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Development of Improved Diagnostics and Therapeutics for Hendra Virus Infections

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Development of Improved Diagnostics and Therapeutics for Hendra Virus Infections

by Glenn Marsh

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Foreword

Hendra virus was discovered in 1994 and has become recognized as one of the most dangerous emerging infectious diseases known to human medicine. The virus occurs naturally in flying foxes (*Pteropus* spp), the reservoir host. Horses become infected by natural transmission of the virus from flying foxes via their excretory contamination of pasture or other aspects of the equine environment. Horses usually show disease and human infections occur where there is close contact with body fluids or excretions of sick horses. Hence horses are the intermediate or amplifier host in terms of zoonotic human exposure.

An equine vaccine for Hendra virus was released in late 2012 and therefore the need for a serological assay that could differentiate infected from vaccinated animals (DIVA) was considered to be necessary. Serological tests used prior to this project for the detection of antibodies to Hendra virus include virus neutralisation assays and ELISA assays using detergent disrupted whole virion preparations. In addition to these assays other experimental assays have been developed included two microsphere-based assays, one for antibody detection and the other a surrogate neutralisation assay. Both of these bead based assays utilise the soluble G glycoprotein antigen, the same antigen that the lead vaccine candidate contains.

This project investigated the use of other viral proteins as antigens for serological tests, allowing for the DIVA approach. N, P and M proteins were expressed in a combination of *E. coli*, yeast, baculovirus and mammalian cells, and tested in both ELISA and bead-based Luminex assays. None of these antigens proved to provide the level of specificity and sensitivity that the G glycoprotein based assays demonstrated, although several assays were developed which can be utilised if necessary. Following the release of the vaccine and assessment to these newly developed assays, policy is changing both within Australia and internationally to consider vaccinated horses to be protected from infection and therefore if an owner can prove a horse to be vaccinated then there will be no need for serological testing.

This report is an addition to RIRDC's diverse range of over 2000 research publications and it forms part of the National Hendra Virus Research Program, which aims to minimise the impact of Hendra virus.

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Craig Burns
Managing Director
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What the report is about

In 2011 a significant increase in the number of Hendra virus infections of horses was observed. In response to this unprecedented number of Hendra virus spill-over events, the New South Wales, Queensland and federal government committed funds to research leading to strategies that minimise the impact of Hendra virus.

This report describes a project that was funded to develop serological assays to differentiate infected from vaccinated animals for Hendra virus. With the recent release of an equine vaccine for Hendra virus in Australia it was considered that it would be necessary to test horses on occasion to demonstrate that they were vaccinated and not infected with Hendra virus.

Who is the report targeted at?

This report is targeted at the National Hendra virus taskforce, state and federal authorities responsible for dealing with equine cases of Hendra virus, staff in veterinary diagnostic laboratories and interested individuals.

Where are the relevant industries located in Australia?

The most relevant industry for this project is the horse industry, in particular horse owners within Queensland and New South Wales regions where Hendra virus cases occur. Horse owners who have their horses vaccinated are most relevant to this report.

Aims/objectives

The objective of the research proposal was to provide improved response capability in the event of future Hendra virus outbreaks, to support the equine industry by facilitating national and international horse movements in the post-Hendra virus vaccine environment, and to minimize the impact of Hendra Virus on potentially exposed individuals.

Different expression systems, including bacterial, baculovirus, yeast and mammalian, were assessed for the production of viral proteins (N, P and M) for use in serological tests. Proteins that express well and can be detected with well characterised sera were then assessed in both ELISA and Luminex formats for suitability for use in a diagnostic setting.

Using a panel of known sera from infected, vaccinated, vaccinated and exposed and sero-negative animals were then assessed against each of the antigens in the different formats. The panel of sera used here has been collected over many years at the Australian Animal Health Laboratory (AAHL) from both field cases and experimental animal infections. These results were compared to virus neutralisation results, the “gold standard” for detection of antibodies to Hendra Virus, which can only be carried out at AAHL.

Methods used

The methodology for this project is based on standard protein expression techniques coupled with either ELISA testing or bead-based immunoassays on the Luminex platform. Hendra Virus proteins assessed for suitability in these assays included the N (nucleocapsid), P (phosphoprotein) and M (matrixprotein). These proteins have been shown in the past to be the most immunogenic of the Hendra Virus protein in natural or experimental infections in horses.

Following expression of the viral proteins, assays were developed with each protein in both the ELISA format and bead-based Luminex platform. These new assays were assessed using a panel of sera from

experimentally infected horses, naïve horses and field cases of Hendra virus. Assay results were compared to the 'gold standard' virus neutralisation test.

Results/key findings

The three chosen antigens (N, M and P) were expressed in a combination of *E. coli*, yeast, baculovirus and mammalian cells. These antigen were chosen based on previous studies that demonstrated them to be immunogenic in Hendra virus infections, compared to F and L which do not always elicit a strong antibody response. N was the easiest antigen to generate reasonable quantities of, with M and P being much harder to express and purify in reasonable quantities.

Initially these antigens were assessed in the ELISA format against a panel of sera from experimentally infected animals (horses and ferrets). Each of these antigens was demonstrated to provide evidence of prior Hendra virus infection, although each antigen failed to give a similar response to that observed for the G glycoprotein. Antigens were that transferred to the Luminex bead system, with all antigens performing similar to that observed in the ELISA format.

These assays were then assessed using a panel of field horses that had previously been demonstrated to be negative to antibodies to Hendra virus using the G glycoprotein and the virus neutralisation test. Of these samples (over 100 tested), approximately 15% of samples gave a positive response to at least one antigen with some giving positive reaction with multiple antigens. The N protein was the most likely to be positive for any sample. This was considered to be due to cross-reacting antibodies to closely related viruses. An example of this is with Cedar virus, a non-pathogenic henipavirus also found in Australian fruit bats. Antibodies generated to the N protein of this virus and Hendra virus have been demonstrated to cross-react and therefore this result is not unexpected.

As a work around to this problem, IgM assays were developed for the N antigen, allowing for determination of a current infection with a henipavirus. Although not ideal, it is suitable in an outbreak setting to test in-contact vaccinated horses. A competitive ELISA was also established for the N protein, with this assay being more specific to Hendra virus antibodies as opposed to closely related viruses. Western blot assays have also been assessed for M and P antigens and were demonstrated to be useful in a DIVA format, but are not high throughput and require significant interpretation.

Implications for relevant stakeholders for:

The implication of this project is that DIVA testing of horses for Hendra virus infection post vaccination is not an easy task. Assays have been developed to achieve this but they require a significant level of interpretation and are not suitable for mass screening of horse samples. Also based on the types of assays developed they were deemed inappropriate to be rolled out to the state laboratories and it was decided that the AAHL would keep these as internal tests with the state laboratories forwarding samples as required.

Additionally, separate to this project, significant discussions were held in regards to the need for DIVA testing for Hendra virus. Based on the experimental challenge results in vaccinated horses, it is considered that vaccinated animals are fully protected from infection with Hendra virus. No horse has ever been demonstrated to be infected following vaccination, either experimentally or in the field. Therefore current feeling both within Australia and internationally is that if an owner can demonstrate a horse has been vaccinated, then there will be no need to prove that it has not been infected with Hendra virus.

Recommendations

Current recommendation is that the tests developed in this project are maintained at AAHL to be used if deemed necessary. However, this will not be a common request as if the horse has previously been vaccinated then it will just be considered to be protected, making testing unnecessary.

Introduction

Hendra virus was discovered in 1994 (8-10) and has become recognized as one of the most dangerous emerging infectious diseases known to human medicine. The virus occurs naturally in flying foxes (*Pteropus* spp), the reservoir host (4, 11). Horses become infected by natural transmission of the virus from flying foxes via their excretory contamination of pasture or other aspects of the equine environment. Horses usually show disease and human infections occur where there is close contact with body fluids or excretions of sick horses (8-10). Hence horses are the intermediate or amplifier host in terms of zoonotic human exposure. Seven people have been diagnosed with Hendra virus disease, which has been fatal in four of these cases. Hendra virus disease in horses has been increasing in incidence, with cases each year since 2006 and a significant increase in cases since 2011 (2).

Control has focused on risk communication among people in the horse industry together with use of personal protective equipment and practices (3). Use of such personal protective equipment and practices is obligatory from an occupational health and safety perspective but meets passive resistance due to its perceived inconvenience. Strategies that do not rely entirely on appropriate human behaviour are being sought. A vaccine that protects against disease in horses and substantially reduces viral shedding and hence the risk of transmission to people is now available (7). Although introduction of the vaccine will reduce the risk of transmission from horses-to-humans improved, it is unlikely that 100% vaccine coverage will occur. Therefore, diagnostics are still an important component of Hendra virus disease control.

Current serological assays for the detection of antibodies to Hendra virus include virus neutralisation assays and ELISA assays using detergent disrupted whole virion preparations. In addition to these assays other experimental assays have been developed included two microsphere-based assays, one for antibody detection and the other a surrogate neutralisation assay (1). Both of these bead based assays utilise the soluble G glycoprotein antigen, the same antigen that the lead vaccine candidate contains. An improved, more specific ELISA assay is also undergoing testing and validation which utilises this same antigen. These assays, although extremely sensitive and specific, will detect antibodies only to the vaccine antigen and not to any other virus antigen and therefore will be essential for monitoring of antibody levels following vaccination but are not suitable for DIVA purposes.

Additional assays have been developed by several groups, including our group, which use pseudotyped-viruses for detection of neutralising antibodies. Viruses that have successfully been pseudotyped with henipavirus F and G glycoproteins include vesicular stomatitis virus (5, 6). These assays have been demonstrated to be highly sensitive, even more sensitive than traditional virus neutralisation assays using “live” henipaviruses, and are safe to perform at lower levels of biocontainment as the viruses lack essential genes to complete full virus replication cycles. However, these assays will also detect antibodies produced to the vaccine and therefore are unsuitable for DIVA purposes.

Several viral proteins, in particular the nucleocapsid, the phosphoprotein and the matrix proteins have been shown to be highly immunogenic in Hendra virus infected animals. Detection of antibodies to these antigens will make a good DIVA strategy and will also provide some flexibility in detecting a novel henipavirus if it was to appear. The nucleocapsid protein of paramyxoviruses is one of the most conserved among related viruses and, therefore the use of this antigen in serological testing has the potential to detect novel antibody responses to Hendra-like viruses.

Objectives

The objective of the research was to provide improved response capability in the event of future Hendra virus outbreaks, to support the equine industry by facilitating national and international horse movements in the post-Hendra virus vaccine environment, and to minimize the impact of Hendra virus on potentially exposed individuals.

Since antibody produced in response to the sG antigen embodied within the candidate Hendra virus vaccine would be detectable by all currently validated serological tests, a DIVA test reactive to antibodies generated within the infected horse against other Hendra virus proteins will be developed. Horses naturally infected with live Hendra virus will develop antibodies to a wide array of viral proteins in addition to G protein; detection of such antibodies is consistent only with prior exposure to live virus.

Different expression systems, including bacterial, baculovirus and mammalian, were assessed for the production of viral proteins (N, P and M) for use in serological tests. Proteins that express well and can be detected with well characterised sera were then assessed in both ELISA and Luminex formats for suitability for use in a diagnostic setting.

Using a panel of known sera from infected, vaccinated, vaccinated and exposed and sero-negative animals were then assessed against each of the antigens in the different formats. The panel of sera used here has been collected over many years at AAHL from both field cases and experimental animal infections. These results were compared to virus neutralisation results, the “gold standard” for detection of antibodies to Hendra virus, which can only be carried out at AAHL.

Methodology

The methodology for this project is based on standard protein expression techniques coupled with either ELISA testing or bead-based immunoassays on the Luminex platform. Hendra and Nipah virus proteins that were assessed for suitability in these assays include the N (nucleocapsid), P (phosphoprotein) and M (matrixprotein). These proteins have previously been shown to be the most immunogenic of the Hendra virus proteins in natural or experimental infections in horses.

Different expression systems were tested for the expression of the Hendra virus proteins, including bacterial (*E.coli*), insect cell/baculovirus and mammalian cell. In each case, proteins were his-tagged and purified by affinity purification on an immobilized metal ion affinity chromatography (IMAC) column. Different expression systems have their advantages and disadvantages, which is why different systems were tested. A major concern, in particular with the mammalian cell expression, was the generation of antibodies from the vaccine preparation. The Hendra virus soluble G antigen, which is the basis for the licensed vaccine candidate, is expressed in mammalian cells. The current formulation does not see this protein being purified to any degree and therefore vaccinated horses will produce antibodies to cell protein (from the CHO cell that the protein is expressed from). These antibodies could then react with cellular proteins that could be carried over in the antigen preparations.

Expression of high quality antigens in sufficient quantities and reproducibly is critical to the success of these tests, which is another reason for assessing different antigen expression systems. Bacterial expression is by far the easiest system and was the first assessed however post-translational modification of proteins from bacteria is different than mammalian cells and therefore the proteins may have epitopes that are masked or modified compared to what an infected horse is exposed to.

Following the successful expression and purification of the different proteins, they were then tested for suitability in the 2 different assay formats. Luminex has the advantage over ELISA in that multiple assays against different antigens can be performed in the same well on a 96 well plate allowing for tests for vaccine induced antibodies at the same time as antibodies from infection. This allows for the assay to be more high-throughput and to provide greater information. Luminex based assays are also generally more sensitive than standard ELISA based methods. Antigens were tested against a panel of sera that has been collected at AAHL from the following groups of animals; a) naïve animals, b) naturally infected animals, c) experimentally infected animals, d) vaccinated animals and e) vaccinated and challenged animals. This will allow for a full range of different sero-states that may be present in any population. Sera was derived from other species of animals and not just horses and it is likely that these tests will be needed to test different animals of infected properties in the future.

Results

The three chosen antigens (N, M and P) were expressed in a combination of *E. coli*, yeast, baculovirus and mammalian cells. These antigen were chosen based on previous studies that demonstrated them to be immunogenic in Hendra virus infections, compared to F and L which do not always elicit a strong antibody response. N was the easiest antigen to generate reasonable quantities of, with M and P being much harder to express and purify in reasonable quantities Figure 1-3 demonstrate expressed proteins.

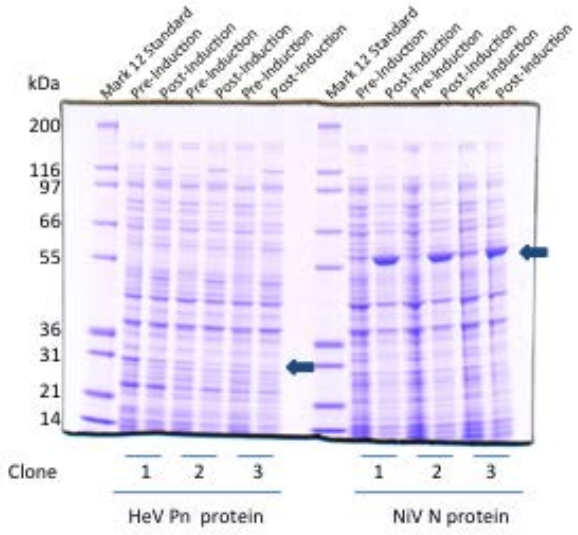


Figure 1. Coomassie stained polyacrylamide gel showing small-scale expression of recombinant henipa virus proteins expressed in *E. coli*.

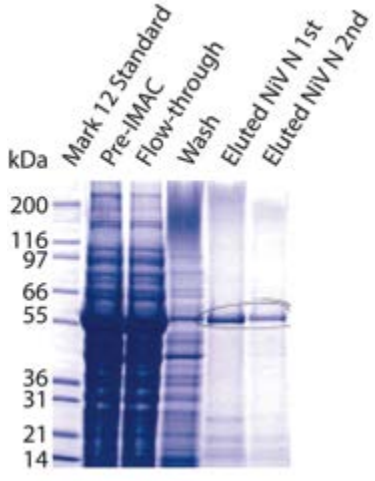


Figure 2. Coomassie Blue-stained gel of IMAC-purified recombinant Nipah virus N protein expressed in *E. coli*.

Both the first and second elutions produced partially pure NiV N protein.

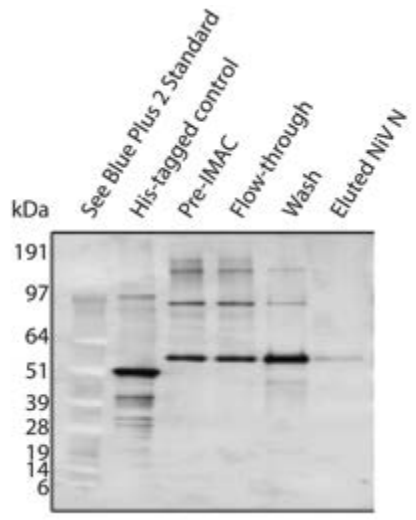


Figure 3. Immunoblot of IMAC-purified recombinant Nipah virus N protein expressed in *E. coli*.

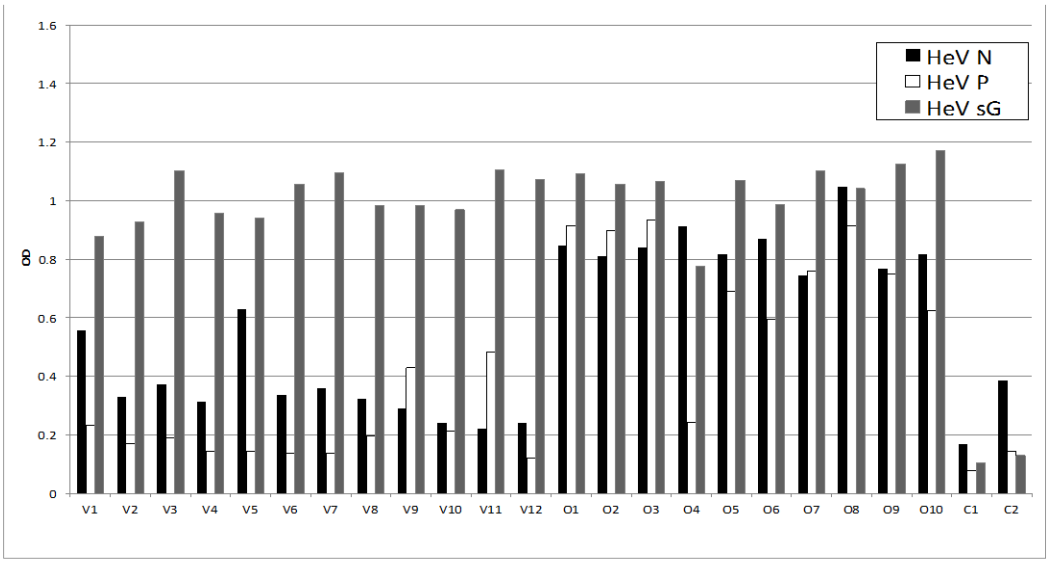
Penta-His Mab (Qiagen) was used diluted 1/1,500; sheep anti-mouse HRP conjugate (Silenus) was used diluted 1/3,000. Substrate was ECL Plus (GE) and image was acquired using a Typhoon FLA9000 (GE). Confirms presence of His-tagged protein NiV N.

Initially these antigens were assessed in the ELISA format against a panel of sera from experimentally infected animals (horses and ferrets). Each of these antigens was demonstrated to provide evidence of prior Hendra virus infection, although the magnitude of the response was reduced on many occasions compared to that observed for the G glycoprotein. Antigens were that transferred to the Luminex bead system, with all antigens performing similar to that observed in the ELISA format.

These assays were then assessed using a panel of field horses that had previously been demonstrated to be negative to antibodies to Hendra virus using the G glycoprotein and the virus neutralisation test. Of these samples (over 100 tested), approximately 15% of samples gave a positive response to at least one antigen with some giving positive reaction with multiple antigens (Figure 1). Setting cut offs for these assays for positive and negatives proved difficult because of this, as some negative samples had higher reading compared to known positive samples. The N protein was the most likely to be positive for any sample. This was considered to be due to cross-reacting antibodies to closely related viruses. An example of this is with Cedar virus, a non-pathogenic henipavirus also found in Australian fruit bats. Antibodies generated to the N protein of this virus and Hendra virus have been demonstrated to cross-react and therefore this result is not unexpected. In fact, when sera from Cedar virus infected ferrets was tested in these assays, the samples routinely were scored as positive. These same samples are negative on G glycoprotein based assays and virus neutralisation assays.

As a work around to this problem, IgM assays were developed for the N antigen, allowing for determination of a current infection with a henipavirus. Although not ideal, it is suitable in an outbreak setting to test in-contact vaccinated horses. A competitive ELISA was also established for the N protein, with this assay being more specific to Hendra virus antibodies as opposed to closely related viruses, however still resulted in some negative samples scoring as positive. Western blot assays have also been assessed for M and P antigens and were demonstrated to be useful in a DIVA format, but are not high throughput and require significant interpretation.

a



b

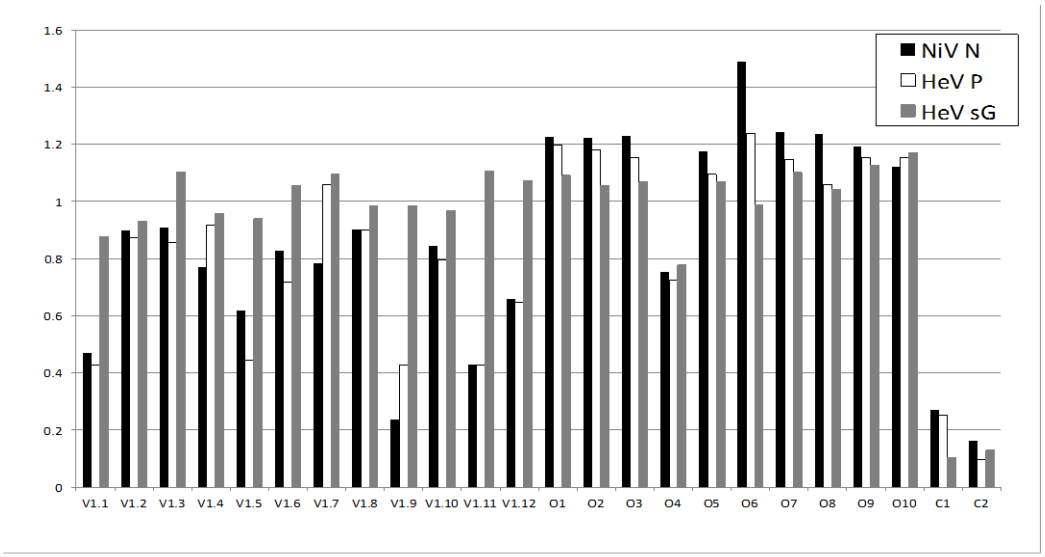


Figure 4. Graph of ELISA results for HeV N, HeV P, HeV sG and NiV N antigens.

ELISA assays were carried out testing antibody responses to the various antigens using sera obtained from vaccinated (v), outbreak (o) and control horses. Cut off values for the ELISA were initially set at OD_{450} of 0.4, however several horse tested had OD reading greater than this with no other evidence of prior Hendra virus infections.

Implications

The implication of this project is that DIVA testing of horses for Hendra virus infection post vaccination is not an easy task. It is very difficult in some cases to truly distinguish between vaccinated and infected horses. A horse which tests positive only to G antibody certainly has a response “consistent with vaccination”, however antibody responses to the other viral proteins are not always present in experimentally challenged animals and ‘field horse occasionally test positive to N and P, with no evidence of prior Hendra virus infection. These antibody responses could indicate horses are exposed to other closely related paramyxoviruses that do not cause an as yet identified disease or a virus that does not cause disease.

Data from the ferret model, in which Hendra virus infection has been confirmed, even though at comparatively low levels leading to survival do not always develop detectable antibodies to HeV N or P, and antibody to HeV F protein is only found in some animals. Due to the limited number of viral proteins, there is very limited scope to test whether other proteins will be suitable. F and L where not tested in this project, F has been tested previously and is not very immunogenic, L has not been tested but for paramyxovirus is rarely immunogenic and is expressed in very low quantities in infected animals.

Assays have been developed that achieve a DIVA diagnosis but they require a significant level of interpretation and are not suitable for mass screening of horse samples. Also based on the types of assays developed they were deemed inappropriate to be rolled out to the state laboratories and it was decided that the AAHL would keep these as internal tests with the state laboratories forwarding samples as required.

Additionally, separate to this project, many countries have now ruled that horses from Australia do not need screening for antibodies to Hendra virus prior to import if vaccination can be proven. All major racing countries globally have agreed to this approach. This approach has been decided on based on the experimental challenge results in vaccinated horses as it is considered that vaccinated animals are fully protected from infection with Hendra virus. No horse has ever been demonstrated to be infected following vaccination, either experimentally (7) or in the field. Therefore current feeling both within Australia and internationally is that if an owner can demonstrate a horse has been vaccinated, then there will be no need to prove that it has not been infected with Hendra virus.

Recommendations

Current recommendation is that the tests developed in this project are maintained at the Australian Animal Health Laboratory to be used if deemed necessary. However, this will not be a common request as if the horse has evidence of up-to date vaccination status then it may be considered to be protected, making testing unnecessary.

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