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ABSTRACT

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Hendra and Nipah viruses (HeV and NiV) are closely related zoonotic pathogens of the *Paramyxoviridae* family. Both viruses belong to the *Henipavirus* genus and cause fatal disease in animals and humans, though only HeV is endemic in Australia. In general and due to the acute nature of the disease, agent detection by PCR and virus isolation are the primary tools for diagnostic investigations. Assays for the detection of antibodies against HeV are fit more readily for the purpose of surveillance testing in disease epidemiology and to meet certification requirements in the international movement of horses. The first generation indirect ELISA has been affected by non-specific reactions which must be resolved using virus neutralisation serology conducted at laboratory bio-safety level 4 containment (PC4). Recent developments have enabled improvements in the available serology assays. The production of an expressed recombinant truncated HeV G protein has been utilised in ELISA and in Luminex-based multiplexed microsphere assays. In the latter format, two Luminex assays have been developed for use in henipavirus serology: a binding assay (designed for antibody detection and differentiation) and a blocking assay (designed as a surrogate for virus neutralisation). Equine and canine field sera were used to evaluate the two Luminex assays relative to ELISA and virus neutralisation serology. Results showed that Luminex assays can be effective as rapid, sensitive and specific tests for the detection of HeV antibody in horse and dog sera. The tests do not require PC4 containment and are appropriate for high throughput applications as might be required for disease investigations and other epidemiological surveillance. Also, the results show that the Luminex assays detect effectively HeV vaccine-induced antibodies.

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1. Introduction

Hendra virus (HeV) and Nipah virus (NiV) belong to the *Henipavirus* genus within the *Paramyxoviridae* family (Eaton et al., 2007). HeV was detected first following an outbreak of a severe and fatal respiratory disease in a large racing stable in the suburb of Hendra, Brisbane in 1994. Since the initial HeV outbreak, sporadic spill-over events have occurred annually in Australia across Queensland and northern New South Wales. The natural reservoir of these zoonotic agents is within the genus *Pteropus* (Haplin et al., 2011), commonly known as fruit bats or flying foxes. This disease is usually fatal in horses with over 80 horses having died or been euthanised due to infection with HeV; furthermore four of the seven humans known

to be infected with HeV have died (Marsh and Wang, 2012). In 2011, a healthy dog on a HeV affected Qld property was also found to have high levels of neutralising antibody against HeV (Croser and Marsh, 2013). More recently in November 2012, a commercial equine vaccine against HeV (Equivac HeV, Zoetis Australia P/L) was released for use in Australia (Mendez et al., 2013; Broder et al., 2013). However, a henipavirus vaccine for humans will take many more years to develop (Middleton, 2012).

Initially NiV emerged in pigs in Malaysia in 1998 (Chua et al., 2000); by April 1999, 106 human deaths had occurred in Malaysia and Singapore (Marsh and Wang, 2012). No further outbreaks of NiV have been reported in Malaysia, however, in separate outbreaks the virus continues to spill over and cause disease in other countries such as Bangladesh and India.

The henipavirus genome is a non-segmented, negative-strand RNA. The genes encode six major structural proteins; the nucleocapsid (N), phosphoprotein (P), matrix protein (M), fusion protein (F), attachment glycoprotein (G) and the large polymerase (L) (Wang et al., 2001). The two major membrane-anchored

Abbreviations: HeV, Hendra virus; NiV, Nipah virus; %P, percent positive; %I, percent inhibition; MFI, median fluorescence intensity.

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glycoproteins are required for infection of a permissive host cell. The F glycoprotein mediates pH-independent membrane fusion between the virus and its host cell (Bossart et al., 2005). The G glycoprotein is the attachment protein which binds the host cell via the Ephrin B2 or Ephrin B3 receptors (Bossart et al., 2008). The G protein of NiV and HeV share 83% nucleotide homology (Wang et al., 2001) and cross-reactive antibodies against the G protein have been observed between the two viruses (Bossart et al., 2005).

Laboratory diagnosis of equine infection following a HeV spillover event is critical to management of potentially exposed persons and animals located on infected premises. Currently, all diagnostic submissions for HeV exclusion received at CSIRO's Australian Animal Health Laboratory (AAHL) are tested by PCR and virus isolation. Due to the fulminant and lethal course of the disease, serology is less frequently definitive in the diagnosis of acute infection. However the technique is appropriate for "proof of freedom" of animals on affected properties, surveillance and regulation testing of horses prior to international transport. At the Australian Animal Health Laboratory (AAHL), HeV serology presently is conducted by indirect ELISA using either inactivated virus (Daniels et al., 2001; OIE, 2009) or the more recently introduced recombinant-expressed protein (Wang and Daniels, 2012; Colling et al., 2013). The latter employs a form of the G protein (sG), truncated for enhanced solubility (Bossart et al., 2005). Currently, all serum reactors (positive and indeterminate) in the iELISAs are resolved by a virus neutralisation assay which must be performed under strict bio-containment procedures in a PC4 laboratory. The interpretation and validation of the HeV iELISAs are complicated by the lack of a large number of test results for positive sera and by the frequency of non-specific reactions, particularly in the whole virus ELISA. These also must be resolved for specificity by virus neutralisation serology. The development of a rapid and safe microsphere immuno-assay (Luminex assay) which can be performed in a PC2/PC3 laboratory, will aid in diagnostic surveillance of this disease.

Two Luminex-based fluorescent microsphere assays have been developed using an approach described previously by Bossart et al. (2007) for detection of antibody against henipaviruses. The target antigen for both assays is recombinant-expressed sG, but the assays are designed separately in total antibody-binding and restricted receptor-blocking formats. The Luminex binding assay was used for antibody detection and differentiation of HeV and NiV whereas the Luminex blocking assay was designed as a surrogate for virus neutralisation. The detection of HeV-specific antibodies in sera from convalescent horses following HeV infection in Australia using the henipavirus Luminex binding and blocking assays was first described by Bossart et al. (2007). In addition, these Luminex assays have been used for further serological studies to detect henipavirus antibodies in bats and other species internationally including; West African fruit bats and domestic pigs (Hayman et al., 2008; Hayman et al., 2011; Peel et al., 2012, 2013), Pteropid bats in Papua New Guinea (Breed et al., 2010) and *Pteropus vampyrus* bats in Indonesia (Sendow et al., 2013). Recently, the Luminex microsphere assay was used to assess HeV infection in the mouse model (Dups et al., 2012) and to confirm HeV infection in human cases by Queensland Health (Playford et al., 2010).

In 2011, a year with an unusually high occurrence of HeV infections (18 outbreaks) in Australia (Mahalingam et al., 2012), three dogs from a HeV infected property undergoing quarantine in Mount Alford, Queensland were assessed by HeV ELISAs and HeV virus neutralisation serology at AAHL (Croser and Marsh, 2013). This was the first report of a dog infected naturally with HeV in Australia.

In this study, the Luminex assays were characterised further for use in detection of HeV specific antibodies in sera from infected and non-infected animals including horses and dogs; results have been

evaluated against assessments using ELISA and virus neutralisation serology assays.

2. Materials and methods

2.1. Animal sera

All field horse and dog sera tested were derived as diagnostic samples submitted to the AAHL; sera were neat or diluted 1:5 in PBS containing 0.5% Tween 20 and 0.5% Triton-X100, and heat treated at 56 °C for 30 min prior to use. A range of horse, pig, goat, rabbit and guinea pig anti-sera for use in analytical specificity (HeV-uninfected) assessments were variously derived from naturally or infected experimentally animals. Sera from HeV vaccinated horses (AAHL job 12-03417) were received for HeV serology assessment, though without details of vaccination time or doses.

2.2. Henipavirus Luminex binding and blocking assays

The multiplex microsphere assays have been described previously by Bossart et al. (2007). Briefly, for both assays, HeV or NiV soluble G (sG) proteins (Bossart et al., 2005) were coupled to individual microsphere sets. In both assays a predetermined number of polystyrene or magnetic beads (Fisher Biotec Pty Ltd, Australia) were added to each well and then mixed with test sera at a dilution of 1:100 (binding assay) or 1:50 (blocking assay). In the binding assay, bound antibody was detected using biotinylated Protein A (Pierce, Rockford, USA) together with biotinylated Protein G (Pierce, Rockford, USA) followed by streptavidin–phycoerythrin (Qiagen Pty Ltd, Australia). Results were recorded as median fluorescent intensity (M.F.I.), or transformed as a percentage relative to the MFI for the positive control (%P): $[(\text{MFI test serum})/(\text{MFI positive control serum})] \times 100$.

For the receptor blocking assay, the presence of HeV antibodies in the serum was detected by the ability to block biotinylated Ephrin B2 (Sapphire Bioscience Pty Ltd, Australia) which otherwise binds directly to soluble G protein-coated beads (Bossart et al., 2008). Streptavidin–phycoerythrin was added for detection of bound Ephrin B2. Low MFI values indicated henipavirus antibodies blocked successfully the binding of the receptor to sG. The results were recorded as a percentage inhibition and raw MFI readings were converted to percentage inhibition using the following formula: $(1 - [(\text{MFI test serum})/(\text{MFI negative serum})]) \times 100$.

Both assays were read using a Bio-Plex Protein Array System integrated with Bio-Plex Manager Software (v 4.1) (Bio Rad Laboratories, Inc., CA, USA) for data acquisition and analysis.

2.3. HeV ELISAs

2.3.1. HeV antibody indirect ELISA (HeV iELISA)

This ELISA, using detergent disrupted/inactivated virus antigen derived from whole cell lysates of HeV-infected Vero cells has been previously described by Daniels et al. (2001). In brief, NUNC Maxi-sorb plates were coated with HeV infected and non-infected (mock antigen) cell lysates diluted in carbonate buffer (pH 9.6) for 1 h at 37 °C. Coating and subsequent binding steps were followed by a four cycle rinse with wash buffer (PBS + 0.05% Tween 20). Sera were assessed at a 1:100 dilution in wash buffer with added 1% skim milk powder for an incubation period of 1 h at 37 °C. Bound antibody was reacted with a Protein A/G-HRP conjugate (Pierce, Rockford, USA), 30 min at 37 °C. TMB substrate (Sigma-Aldrich Pty Ltd, Australia) was reacted for 7–10 min before addition of 1 M H₂SO₄. Plates were read for absorbance at optical density 450 nm. After background subtraction, a threshold optical density 0.2 was assigned to differentiate positive reactor from negative sera. All reactive sera were retested in virus neutralisation serology. Significant reactions on

Fig. 1. Limit dilution titration of Luminex binding (○) and blocking assays (■) for HeV sG using a positive serum (Tho) with a virus neutralisation titre 1024.

the mock antigen in association with above threshold OD detections were qualified for interpretation as “non-specific reactors”.

2.3.2. HeV antibody indirect ELISA (HeV sG-iELISA)

The ELISA used HeV sG antigen coated at a concentration of 0.23 µg/ml. After a coating incubation (1 h at 37 °C), the plate was blocked with skim milk for 30 min and washed with PBST. Test sera were diluted 1:100, added to the plate and shaken for 1 h at 37 °C. Bound antibody was detected by using anti equine-HRP (Sigma-Aldrich Pty Ltd, Australia) or Protein A/G-HRP conjugate (Pierce, Rockford, USA) and a TMB substrate. Plates were read for absorbance at optical density 450 nm. After subtraction of background (taken as the OD from the negative control serum), ODs were transformed to a ratio relative to a low positive control serum (average OD approximately 0.5) and a signal to positive S/P ratio was calculated. A threshold S/P of 0.4 was assigned to differentiate positive reactor from negative sera. This assay is specific for equine sera due to the anti-equine conjugate used in the test. As for the HeV iELISA, all positive reactor sera in the sG-iELISA were retested in virus neutralisation serology.

2.4. HeV virus neutralisation serology

A standard virus neutralisation in microplate format was used for assessing sera for neutralising antibody against HeV (Bossart et al., 2007; OIE, 2009). The test used Vero cells and a virus concentration of 100 TCID₅₀/well. Sera were assessed from an initial dilution of 1:2 and were incubated with virus in a 96 well plate for 30 min at 37 °C. Vero cells were added and the plates incubated for 4 days at 37 °C in a CO₂ incubator. Cell monolayers were scored for the presence of cytopathic effect (CPE) and serum neutralisation titres were determined as the reciprocal of the serum dilution where no CPE was evident.

3. Results

3.1. Analytical sensitivity for the Luminex assays

To determine the analytical sensitivity for the henipavirus Luminex binding assay, a titration of a seropositive field infection serum (Tho), with a virus neutralisation titre of 1:1024 was performed (Fig. 1). The lower asymptote end point of the curve was approached at an MFI value of approximately 865 reaching this point at a dilution of 1:3200. Analytical sensitivity was derived from a third order polynomial regression curve fitted to the four

Fig. 2. Detection of henipavirus antibodies in a panel of sera from a range of *Paramyxoviruses*, *Flaviviruses* and *Alphaviruses* using the Luminex binding and blocking assays.

lowest data points ($r^2 = 1$) and applying assay threshold MFI value of 1500. Using HeV Luminex binding assay, the end-point dilution for positive signal detection was 1:2125. This represents an analytical detection range of approximately 2 times the assigned virus neutralisation titre (1024). The positive serum (Tho) was designated as a positive control for use in subsequent assays. In the binding assay and for the purpose of a normalising data, MFI values for test sera were transformed relative to the MFI value for the positive serum to yield a percentage positive (%P) value. The threshold was set at 5%P, being approximately 1500 MFI.

The serum was tested similarly in the Luminex blocking assay, reaching the lower asymptote at a dilution of 1:800. Using a positive/negative threshold of 15 percent inhibition (%I), detection to threshold spanned a 1:475 dilution range which is less than the virus neutralisation range by approximately half. Relative to the binding assay, the detection range for the blocking assay was reduced by a factor of 4.48. Taking into account the initial dilution (1:100 for binding assay and 1:50 for blocking assay), the binding assay had an analytical sensitivity approximately 9 times that of the blocking assay.

3.2. Analytical specificity of Luminex assays

In order to assess the analytical specificity of the henipavirus Luminex binding and blocking assays anti-sera against a range of *Paramyxoviridae*, *Flaviviruses* and *Alphaviruses* were tested.

In the binding format of the assay, one serum not raised against Henipavirus was marginally above the 5% provisional threshold with a reaction level of 7%P (Fig. 2). This reactive serum was an experimentally produced equine antiserum against eastern equine encephalitis virus. In the blocking format all non-Henipavirus antisera were not reactive relative to the set threshold of 15%I. Antiserum against NiV cross reacts to a high level in both binding and blocking assays.

The HeV iELISA and sG-ELISA were also assessed using this panel of sera. Frequently interpretation of results for the HeV iELISA

(using the whole virus antigen preparation) was influenced by significant non-specific reactions evident as high OD values (greater than 0.2) against the mock antigen. The HeV sG-iELISA showed a high level of analytical specificity for HeV antibodies as all non-Henipavirus sera were negative.

3.3. Diagnostic specificity of Luminex assays

Two panels of HeV antibody negative sera as determined by virus neutralisation and/or ELISA serology were tested in Luminex binding and blocking assays to provide evidence for diagnostic specificity.

One hundred and thirty five horse field sera which previously had tested negative in the HeV sG-iELISA were examined in the Luminex binding and blocking assays. The results in the binding assay (Table 1) showed that sera had a mean %P value of 0.4 with standard deviation of 0.12. In the receptor blocking assay, test sera displayed 0.8 ± 1.42 (mean $\pm$ standard deviation) percent inhibition; the positive control sera gave results in the range 93 to 95%. During the course of this study, the use of polystyrene beads was changed to magnetic beads. The results observed here showed good correlation between using magnetic or polystyrene beads (Table 1).

An additional panel ($n = 145$) of HeV virus neutralisation negative sera, grouped by an indeterminate result following testing in the HeV iELISA were further assessed by HeV Luminex assays. The binding assay showed test sera to have an average 1.2 ± 0.82 (mean $\pm$ standard deviation) %P with all results below the provisional 5%P threshold, however one serum reacted to 4.1%P. This serum panel were also examined by the Luminex blocking assay, giving an average %I of 2.42 with a standard deviation of 2.98 and a maximum of 12.4. The results for these sera which were problematic when tested by ELISA differ from results testing other negative sera; suggesting that the blocking format may be more affected by serum sample characteristics than the binding assay.

In addition, sera with an assigned negative status (designated by clinical and/or serological data for virus neutralisation and/or ELISA), total testings were made, respectively, of 277 (binding assay) and 267 (blocking assay) sera (Fig. 3). Results provided support for the assigned provisional cut-off thresholds; 5%P control for the binding assay and 15%I for the receptor blocking assay.

3.4. Use of Luminex assays for post infection and post vaccination sera

The Luminex assays were assessed further using sera derived from horses naturally infected with HeV during the original outbreak in 1994, and from other HeV outbreaks. A total of twenty one post infection sera were examined by ELISA, virus neutralisation and the Luminex assays. All sera tested were positive by virus neutralisation and above the cut off value of 1000 MFI using the Luminex binding assay. The results showed the higher the virus neutralisation titre, the higher the Luminex binding and blocking results using both HeV sG and NiV sG coated beads (Table 2).

Fifty four sera from horses that had been vaccinated with the commercially available Hendra virus vaccine (Equivac HeV) which contains the soluble G protein were assessed by the ELISA, virus neutralisation and Luminex binding and blocking assays. The HeV iELISA produced inclusive results due to binding in the mock antigen wells, whereas the sG-iELISA returned positive results for all the sera. Using the Luminex assays, the vaccinated horse sera displayed high levels of greater than 50.3%P control in the binding assay and above 22%I for the blocking assay using the beads coated with soluble G (Fig. 4). Serum from one horse (12-03417-0001) produced low results in the Luminex and ELISA however this correlated with the virus neutralisation negative result for this serum.

Fig. 3. Distribution of Luminex binding and blocking assay results for sera previously determined to be henipavirus antibody negative (binding assay $n = 277$, blocking assay $n = 267$), 21 post-infection sera and 54 post vaccination sera. Status assigned by clinical and/or serological data (virus neutralisation and/or ELISA). Luminex binding and blocking assay thresholds were set at 5%P and 15%I, respectively.

Fig. 4. Correlation of HeV virus neutralisation antibody with HeV sG iELISA, Luminex binding and Luminex blocking assay for sera collected from fifty four horses following vaccination with Equivac HeV. Note sera with titres of greater than 256 were assigned a nominal titre of 512 for the purpose of representation in the plot.

Table 1

Results using magnetic or polystyrene beads in Luminex binding and blocking assays of horse field sera determined as negative by HeV sG iELISA.

	Luminex binding assay (MFI)		Luminex blocking assay (% inhibition)	
	Magnetic HeV sG	Polystyrene HeV sG	Magnetic HeV sG	Polystyrene HeV sG
No serum control	43	43	0	0
Neg horse serum	118	92	–1	–1
Pos serum (Tho)	24,989	23,599	93	96
Pos serum (Tam)	26,666	25,882	95	97
Test sera (average $\pm$ standard deviation) ¹	99 $\pm$ 30 ¹	66 $\pm$ 29 ¹	0.8 $\pm$ 1.42 ²	0.52 $\pm$ 1.27 ²

¹ 135 normal horse sera were assessed in the Luminex binding assay.² 126 normal horse sera were assessed in the Luminex blocking assay.

3.5. Review of assay thresholds

Receiver-operator curve (ROC) analysis using assigned positive or negative status of all sera assessed by Luminex binding and blocking assays was used to evaluate provisional thresholds. Results are summarised in Table 3 suggest that an improvement in assay performance could be obtained by marginal alterations to the provisional thresholds, specifically, the binding assay from 15 to 12.45 and the blocking assay from 5 to 3.67. However confidence intervals have considerable overlap and the different thresholds affected the result determination for only one serum. As a working resolution, a zone of equivocal determination for results falling between 10 and 15 for the binding assay and from 3 to 5 in the blocking assay was defined. Samples with results falling within these zones would be subject to qualified reporting and/or additional assessment.

3.6. Use of Luminex assays for naturally infected dog sera

The two serum samples taken on the 20th and 26th July 2011 (2011/Mount Alford, QLD no. 1 and 2011/Mount Alford, QLD no. 2) from a naturally HeV-infected dog were examined together with dog sera from other HeV infected premises obtained throughout 2011. The Luminex binding assay showed that a high level of HeV specific antibodies were present in the two blood samples taken from the HeV infected dog with results of 22,182 MFI and 12,241 MFI (Table 4). All other dog sera tested in the Luminex binding assay had MFI values below 230. In the Luminex blocking assay the

two blood samples taken from the HeV infected dog had 88% and 61% inhibition while results for all the other dog samples tested showed very low levels of inhibition below 3%. All the dog samples were also examined by HeV iELISA, sG-iELISA and virus neutralisation. Many of the dog sera tested in the HeV iELISA produced non-specific reactors preventing conclusive determination by that assay. The HeV sG-iELISA also produced some non-specific positive results as determined by the correlating Luminex and HeV virus neutralisation assays showing a negative antibody status. In comparison to the ELISAs, the Luminex assays look to be a useful tool for testing naturally HeV infected dog sera.

4. Discussion

Serology for BSL 4 agents can be problematic in the absence of appropriate containment facilities; hence the development of assays that do not require live virus is advantageous. Assays applied at the time of initial emergence of the disease will generally require technical and interpretive refinement as observations and data accumulate. However, the validation of these assays for new or emerging viruses frequently presents additional challenges due to the lack of well characterised sera. This is exacerbated in such fulminant disease as HeV by the high early mortality and a policy of immediate euthanasia upon confirmed detection. Serology procedures applied to date at the Australian Animal Health Laboratory (PC3 and PC4 containment) have included virus neutralisation, ELISA and more recently the Luminex-based fluorescent bead assays. The availability of recombinant expressed proteins has

Table 2

Assessment of sera from horses naturally infected with HeV by ELISA, virus neutralisation, Luminex binding and blocking assays.

HORSE SERA year/location/number	HeV iELISA (OD)	HeV sG iELISA (S/P)	HeV neutralisation (titre)	HeV sG Luminex binding (% Pos)	HeV sG Luminex blocking (% inhibition)
2008/Redlands, QLD/no. 1	1.8	1.83	2048	104	93
2008/Redlands, QLD/no. 2	0.99	1.76	4096	103	91
2008/Redlands, QLD/no. 3	0.57	1.38	128	96	76
2008/Proserpine, QLD/no. 1	1.79	1.63	512	103	77
2008/Redlands, QLD/no. 4	1.7	1.8	2048	105	95
2008/Redlands, QLD/no. 5	1.58	1.87	2048	106	95
2008/Redlands, QLD/no. 6	1.8	1.71	4096	104	85
2008/Hendra, QLD/no. 1	0.44	0.03	16	6	29
2008/Redlands, QLD/no. 7	0.97	1.44	2048	97	81
2006/QLD/no. 1	0.55	0.02	20	4	15
2009/Cawarral/no. 1	0.86	2.18	64	32	61
2009/Cawarral/no. 2	1.32	2.79	1024	78	67
2009/Cawarral/no. 3	1.38	1.81	16	104	90
2009/Cawarral/no. 4	1.52	2.8	16	103	89
1994/Hendra, QLD/no. 1	1.40	1.58	640	104	95
1994/Hendra, QLD/no. 2	1.63	1.66	640	104	95
1994/Hendra, QLD/no. 3	1.66	1.71	1280	104	92
1994/Hendra, QLD/no. 4	0.61	0.15	20	35	18
1994/Hendra, QLD/no. 5	1.15	1.52	640	104	94
1994/Hendra, QLD/no. 6	1.11	1.54	640	100	88
1994/Hendra, QLD/no. 7	1.47	1.64	640	103	93

Assay positive detection thresholds are: HeV iELISA OD > 0.2; HeV sG ELISA S/P > 0.4; HeV virus neutralisation titre $\geq$ 2; HeV Luminex binding % $\geq$ 5%; HeV Luminex blocking % $\geq$ 15%.

Table 3

Assessment of assay thresholds using results from ROC analysis.

(a) Luminex blocking assay				
Criterion	Sensitivity	95% CI	Specificity	95% CI
> 12.45	100.00	83.9–100.0	100.00	98.6–100.0
> 15	95.24	76.2–99.9	100.00	98.6–100.0
(b) Luminex binding assay				
Criterion	Sensitivity	95% CI	Specificity	95% CI
>3.67	100.00	83.9–100.0	99.28	97.4–99.9
>5	95.24	76.2–99.9	99.64	98.0–100.0

allowed for improvements, particularly to reduce the frequency of non-specific reactions encountered in ELISAs using crude disrupted virus. As a further refinement to testing procedures, this study has shown that the henipavirus Luminex binding and blocking assays are effective for HeV serology in the assessment of equine and canine sera and are advantageous particularly in the resolution of indeterminate ELISA results without further recourse to virus neutralisation serology. While progressive validation of this approach remains dependent on the availability of infrequent positive samples generated in episodic outbreaks, the Luminex assay have been shown to perform better than the conventional ELISAs currently in use at AAHL in terms of both sensitivity and specificity.

The results supplement other serological studies in Australia and Africa which have utilised Luminex serology assays to demonstrate evidence for henipavirus infection (Bossart et al., 2005, 2007; Hayman et al., 2011; Peel et al., 2012, 2013; Dups et al., 2012; Playford et al., 2010). In particular, this study has explored more extensively the relative performance characteristics of ELISAs and Luminex and evaluated more recent developments in assessments of post-infection canine sera and post vaccination equine sera.

The Luminex binding assay has a more conventional indirect detection format, allowing recognition of both neutralising and non-neutralising antibodies targeting the HeV G protein (Bossart et al., 2007). Analytical sensitivity relative to the HeV virus neutralisation favoured the Luminex by a factor of approximately 2, though this margin is of no practical value for predicting diagnostic performance. The Luminex receptor-blocking assay has a narrower specificity, being limited to antibodies in the test sample which may interfere with binding of the labelled ephrin B receptor. Hence the assay is described as a neutralisation test surrogate and

has been shown to be uninfluenced by binding of non-neutralising monoclonal antibodies against G protein (Bossart et al., 2007). The assay therefore has potential as a confirmatory test for specificity of results from the binding assay or as a test that would remove the need for confirmatory virus neutralisation serology. Evidence for this higher specificity is also suggested from analytical specificity results presented. However the test has a detection range approximately half that of the virus neutralisation; relative to the binding assay analytical sensitivity is reduced by a nine-fold factor. Nevertheless the relevance of this margin to diagnostic performance is difficult to assess, particularly given the dearth of post infection antibody-positive sera. Other than for the expected cross-reactions with NiV, both assays showed a high level of analytical specificity. Diagnostic specificity was assessed in Luminex binding and receptor blocking assays using a total of 277 (binding) and 267 (blocking) sera with an assigned negative status as determined by virus neutralisation and/or ELISA. All produced low MFI results and allowed provisional thresholds to be set at 5% percent positive control and 15% for the receptor blocking assay.

The commercial release in 2012 of the Equivac HeV vaccine for use in Australian horse populations (companion, farm and racing) has resulted necessarily in a modification to the “fitness for purpose” of serology assays which incorporate the HeV G protein, including the Luminex and ELISA assays described in this publication. The vaccine induces detectable antibody against G protein as is evident in this assessment of 54 vaccinated horses which showed an average of 93%P control. As a consequence and for external reporting, in reports of results for antibody against G protein, a comment is inserted “The currently available serology assays do not distinguish between antibodies due to natural infection and those due

Table 4

Evaluation of HeV detectable antibody in canine field sera collected from dogs located in proximity to a confirmed equine HeV infection.

DOG SERA year/location/number	HeV iELISA OD (mock Ag OD)	HeV sG-i ELISA (S/N)	Virus neutralisation	Luminex binding (MFI)	Luminex blocking (% inhibition)	Interpretation
2011/Mount Alford, QLD/no. 1	2.82 (0.09)	3.02	Positive with a titre >1:16	22,182	88	Positive
2011/Mount Alford, QLD/no. 2	2.53 (0.22)	3.17	Positive with a titre of 128	12,241	61	Positive
2011/Biddadaba, QLD/no. 1	0.34 (0.11)*	0.05	Negative	105	3	Negative
2011/Biddadaba, QLD/no. 2	0.31 (0.14)*	0.02	Negative	74	0	Negative
2011/Biddadaba, QLD/no. 3	0.22 (0.15)*	0.02	Negative	43	0	Negative
2011/Biddadaba, QLD/no. 4	0.25 (0.11)*	0.03	Negative	89	0	Negative
2011/Biddadaba, QLD/no. 5	0.23 (0.06)*	0.02	Negative	83	1	Negative
2011/Biddadaba, QLD/no. 6	0.33 (0.12)*	0.02	Negative	95	1	Negative
2011/Wardell, NSW/no. 1	0.16 (0.05)*	0.03	Negative	144	1	Negative
2011/Zillmere, QLD/no. 1	3.08 (2.96)*	1.84**	Negative	97	1	Negative
2011/Zillmere, QLD/no. 2	3.32 (3.42)*	1.94**	Negative	109	1	Negative
2011/Zillmere, QLD/no. 3	2.88 (2.52)*	2.64**	Negative	107	1	Negative
2011/Chinchilla, QLD/no. 1	0.1 (0.05)	0.06	Negative	110	2	Negative
2011/Currumbin Valley, QLD/no. 1	0.38 (0.15)*	0.17	Negative	80	1	Negative
2011/Tintenbar, NSW/no. 1	0.59 (0.44)*	0.18	Insufficient sera***	57	0	Negative
2012/Mackay, QLD/no. 1	0.25 (0.06)*	0.02	Indeterminate: sample toxicity at dilutions less than 1:16***	33	2	Negative
2012/Mackay, QLD/no. 2	0.2 (0.05)*	0.01	Negative	229	1	Negative

* Inconclusive result due to binding in mock antigen well.

** Non-specific sG ELISA result.

*** These animals were negative for HeV RNA by PCR.

to vaccination. Any positive result must be interpreted in the context of the animal's vaccination history". This limitation is presently being addressed at this laboratory through the development of ELISA and Luminex assays which are specific for alternative target proteins such as M, F and/or N. The successful development of these assays will provide the necessary tools for differentiating infected from vaccination-derived antibody, commonly referred to as the DIVA (differentiating infected and vaccinated animals) approach. Instances which may be affected by the qualification applied to the results of assays detecting anti-G antibody would include assessment of vaccinated (or possibly vaccinated) animals for the absence of HeV exposure. This is expected to become necessary most frequently should an outbreak occur in the vicinity of vaccinated animals. For the international movement of vaccinated horses, the preferred option is considered to be the recognition of a vaccination certificate without recourse to laboratory testing; recently this process has been allowed by Hong Kong.

While the zoonotic potential of HeV has been evident since the initial outbreak and subsequent laboratory based studies (Williamson and Torres-Velez, 2010), the detection in 2011 and 2012 of natural HeV infection in dogs has been a significant development in the epidemiology of this virus. As there is evidence that these infections can be asymptomatic, laboratory testings for agent and antibody will be key investigative approaches. This study provides some evidence for the potential use of Luminex in canine HeV antibody assessment; in addition it should be noted that indirect ELISA-based approaches may be prone to non-specific binding effects which lessen confidence in results. However Luminex binding and blocking assay results correlated well with results using virus neutralisation. While validation studies are required to establish fully the relative merits of the assays, these early determinations are favourable for the use of Luminex.

The results obtained in this study involving horses and dogs supplement the findings by Bossart et al. (2008) to demonstrate the potential for henipavirus Luminex assays to become used widely for diagnostic henipavirus serology. Other diagnostic laboratories in Australia are currently developing microsphere immune-assays for detection of HeV which could allow the use of this assay across Australia for diagnostic use in the future. Advantages, evident for the henipavirus Luminex binding and blocking assays include short testing time, a high level of sensitivity and specificity, the absence of a requirement for PC4 bio-containment and multiplexing capability allowing simultaneous investigation for several disease agents. A challenge is presented in the context of applying the novel assay (Luminex) for detection of an emerging disease for which very few retrospective positive samples are available to determine validation and fitness. The frequent requirement to assess acute disease samples for antibody which may not have developed serves to emphasise the need for both agent and antibody detection in diagnostic evaluations. Significantly, this assessment of Luminex assays used sera that were determined as positive in virus neutralisation and/or ELISA and it is therefore not possible to infer the diagnostic characteristic of the assay under these limitations. With performance characteristics that are equal to or better than ELISA and virus neutralisation, it is predicted that Luminex assays will become a versatile tool in disease investigation, epidemiological, and surveillance studies for the detection of henipavirus specific antibodies in the future.

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