APPLICATION OF ENZYME-LINKED IMMUNOSORBENT ASSAYS FOR THE DIAGNOSIS OF EMERGING EQUINE PARAMYXOVIRUS INFECTION

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Enzyme-linked immunosorbent assays (ELISAs) for the diagnosis of infections with Nipah or Hendra viruses, developed by the Australian Animal Health Laboratory (AAHL), were evaluated for their diagnostic application in Japan. The original ELISA protocols for the two viruses differed, with the method for Nipah virus developed to detect antiviral antibody in swine sera and that for Hendra virus developed to detect the antibody in horse sera. However, preprocessing of horse sera was not necessary for the Nipah virus ELISA as there was no interfering nonspecific reactivity. Cross-reactivity between antibodies against the Nipah and Hendra viruses demonstrated a two times higher OD (450 nm) in a homologous reaction than in a heterologous reaction. While the diagnostic threshold of antibody levels in the Hendra virus ELISA was dependent on OD value, results were easily influenced by the concentration of virus antigen in the test sample. For the diagnosis of either virus infection in horses, more rigorous standards for diagnostic thresholds of antibody titers are recommended. This is a good application model for the diagnosis of these emerging diseases.

Key words: antibody detection, diagnosis, ELISA, Hendra virus, Nipah virus

INTRODUCTION

The first of a number of new paramyxoviruses to be recognized was the Hendra virus. This virus was identified as the cause of a 1994 outbreak of acute respiratory disease in two humans and 21 thoroughbred race horses in Hendra, a suburb of Brisbane, Australia (Westbury, 2000; Anon, 2005). The infection resulted in the death of one human and 14 horses, while a further 7 horses with subclinical infection were euthanized. The clinical features and findings from both human and equine autopsies were consistent with interstitial pneumonia (Westbury, 2000; Hooper *et al*., 2001). Viral material isolated from the equine lung and human kidney tissue taken at autopsy was incorrectly identified at the time as equine morbillivirus, on the basis of a weak one-way cross reaction with the rinderpest virus. A second, smaller outbreak occurred approximately 12 months

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later. A farmer from Mackay, around 1000 km north of Brisbane, died from severe encephalitis that was later shown to have been caused by infection with the Hendra virus (Westbury, 2000). Around 13 months before, the farmer had assisted in the necropsy of two horses that had died from acute respiratory distress and rapid onset of neurological symptoms. While he became ill soon after, he had subsequently recovered. Retrospective investigation established that the outbreak in Mackay, which predated the Brisbane outbreak by a month, was also due to Hendra virus infection (Westbury, 2000). A third incident, in which a horse died following infection with the Hendra virus, occurred in Cairns, Northern Queensland, in January 1999, while in December 2004, the Hendra virus was found to be responsible for the death of a horse in Townsville, Northern Queensland (Anon, 2005).

The second of the new paramyxoviruses to be identified was the Nipah virus. This virus was responsible for a major outbreak of disease in pigs and humans in Peninsula Malaysia, between September 1998 and April 1999, and resulted in 283 human cases with 109 fatalities. The outbreak began in late 1998 with sporadic cases of encephalitis among pig farmers in Ipoh, in the state of Perak. The outbreak was initially ascribed to the Japanese encephalitis virus (JEV), and a large scale JEV immunization program was undertaken. However, the disease subsequently spread within Malaysia, presumably through the movement of infected animals, suggesting a cause other than JEV. A new paramyxovirus, closely related to the Hendra virus, was then isolated from human brain tissue of a fatal case in March 1999. The virus was termed Nipah as the dead patient originated from the Kampung Sungai Nipah area (Chua *et al*., 1999; Chua, 2003). Worryingly, two horses which had been bred in the Iskandar Polo Club, located close to the original outbreak, were found to have antibodies against the Nipah virus. One of the horses was confirmed as infected with the virus using immunohistochemistry of brain and spinal cord tissue (Hooper *et al*., 2001).

These emerging paramyxoviruses were considered as major threats to the horse racing and horse riding industries throughout the region. If the viruses were capable of passing readily between horses, all racing events would have to be prohibited, while the zoonotic nature of the infections would impact on participation in horse-related industries. It is obvious, therefore, that rapid and robust diagnostic tests for these paramyxoviruses are required. Unfortunately, the viral antigens used in currently available ELISAs are required by international agreement to be prepared in a secure BSL-4 facility, currently unavailable in Japan. Viral antigens, as well as known positive and negative sera, are therefore imported from the Australian Animal Health Laboratory (AAHL), Victoria, Australia (Daniels *et al*., 2001; Eaton *et al*., 2004). The present study reports on the practical application of these ELISAs as diagnostic tools for emerging paramyxoviral horse diseases in the country.

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<div align="center">MATERIAL AND METHODS</div>

Serum samples

Fifty serum samples were collected from horses owned by the Equestrian Park of Japan Racing Association (JRA).

Antigen preparation

All antigens were prepared in the BSL-4 secure facility, AAHL. Nipah and Hendra viruses were inoculated into Vero cells cultured in roller bottles, and incubated for 48 h at 33°C. When the majority of cells developed syncytia, culture media was removed and monolayers were washed once with cold PBS. Infected cells were scraped off into ice cold PBS using a large rubber scraper. The cells were then centrifuged at 1000 rpm for 5 min, and NP40 was added for cell lysis. The resulting tissue was then further processed using a rotating Dounce homogenizer, with 10 strokes of a Teflon plunger (drill speed 4). Cell nuclei were then pelleted by centrifugation at 2,000 rpm for 10 min. The resulting supernatant was collected and TNE (10 mM Tris, 10 mM NaCl, 1.5 mM EDTA, pH 7.2) buffer was added to make up to a final volume of 10 mL. This was then divided into 1 mL aliquots and at once frozen at -80°C. The freezed aliquots were then inactivated by gamma-irradiation (6 mega rads) and stored at -80°C until use. Paramyxovirus negative antigens were prepared from noninfected Vero cells using the same technique.

Reference antisera

Antisera were also supplied by AAHL. Positive control serum for the Nipah virus was prepared using a stock of anti-Nipah virus pig serum from reference animal LAF pig 6, number 9909231102. Negative control serum was prepared from pooled negative pig serum derived from animal number 9808051111. Positive control serum with low levels of anti-viral antibody was prepared by mixing positive and negative control sera (final OD was adjusted to 0.3 ~ 0.4). Positive control serum for the Hendra virus was derived from a stock of anti-Hendra virus horse serum which was confirmed as positive using serum neutralization. Also, corresponding negative control serum was derived from stock horse serum confirmed as negative for antibody to the Hendra virus by serum neutralization. As for the Nipah ELISA, serum with low levels of antibody was prepared by mixing positive and negative control sera (final OD was adjusted to 0.3 ~ 0.4).

ELISA protocol

For anti-Nipah virus antibody detection, the ELISA method developed by AAHL was as follows. Nipah virus antigen was diluted 1:1,000 in PBS; 50 μL aliquots were placed in each well of a 96 well immuno plate (Nunc Maxisorp), and incubated at 37°C for 1 h. Five microliters of each serum sample were added to 20 μL of PBS containing 0.5% Tween 20 and 0.5% triton X-100, and then inactivated at 56°C for 30 min. Twenty five microliters of 100 times diluted, uninfected Vero cell antigen was added to the treated serum, and incubated at

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room temperature for 30 min. To make the final dilution of the treated serum to 1:100, 450 μL of blocking solution (PBS containing 0.05% Tween 20, 5% skim milk and 5% chicken serum) was added and incubated at room temperature for 30 min. Antigen adsorbed plates were washed 4 times with 250 μL per well of PBS containing 0.05% Tween 20 (PBST) and blocking was performed using 100 μL per well of blocking solution at 37°C for 30 min. The plates were then washed using the same method as above, and the 1:100 diluted sera were applied at 100 μL per well, then incubated at 37°C for 1 h. Each serum sample was applied to each of 2 wells on the plate. The plates were then washed using the same method as above, and protein A peroxidase conjugate (1:10,000 in 1% skim milk/PBST) applied at 100 μL per well and incubated at 37°C for 1 h. The plates were then washed as above, and 100 μL TMB substrate (3,5',5,5'-Tetramethyl benzidine) was added to each well, followed by incubation at room temperature for 10 min. Color change was stopped using 100 μL 1 M sulphuric acid applied to each well, and optical density (OD) was measured by ELISA reader at 450 nm.

The Hendra virus ELISA method developed by AAHL was as follows. Hendra virus antigen was diluted 1:300 in PBS and 50 μL was applied to each well of a 96 well immuno plate (Nunc Maxisorp), and incubated at 37°C for 1 h. The plates were then washed 4 times with 250 μL per well of PBST. Five microliters serum samples were diluted 1:100 times in PBS including 1% skimmed milk, and 50 μL samples were added to each well on the plates and incubated at 37°C for 30 min. Each serum sample was applied to each of the 2 wells. The plates were washed using the same method as above, then 50 μL protein A peroxidase conjugate (1:10,000 in 1% skimmed milk in PBST) was added to each well and incubated at 37°C for 30 min. The plates were then washed using the same method as above. TMB substrate was applied to each well (50 μL), then incubated at room temperature for 15 min. The color change was stopped using 50 μL 1 M sulphuric acid and the resulting change in OD read at 450 nm.

Modified ELISA methods to detect antibodies to both viruses were developed as follows. For the Nipah virus ELISA, sera were not processed by either inactivation or blocking prior to use, but were instead diluted 1:100 with 1% skimmed milk in PBST, then 100 μL applied to each well of the antigen adsorbed plate. The rest of the procedure followed the original protocol. For the Hendra virus ELISA, the method was modified as follows. Sera diluted 1:100 were applied at 100 μL per well and incubated at 37°C for 1 h. Protein A peroxidase conjugate (1:10,000 with 1% skimmed milk in PBST) was applied at 100 μL per well and plates incubated at 37°C for 60 min. TMB substrate was applied at 100 μL per well and plates incubated at room temperature for 15 min. Color change was then stopped using 100 μL 1 M sulphuric acid per well.

In both the original and modified Nipah virus ELISAs, plates were read at 450 nm against a TMB blank, and the results were calculated as the average OD of duplicate samples of both the Nipah and Vero antigens. These values were then used to calculate S/N ratios for each sample (S/N ratio = average OD of test sample against negative control sample). Those samples with an OD less than 0.2 against the Nipah antigen were considered to be nonreactors, as were those

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samples with an S/N ratio greater than 2.0 but an OD less than 0.2. Conversely, those samples with an S/N ratio greater than 2.0 and with an OD greater than 0.2 against the Nipah antigen were considered to be reactors. Each positive sample identified in the ELISA should then be confirmed in a serum neutralization test (SNT).

In the Hendra virus ELISA, plates were read at 450 nm against a TMB blank, and the average OD of duplicates for both the Hendra and Vero antigens calculated. The test was considered valid if the mean OD for the high positive serum was greater than 0.7 OD, if the low positive was greater than 0.25 OD, and if the negative control was less than 0.2 OD. Using these parameters, samples with an OD greater than 0.2 against the Hendra antigen were considered positive, while those with an OD less than 0.2 were considered to be negative. A sample with an OD greater than 0.2 against both the Hendra and Vero antigens was considered to be a nonspecific reactor.

The effect of blocking buffer on the detection of Nipah virus antibody in pig serum

The potential to reduce nonspecific reactivity was assessed using three commercially available blocking solutions (SuperBlocking Blocking Buffer, SEA BLOCK Blocking Buffer containing trace amounts of colloidal carbon, and Blocker TM BSA (10×), all from Pierce Biotechnology Inc. Rockford, U.S.A.), as compared to the standard blocking buffer described in the original ELISA method.

Cross-reactivity between the Nipah and Hendra virus ELISAs

To investigate the potential for cross-reactivity to either of the viruses using the diagnostic ELISAs, tests were performed as above, using positive control swine and horse sera. Homologous and heterologous antibodies were then included in the ELISAs, so that the Nipah ELISA technique was used on Hendra virus positive samples and *viceversa*.

RESULTS

Evaluation of the Hendra and Nipah virus ELISAs

All of the 50 horse samples assayed using either the original or modified ELISA techniques were negative for anti Hendra virus antibody. A representative subset of these results, from 20 horses is shown in Fig. 1. The ODs of the positive control samples using the original ELISA method proved inadequate. However, the results of the modified ELISA method did reach an acceptable OD for the positive controls. When the same 50 samples were used in the original and modified Nipah virus ELISAs, the OD values of the nonprocessed sera were higher than those of the processed samples (Fig. 2). However, the OD values of the nonprocessed sera were still lower than 0.035, which was clearly a negative value.
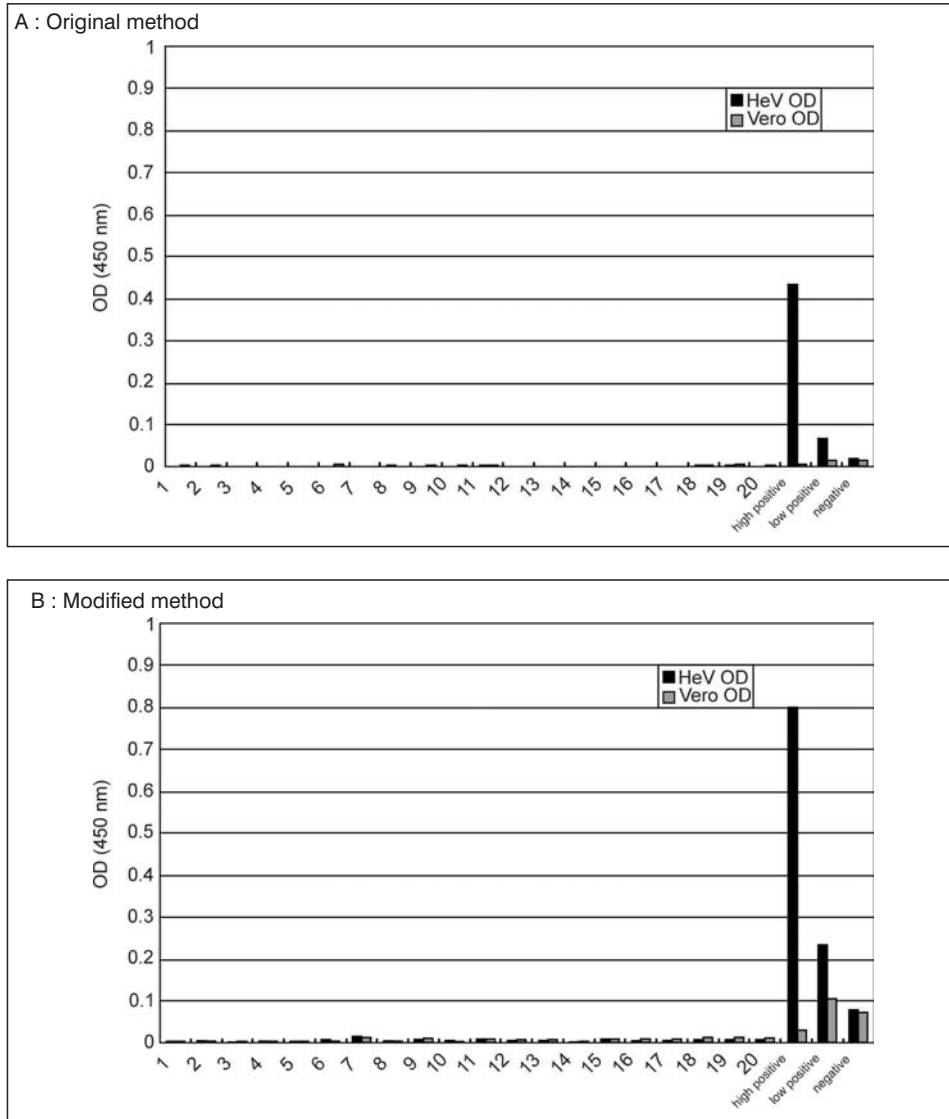
Figure 1. Comparison of two Hendra virus ELISA methods. Method A was developed by
AAHL, while Method B was modified by increasing the total volume of serum, protein
A conjugate, substrate, and stopping solution from 50 μl to 100 μl/well

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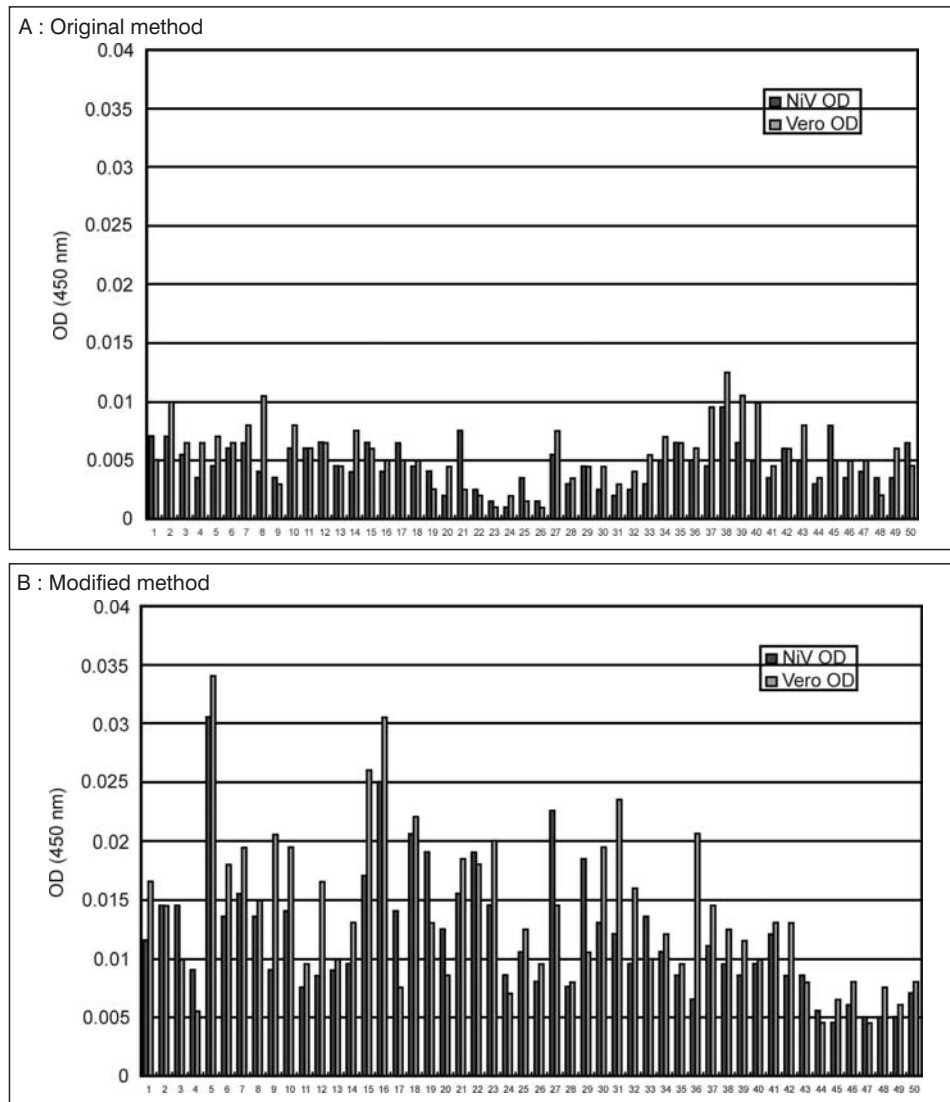for the diagnosis of emerging equine paramyxovirus infection

Figure 2. Comparison of the non-specific reactivity of horse serum in the original and modified Nipah virus ELISAs. Fifty horse serum samples collected in Japan were processed by both methods, with results expressed as units of OD

The effect of blocking solutions on Nipah virus antibody detection in pig serum

The effect of suppression of nonspecific reactivity using a standard blocking solution (5% skimmed milk and 5% chicken serum in PBST) and 3 kinds of

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commercially supplied blocking solution are shown in Fig. 3. The OD values of both the positive and negative control samples were higher when the ELISA protocol used commercially supplied blocking solutions. As a result of the observed higher ODs against the vero antigen, the required S/N ratios were not reached in the positive control samples. The original blocking solution therefore proved superior in suppressing nonspecific reactivity in the Nipah virus ELISA.
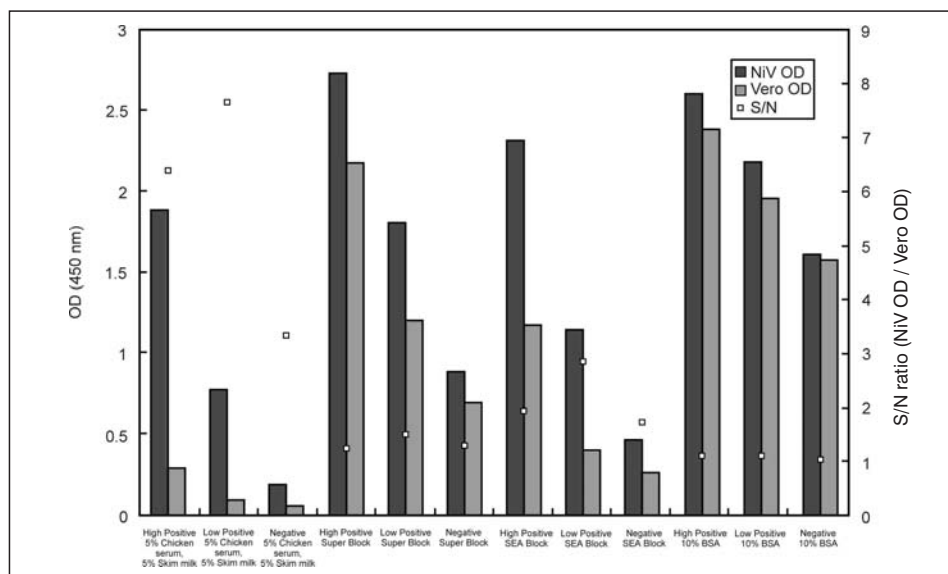

Figure 3. Comparison of the effects of different blocking solutions on the Nipah virus ELISA. Results are expressed as units of OD.
1. 5% chicken serum and 5% skimmed milk
2. Super block (containing colloidal carbon)
3. Sea block (containing non-mammalian substance)
4. Blocker TM BSA (x 10)

Cross-reactivity between ELISAs for the Nipah and Hendra viruses in swine and horse serum

The results of ELISAs for the Nipah and Hendra viruses using control swine sera are shown in Fig. 4, and demonstrate that the OD values obtained in the homologous ELISA reactions were approximately twice as high as in the heterologous ELISAs. The corresponding results using control horse sera are shown in Fig. 5, where the OD value obtained in the homologous ELISAs were also approximately twice as high as the heterologous reactions, regardless of which ELISA method was used.

The effect of varying the dilution of Hendra virus antigen on ELISA results

The results of the effects of varying the dilution of Hendra virus antigen on ELISA results were shown in Fig.6. The appropriate dilution of Hendra virus antigen showed from ×400 to ×800 in this study. Other dilution of the antigen brought the outside range of approval of ELISA test.

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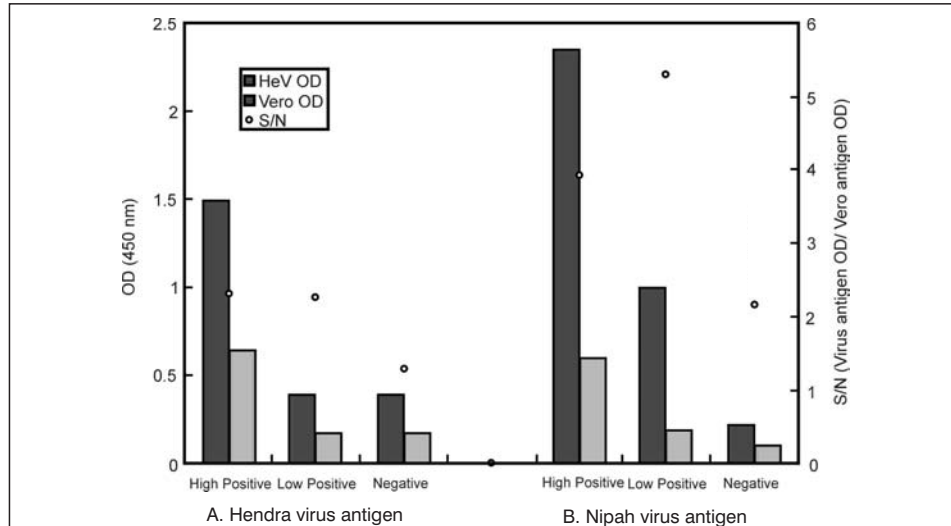for the diagnosis of emerging equine paramyxovirus infection

Figure 4. ELISA results, expressed as OD units, of high and low positive, and negative control samples for the Nipah and Hendra infections
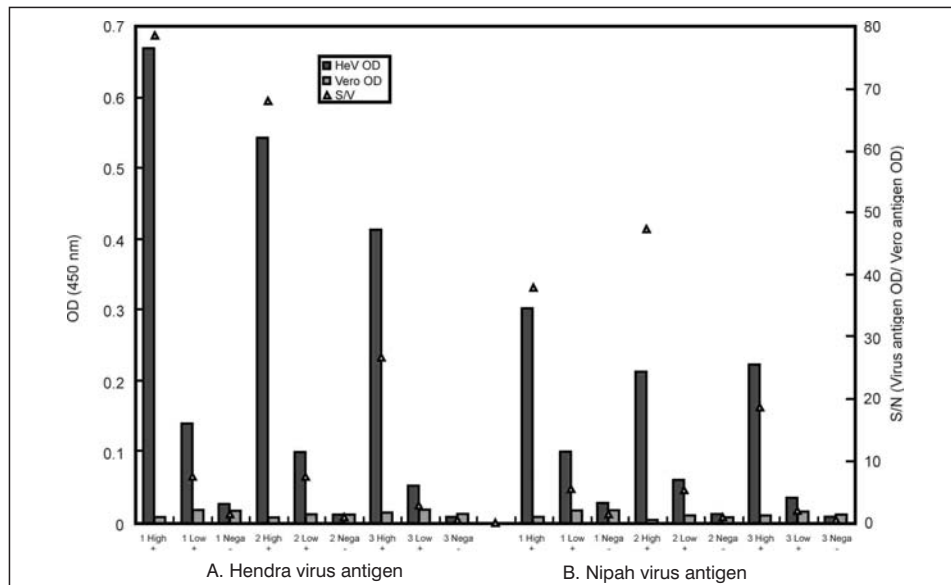

Figure 5. ELISA results from testing Hendra virus antibody positive horse sera using both the Hendra and Nipah virus ELISAs, and the effect of processing the horse serum prior to use. Results are expressed as units of OD.
1. Serum was not processed and blocking buffer was not used
2. Serum was not processed and blocking buffer was used
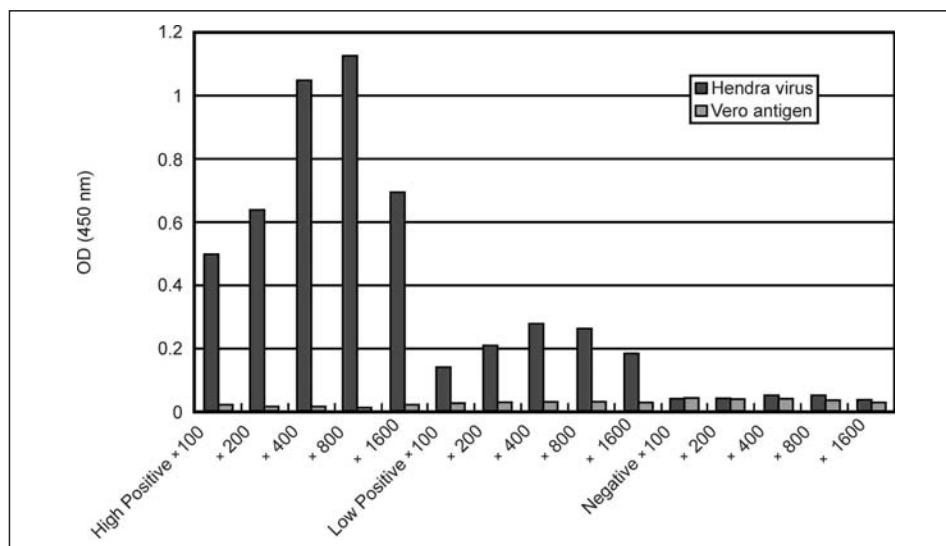3. Serum was processed and blocking buffer was used

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Figure 6. The effect of varying the dilution of Hendra virus antigen on ELISA results.
Results are expressed as units of OD

DISCUSSION

Identification of the two previously unknown paramyxoviruses as the causes of outbreaks of severe disease in horses, swine and humans in Australia and Malaysia, is a cause for concern in these and other countries. Of the two, the Nipah virus has been responsible for the majority of human deaths to date, with over one hundred people dying of viral encephalitis in Malaysia between 1998 and 1999. With a mortality rate of 40% (Chua, 2003), the Nipah virus presents a significant disease risk for humans. While the number of human cases has been much lower, outbreaks of Hendra virus infection in humans and horses in Australia in 1994, 1995 and 2004 are also a source of concern, not only as evidence of a novel zoonotic disease (Westbury, 2000), but also because of the potential economic impact on various horse related industries. Since the various outbreaks, the Nipah and Hendra viruses have been classified as paramyxoviruses (*henipavirus*) (Wang and Eaton, 2002) and both are now known to be capable of causing disease in horses (Hooper *et al*., 2001).

Understandably, those involved in industries such as horse racing or in recreational horse riding in the country are anxious that robust and practical diagnostic methods for these two diseases are available. For this reason, the authors have imported viral antigens, as well as anti sera known to be either positive or negative for these two diseases, to evaluate the suitability of ELISA techniques developed in AAHL to local studies of emerging diseases.

The Hendra virus ELISA developed in AAHL was unable to reach the required level of sensitivity in our laboratory (Fig. 1). This was potentially due to

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the original protocol including the use of a micro plate shaker (approximately 500 rpm) to mix the antigen, test serum, protein A peroxidase conjugate, and substrate; this shaker was not available for use in the present study. While this may have been responsible for the low ODs in the Hendra virus ELISA using the AAHL method, attempts were made to reduce any potential effects by adjusting the total volume of the test serum, protein A conjugate, substrate and sulphuric acid solution to 100 μL as used in the AAHL Nipah virus ELISA. Following this modification, the ODs of the positive control sera did reach the required standard in the Hendra virus ELISA.

The AAHL ELISA protocol for the Nipah virus was originally optimized for use with pig serum. However, it was observed in our laboratory that pig serum had a high level of nonspecific reactivity. In an attempt to reduce this effect, a number of potential blocking buffers were assessed. As the results shown in Fig. 3 demonstrate, high nonspecific reactivity was observed in the tests using three different commercially available blocking solutions. With each, the ODs for the Vero cell negative antigens were almost as high as those obtained for the viral antigens. While the commercially purchased blocking buffers proved ineffective, using a standard buffer containing 5% chicken serum and 5% skimmed milk in PBST did significantly lower the level of nonspecific binding. Although the reasons for this are unclear, it is noted that the best performing of the three commercially available buffers, Sea block, contained no mammalian reagent, and it may be that the use of chicken serum in the standard laboratory blocking buffer is responsible for the observed results.

The necessity to block nonspecific reactivity when testing horse serum was also assessed. While the ODs obtained with nonblocked serum were higher than those of processed serum, the highest OD of the nonblocked serum was still under 0.035 (Fig. 2) and it was considered that blocking nonspecific reactivity was not necessary when using horse serum.

The potential for cross-reactivity when testing sera for either Nipah or Hendra infection was considered a considerable risk factor in the study of these closely related viruses. The results of ELISAs using heterologous or homologous reagents presented in Figs. 4 and 5 show that the ODs obtained in the homologous reactions were approximately twice as high as in the heterogonous reactions for each virus. Despite this difference, use of both homologous and heterogonous reagents is required to ensure the most accurate diagnosis of species-specific viral infection. The threshold OD on which positive diagnoses could be made proved to be different between the Nipah and Hendra ELISAs. Indeed, the required standard for diagnosis was not attained for the Hendra virus because of an inherent variability in the results obtained for the positive and negative control sera (Fig. 5). To determine the reason for this, we studied the effect of changing the antigen dilution, and determined that this significantly affected the ELISA results. Rather than the original dilution of 1:300, the optimal range for the Hendra virus antigen was between 1:400 to 1:800. Other conditions which affected the Hendra ELISA results were the freshness, temperature and concentrations of the reagents. As a result of the variability of the Hendra virus ELISA, it was decided to adopt a similarly rigorous standard for decision making

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as used for the Nipah virus ELISA. A further series of experiments were deemed necessary to determine the optimum standard, using much higher amounts of virus positive horse serum. However, the limited availability of Hendra positive horse serum, and the lack of Nipah positive horse serum, meant these experiments were not performed. Despite this, it is recommended that our proposed standards be adopted in the diagnosis of these viruses, particularly as a similar standard is used by AAHL (Eaton *et al*., 2004).

In the present study, ELISA protocols for the detection of two emerging paramyxoviruses, Hendra and Nipah, were evaluated and found to be considerably sensitive to enable accurate diagnosis. These viruses have the potential to cause severe respiratory and neurological disorders in horses, pigs and humans (Hooper *et al*., 2001), and the development of robust, practical tests should be a priority. Until then, the diagnosis of Nipah virus infections in the country will rely on immunohistochemistry (Tanimura *et al*., 2004) while there is no standard recommended method of diagnosing Hendra virus infection. We consider that this study is a good model for the diagnosis of these emerging diseases in the country where BSL4 facility is not available.

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PRIMENA ELISA TEHNIKE U DIJAGNOSTICI INFEKCIJA KONJA IZAZVANIH PARAMIKSO VIRUSIMA

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SADRŽAJ

U ovom radu su prikazani rezultati primene ELISA tehnike razvijene od strane Australian Animal Health Laboratory (AAHL) u dijagnostici infekcija konja izazvanih Nipah i Hendra virusima. Originalni ELISA protokoli su bili različiti jer je test za Nipah virus bio predviđen za dokazivanje antitela u serumu svinja a test za Hendra virus za konjski serum. Pretretman seruma konja za dokazivanje antitela protiv Nipah virusa nije bio potreban jer ne postoji nespecifična reaktivnost. Unakrsna reaktivnost antitela na Nipah i Hendra viruse utvrđena je pri dvostruko većoj optičkoj gustini (OD 450 nm) u homologim reakcijama u odnosu na hetero-logne. Rezultati testa za Hendra virus bili su zavisni od količine virusnog antigena tako da primena ovih metoda zahteva uvođenje strogih standarda.