious substances shipped on liquid nitrogen in packagings that meet the requirements of Packing Instruction 202 are excluded from the testing requirements of Subsection 6.5 and the marking requirements of 6.0.6.

Note: Packagings of the type known as a “dry shipper” when used to ship infectious substances must meet the testing requirements of Subsection 6.5 and the marking requirements of 6.0.6.

Packages must be at least 100 mm (4 in) in the smallest overall external dimension.

For all packages containing infectious substances other than those containing large body parts or whole organs which require special packaging, an itemized list of contents must be enclosed between the secondary packaging and the outer packaging.

The primary receptacle or the secondary packaging used for infectious substances must be capable of withstanding, without leakage, an internal pressure which produces a pressure differential of not less than 95 kPa (0.95 bar, 13.8 lb/in^2) in the range of -40°C to +55°C (-40°F to 130°F).

All packages containing infectious substances must be marked durably and legibly on the outside of the package with the NAME and TELEPHONE NUMBER OF A PERSON RESPONSIBLE FOR THE SHIPMENT.

Shipments of Infectious Substances of Division 6.2 require the shipper to make advance arrangements with the consignee and the operator to ensure that the shipment can be transported and delivered without unnecessary delay. The following statement required by 8.1.6.11.3 must be included in the Additional Handling Information

area of the Shipper's Declaration: “Prior arrangements as required by the IATA Dangerous Goods Regulations 1.3.3.1 have been made.”

Specific Requirements

Although in exceptional cases, for example, the shipment of large body parts and whole organs, may require special packaging, the great majority of infectious substances can and must be packed according the following requirements:

- Substances shipped at ambient or higher temperatures: Primary receptacles may only be of glass, metal or plastic. Positive means of ensuring a leak-proof seal must be provided, such as heat seal, skirted stopper or metal crimp seal. If screw caps are used, these must be reinforced with adhesive tape.
- Substances shipped refrigerated or frozen (wet ice, prefrozen packs, Carbon dioxide, solid [dry ice]): Ice, Carbon dioxide, solid (dry ice) or other refrigerant must be placed outside the secondary packaging(s) or alternatively in an overpack with one or more complete packages marked in accordance with 6.0.6. Interior support must be provided to secure the secondary packaging(s) in the original position after the ice or Carbon dioxide, solid (dry ice) has been dissipated. If ice is used, the packaging must be leak-proof. If Carbon dioxide, solid (dry ice) is used, the outer packaging must permit the release of carbon-dioxide gas. The primary receptacle and the secondary packaging must maintain their containment integrity at the

temperature of the refrigerant used as well as at the temperatures and pressure(s) of air transport to which the receptacle could be subjected if refrigeration were to be lost.

- Substances shipped in liquid nitrogen: Plastic primary receptacles capable of withstanding very low temperatures must be used. Secondary packaging must also withstand very low temperatures and in most cases will need to be fitted over individual primary receptacles. Requirements for shipment of liquid nitrogen must also be observed. The primary receptacle must maintain its containment integrity at the temperature of the refrigerant used as well as at the temperatures and pressure(s) of air transport to which the receptacle could be subjected if refrigeration were to be lost. Where multiple primary receptacles are contained in a single secondary packaging, they must be separated and supported to ensure that contact between them is prevented.
- Lyophilized substances: Primary receptacles must be either flame-sealed glass ampoules or rubber-stoppered glass vials with metal seals.

APPENDIX 7

PACKING DIAGNOSTIC SPECIMENS WITH A LOW PROBABILITY OF BEING INFECTIOUS – INTERNATIONAL AIR TRANSPORT ASSOCIATION (IATA) PACKAGING INSTRUCTION 650

The following is taken from the IATA Dangerous Goods Regulations (42nd edition, CD-rom version 42.01).
Please refer to the IATA internet site (http://www.iata.org) for further information.

OPERATOR VARIATIONS: AS-02, CO-07/08, CS-07, FX-09, QF-05

The General Packing Requirements of Subsection 5.0.2 must also be met.

General Requirements

Shippers of diagnostic specimens where a relatively low probability exists that infectious substances are present must comply with Packing Instruction 650 of these Regulations. Diagnostic specimens being transported to undergo routine screening tests or for the purpose of initial diagnosis may be considered to fall under the category of those specimens where a low probability exists that infectious substances are present. The shipper must also ensure that

shipments are prepared in such a manner that they arrive at their destination in good condition and that they present no hazard to persons or animals during shipment. In the absence of other dangerous goods, no requirements other than those identified in Packing Instruction 650 are deemed to apply to such shipments. Packages prepared under this Packing Instruction must include:

(a) inner packagings comprising:

- a watertight primary receptacle(s) — for diagnostic specimens the maximum quantity must not exceed 500 ml;
- a watertight secondary packaging — the maximum quantity per outer packaging for diagnostic specimens must not exceed 4 litres;
- an absorbent material — must be placed between the primary receptacle and the secondary packaging. No absorbent material is required when shipping solid substances.

If multiple primary receptacles are placed in a single secondary packaging they must be wrapped individually or for those transported in liquid nitrogen, separated and supported to ensure that contact between them is prevented.

The absorbing material, for example cotton wool, must be sufficient to absorb the entire contents of all primary receptacles.

(b) an outer packaging of adequate strength for its capacity, weight and intended use.
However, each completed package must be capable of successfully passing the drop test described in 6.5.1 except that the height of the drop must not be less than 1.2 m.

The primary receptacle or the secondary packaging used for liquid diagnostic specimens must be capable of withstanding, without leakage, an internal pressure which produces a pressure differential of not less than 95 kPa (0.95 bar, 13.8 lb/in^2) in the range of -40°C to +55°C (-40°F to 130°F). It is not necessary for the primary or secondary packaging to be capable of withstanding 95 kPa pressure differential when solid diagnostic specimens are being shipped.

Packages consigned as freight must be at least 100 mm (4 in) in the smallest overall external dimension.

An itemized list of contents must be enclosed between the secondary packaging and the outer packaging.

Each package and the “Nature and Quantity of Goods” box of the air waybill must show the text - “DIAGNOSTIC SPECIMEN PACKED IN COMPLIANCE WITH IATA PACKING INSTRUCTION 650”.
A Shipper's Declaration for Dangerous Goods is not required.

Specific Requirements

Although exceptional cases, (for example, the shipment of whole organs) may require special packaging, the great majority of diagnostic specimens can and must be packaged according to the following guidelines:

- Substances shipped at ambient temperatures or higher: Primary receptacles include those of glass, metal or plastic. Positive means of ensuring a leak-proof seal, such as heat seal, skirted stopper or metal crimp seal must be provided. If screw caps are used these must be reinforced with adhesive tape.
- Substances shipped refrigerated or frozen (wet ice, pre-frozen packs, Carbon dioxide, solid [dry ice]) or other refrigerant must be placed outside the secondary packaging(s) or alternatively in an overpack with one or more completed packagings. Interior support must be provided to secure the secondary packaging(s) in the original position after the ice or Carbon dioxide, solid (dry ice) has been dissipated. If ice is used the packaging must be leak-proof. If Carbon dioxide, solid (dry ice) is used the outer packaging must permit the release of carbon-dioxide gas. The primary receptacle must maintain its containment integrity at the temperature of the refrigerant as well as at the temperatures and pressure of air transport to which the receptacle could be subjected if refrigeration were to be lost.

- Substances shipped in liquid nitrogen: Plastic capable of withstanding very low temperatures must be used instead of glass receptacles. Secondary packaging must also withstand very low temperatures and in most cases will need to be fitted over individual primary receptacles. Requirements for shipment of liquid nitrogen must also be observed. The primary receptacle must maintain its containment integrity at the temperature of the refrigerant used as well as at the temperatures and pressure of air transport to which the receptacle could be subjected if refrigeration were to be lost.
- Lyophilized substances: Primary receptacles must be either flame-sealed glass ampoules or rubber-stoppered glass vials with metal seals.