Development and validation of an IgM antibody capture ELISA for early detection of Hendra virus

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ABSTRACT

Zoonotic transmission of Hendra virus (HeV) from primary hosts (pteropid bats) to horses, and, occasionally, onward adventitious spread to humans, is associated with high mortality rates in both affected secondary species. The introduction of an effective recombinant G protein vaccine for use in horses has been a major advance for the suppression of disease risk. However, equine HeV vaccination induces neutralising antibody that is indistinguishable from a post infection immune response when using most first line serology assays (eg. VNT and some ELISAs). We have constructed and evaluated an IgM antibody capture (MAC) ELISA which employs yeast expressed HeV nucleoprotein (N). All other serology tests use the G protein which does not detect early infection and is present in the current Hendra virus vaccine and may cause ambiguity in interpretation of results. Thus, this is the first test developed using a N protein which can successfully detect a recent (primarily within the last four weeks) infection of horses with HeV and is not affected by vaccination induced antibody. Testing a limited panel (21 samples) of post infection sera, a normal serum panel (288 samples) and a post vaccination panel (163 samples), we have estimated DSe to be 100 % (95 % CI, 83.9–100.0 %) and DSp to be 98.4 % (95 % CI, 96.8–99.4 %) relative to assigned serology results (VNT, ELISA and Luminex) for the test panels. The HeV IgM MAC ELISA is intended to supplement other molecular and serology test results, with selective use, and is the only serology test which can provide an indication for recent infection which is otherwise not available.

1. Introduction

Hendra virus (HeV, family: *Paramyxoviridae*, genus: *Henipavirus*) disease was first diagnosed in horses and humans in 1994 in the Brisbane suburb of Hendra, Queensland (Mahalingam et al., 2012). Since that occurrence, the virus has been detected only in Australia, although serological evidence for possible broader distribution has been reported (Field et al., 2011). Further serological investigations (Young et al., 1996) implicated Australian pteropid fruit bats (flying foxes) as the likely wildlife reservoir of which, one species, the black flying fox (*Pteropus alecto*), was later most strongly associated with spillover events using epidemiological and laboratory studies (Middleton, 2014; Edson et al., 2019). All seven human cases, with four associated fatalities, have occurred in individuals closely involved with the handling and management of infected horses (Field, 2016). Records of equine infections

identify a total of 65 HeV incidents since 1984 affecting 105 horses; all animals died as a direct result of the disease or related euthanasia (Queensland State Government, 2021). At least one detection of Hendra virus has occurred every year since 2006.

A breakthrough for disease control came in November 2012 with the production of Equivac HeV vaccine (Zoetis Australia P/L) based on an expressed form of HeV G protein (Middleton et al., 2014). HeV G protein is the viral structural protein involved in host receptor-cell binding and is also the target of neutralising antibodies (Eaton et al., 2006). Minimising disease outbreaks through vaccination requires effective coverage, particularly for horses which are co-located in the fruit bat habitat range. Reported vaccination rates in the Australian horse population have been estimated at between 11–17% (Manyweathers et al., 2017); additionally, annual boosters are prescribed to maintain efficacy. While higher vaccine usage in defined transmission zones may moderate

risk, as there is no prospect for eradication, it is predictable that HeV spillover events will continue to affect horses and pose challenges for detection and management.

Recognition of the disease requires a combination of clinical and laboratory expertise. A spectrum of different HeV disease presentations can occur in affected horses, which must be differentiated from a range of other infection and non-infection related etiologies with similar or overlapping syndromes. Molecular detection of HeV is the primary laboratory diagnostic tool for detection/exclusion of HeV in acute disease. The fulminant acute manifestation of lethal disease frequently precludes sufficient development of antibody for serology to be a useful adjunct test. However, scenarios do emerge in which serology is useful in support for PCR or even requisite as a primary diagnostic tool. This can include investigation of milder disease cases, particularly instances in which collection of samples has been delayed beyond the appropriate diagnostic window for PCR. Alternatively, serology can be essential for investigation of presumptively exposed animals identified in epidemiological review of disease outbreaks and in other atypical events. Furthermore, if vaccination histories are not available, and particularly if annual booster vaccinations have not been maintained, clinical and laboratory interpretations may need to be qualified by these constraints (eg. detected antibody may represent evidence for exposure or vaccination). In such cases, serology may enhance the laboratory evidence from molecular testing in support of better clinical diagnosis. However, some frontline serology tests may have reduced value for use in disease diagnosis as vaccination or infection induced antibody cannot be differentiated by tests which target antibody to that protein (eg. VNT and G-protein ELISAs).

In this paper we describe the development and validation of an ELISA to detect equine IgM antibody against HeV nucleocapsid (N) protein that is both uninfluenced by vaccination-derived antibodies and a diagnostic test for recent infection. Detection of IgM antibody has been used as a marker for recent viral infection in horses (Lam et al., 2005; Davidson et al., 2005; Castillo-Olivares et al., 2011; Ching et al., 2015) and the antibody class is generally detectable following seroconversion for several weeks to months. This test is intended to complement other requisite molecular and serology testing, particularly in instances of samples collected for investigation of possible recent infection.

2. Materials and methods

2.1. Serum specimens

All field horse serum specimens were derived from diagnostic samples received at the Australian Animal Health Laboratory. This included 288 sera from horses determined to be negative for antibody to HeV, 163 post HeV vaccination sera and 21 post HeV infection sera (Table 1).

2.2. HeV IgM MAC ELISA

The HeV N-protein IgM antibody capture ELISA (HeV N-MAC ELISA) was modified from published methods (Kurtz and Malic, 1981). In brief, reagent concentration levels were optimised for use in the assay by prior titration. Repeated steps included 50 μL reagent volumes, all incubations prior to substrate were at 37 °C with plate shaking and a three-cycle wash of plates using wash buffer (PBS + 0.05 % Tween 20) separated incubation steps. Coating of plates: ELISA plates (Maxisorp, Nunc, Denmark) were coated with anti-equine IgM (2.5 μg/mL) (KPL, Gaithersburg, MD, USA) in carbonate-bicarbonate buffer (pH 9.0). Antibody capture: serum specimens diluted 1 in 300 in ELISA diluent (wash buffer with added 1% skim milk powder) were added to duplicate wells and incubated. Antigen binding: recombinant yeast expressed HeV N antigen diluted 1 in 1000 in ELISA diluent using 0.5 mg/mL stock (Juozapaitis et al., 2007) was added to alternate well columns, separated by columns with ELISA diluent only. This effectively provided antigen and antigen-free wells for each test serum. Detection: rabbit anti-HeV N

Table 1

Description of the case history of the positive animals naturally infected with HeV which includes 14 different horses (21 positive sera). Horse A had six blood samples taken day 0, 3 (x2), 11, 14 and 16 after infection, Horse B had two blood samples taken day 0 and 6 after infection and Horse C had two blood samples taken 5 days after infection.

Blood sample No.	Blood samples taken after onset of clinical signs	Information about Case	Date Collected	Time since onset of symptoms
1	Horse #1 163,910/1 Sign of Battle 1994/ Hendra, QLD/ no.1	Original outbreak in Hendra 1994	21-OCT-1994	7 days
2	Horse #2 163,910/2 Quegent 1994/ Hendra, QLD/ no.2	Original outbreak in Hendra 1994	21-OCT-1994	7 days
3	Horse #3 163,910/3 Minders Girl 1994/Hendra, QLD/no.3	Original outbreak in Hendra 1994	21-OCT-1994	7 days
4	Horse #4 163,910/4 Hurmino 1994/ Hendra, QLD/ no.4	Original outbreak in Hendra 1994	21-OCT-1994	7 days
5	Horse #5 163,910/5 Big Red 1994/ Hendra, QLD/ no.5	Original outbreak in Hendra 1994	21-OCT-1994	7 days
6	Horse #6 163,910/6 Sir Ambition 1994/ Hendra, QLD/ no.6	Original outbreak in Hendra 1994	21-OCT-1994	7 days
7	Horse #7 163,910/7 Will Tango 1994/ Hendra, QLD/ no.7	Original outbreak in Hendra 1994	21-OCT-1994	7 days
8	06−03803-0004 2006/QLD/no.1	Rej Banner	3-NOV-2006	0 days
9	08−02438-0027 2008/Redlands, QLD/no.7 Horse A (1)	QLD Redlands Vet 08−161435 Tamworth original bleed neurological signs	7-JUL-2008	0 days
10	08−02480-0001 2008/Redlands, QLD/no.6 Horse A (2)	08 161,772 Tamworth Box 2 EDTA plasma recovered horse	10-JUL-2008	3 days
11	08−02480-0003 2008/Redlands, QLD/no.2 Horse A (3)	QLD Redlands Vet 08−161772 Tamworth recovered horse	10-JUL-2008	3 days
12	08−02667-0002 2008/Redlands, QLD/no.4 Horse A (4)	QLD Redlands Vet 08−163515 Tamworth	18-JUL-2008	11 days
13	08−02669-0001 2008/Redlands, QLD/no.5 Horse A (5)	QLD Redlands Vet 08−166801 Tamworth	21-JUL-2008	14 days
14	08−02668-0001 2008/Redlands, QLD/ no.1 Horse A (6)	QLD Redlands Vet 08 165,480 Tamworth	23-JUL-2008	16 days
15	08−02844-0001 2008/ Proserpine, QLD/ no.2 Horse B (1)	SS08S9055 340,349,079 Thomas PURVIS	23-JUL-2008	0 days
16	08−02813-0001 2008/	08 166,026 Thomas	29-JUL-2008	6 days

Table 1 (*continued*)

Blood sample No.	Blood samples taken after onset of clinical signs	Information about Case	Date Collected	Time since onset of symptoms
17	Proserpine, QLD/ no.1 Horse B (2) 08−02503-0001 2008/Redlands, QLD/ no.3	QLD Redlands Vet From companion horse	9-JUL-2008	0 days
18	09−02723-0001−01 2009/ Cawarral/no.1	QLD Carwarral 09−122252	12-AUG-2009	0 days
19	09−02723-0002−01 2009/ Cawarral/no.2	QLD Carwarral 09−122824	14-AUG-2009	2 days
20	09−02844-0007−01 2009/ Cawarral/no.3 Horse C (1)	QLD Carwarral 09−123441 4 Whinney	19-AUG-2009	5 days
21	09−02844-0008−01 2009/ Cawarral/no.4 Horse C (2)	QLD Carwarral 09−123441 4 Whinney	19-AUG-2009	5 days

antibody (provided by Dr Grant Peck, AAHL, Geelong, Australia) diluted 1/5000 in ELISA diluent supplemented with 1% NHS for thirty minutes at 37 °C. Conjugate: Donkey anti-rabbit IgM (Jackson ImmunoResearch Laboratories, Pennsylvania, USA) for thirty minutes at 37 °C. Substrate: TMB (Sigma-Aldrich, St. Louis, USA) for 10 min at room temperature before stopping the reaction by using 1 M H_2SO_4. Plates were read using the absorbance of 450 nm. OD levels in antigen and antigen-free wells for the negative control serum used as an overall baseline for other readings on the plate, while OD levels in antigen wells for each serum were also reduced by any net OD in the corresponding antigen-free well. The residual OD levels for each test serum were converted to a percentage of the positive control (%P). MedCalc (MedCalc Software, Ostend, Belgium) statistics and ROC curves were used to determine cut off values based on optimised diagnostic sensitivity (DSe) and specificity (DSp).

2.3. Indirect antibody ELISA using whole virus - Hendra iELISA

The Hendra iELISA was the first developed indirect ELISA for Hendra virus detection using detergent disrupted/inactivated virus antigen derived from whole cell lysates of Hendra-infected Vero cells and has previously been described by Daniels et al., 2001. A threshold OD of 0.2 was assigned to differentiate positive and negative sera.

2.4. Indirect antibody ELISA using expressed HeV G protein - HeV sG iELISA

The HeV sG iELISA (estimated DSe 84.2 %, DSp 97.1 %) for the detection of antibodies against HeV in horse sera was conducted as described previously (Colling et al., 2018). A negative result had a S/P ratio <0.25 whereas a S/P ratio >0.4 was positive. Results between these S/P ratios were classified as inconclusive.

2.5. Henipavirus Luminex binding assay using expressed HeV g protein

The multiplex microsphere assay (estimated DSe 100 %, DSp 95.2 %) was performed as described previously by McNabb et al., 2014. All results were recorded as median fluorescent intensity (MFI) with a positive result >1500 MFI.

2.6. Virus neutralisation assay - HeV VNT

The HeV virus neutralisation assay (World Organisation for Animal Health (OIE), 2019) was used to detect neutralising antibody against

HeV. From an initial dilution of 1:2, end-point titres are calculated as highest dilution having 50 % microplate well neutralisation. The HeV VNT is the reference assay against which relative DSe and DSp proportions are assigned for other HeV serology assays.

2.7. Assay validation

2.7.1. Analytical specificity

Analytical specificity provides a limited representation of the potential for cross-reactions which may affect assay specificity, particularly regarding more closely related agents. For the analytical specificity estimate, thirteen horse serum samples, IgM antibody positive to related Paramyxoviruses, Flaviviruses, Obiviruses and Alphaviruses (McNabb et al., 2014) were tested in the Hendra IgM MAC ELISA and compared to two Hendra virus infected horses.

2.7.2. Analytical sensitivity

Analytical sensitivity was estimated by interpolation of curves plotted from the HeV IgM MAC ELISA in relation to the HeV sG iELISA and the HeV VNT to determine the limit of detection of antibody present in the serum. For the analytical sensitivity estimate, a positive Hendra virus sample was titrated, and the various serum dilutions were compared using the cut off threshold for each assay.

2.7.3. Diagnostic specificity

Diagnostic specificity is a measure of how well the IgM MAC ELISA is at identifying true negatives which was assessed by testing sera from animals that had previously yielded negative results in the HeV sG iELISA and/ HeV VNT. OD Values for each sample were expressed as a percentage relative to the positive control (%P) which was used to establish the baseline (cut off) for negative results. For the diagnostic specificity estimate, 451 negative equine sera (288 normal horse sera and 163 post vaccination sera) were used to determine the assay threshold value.

2.7.4. Diagnostic sensitivity

Diagnostic sensitivity is a measure of how well the IgM MAC ELISA is at identifying true positives which was assessed by testing HeV positive equine sera. For the diagnostic sensitivity estimate, 21 positive equine sera were all found to be above the assay threshold value of 8.99 %P.

3. Results

3.1. Analytical specificity of the HeV IgM MAC ELISA

The analytical specificity of the HeV IgM MAC ELISA was determined using immune antiserum from thirteen horses previously infected from a range of *Paramyxoviruses, Flaviviruses, Obiviruses* and *Alphaviruses*. The results showed that the HeV IgM MAC ELISA was unaffected by antibodies in these immune sera (Fig. 1) with only the two HeV specific positive sera above the cut off value of 8.99 %P (optimised for DSe/DSp as detailed below).

3.2. Analytical sensitivity of the HeV IgM MAC ELISA

A HeV positive serum sample (Horse #7; Williamson et al., 1998) obtained from experimental equine infection (VNT titre 1600) and collected 8 days post infection was titrated in both a HeV MAC ELISA and a HeV sG iELISA (Fig. 2) to determine the relative limit of antibody detection.

The limit of detection using the HeV IgM MAC ELISA (cut off value of 8.99 %P) occurs at a dilution of 1/61,440. This is compared to the HeV sG iELISA which has a limit of detection (cut off S/P value of 0.25) occurring at a dilution of 1/38,400, giving a factor of 1.6 favouring the IgM ELISA. However, when comparing different assays, it is also useful to factor in the working assessment range defined by the initial to limit
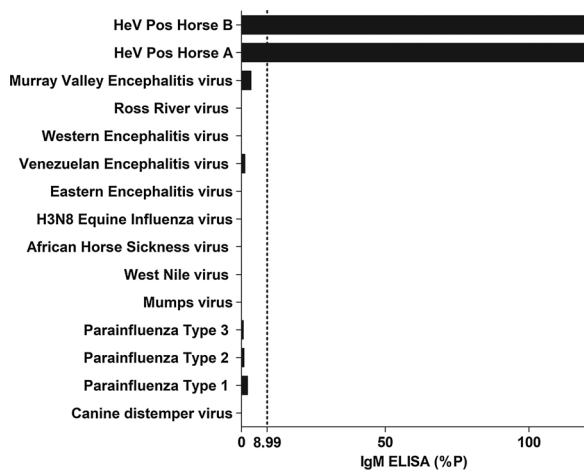
Fig. 1. Detection of anti-equine HeV IgM antibodies present in a panel of sera from a range of Paramyxoviruses, Flaviviruses, *Orbiviruses* and *Alphaviruses*. Horse A and Horse B explained in Table 1.
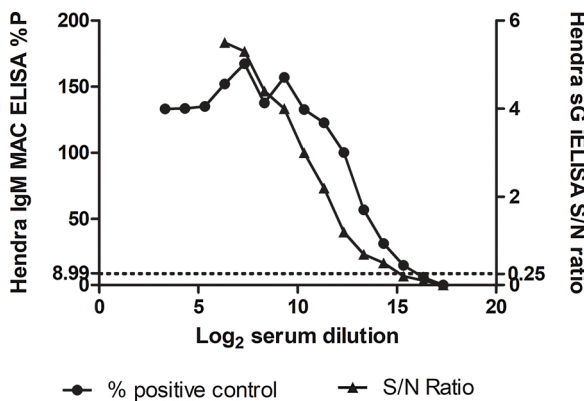

Fig. 2. Limit dilution titration of the positive control for the HeV IgM MAC ELISA %P (Y left axis) and the HeV sG iELISA S/N ratio (Y right axis). The threshold line is represented by a dotted line.

dilutions. As the HeV IgM MAC ELISA has a higher initial dilution requirement (1/300) relative to the HeV sG iELISA (1/100), the analytical detection ratio (end-point titre to initial dilution) is greater for the indirect ELISA (384 to 204.8). Relative to the VNT, the ratio is somewhat higher at 800 (initial dilution 2, endpoint titre 1600). While the comparison can be affected by sample selection (eg. in samples having greater or lesser relative proportions of IgG and IgM), results with the tested sample suggest comparably close analytical sensitivity for the ELISAs, with the VNT more sensitive.

3.3. Threshold-optimised diagnostic sensitivity (Se) and diagnostic specificity (sp) of the HeV IgM MAC ELISA

Paired diagnostic sensitivity and specificity estimates were evaluated relative to varied assay thresholds using a test-negative population (HeV sG iELISA and/or virus neutralisation assays) of 451 equine sera (288 normal horse sera and 163 post vaccination sera) and a test positive group of 21 equine sera obtained during the original and later Hendra outbreaks in horses. Using the combined inhibition values related to the positive control (%P) of the 451 negative sera in relation to the 21 positive sera, a MedCalc statistical approach produces a receiver operating characteristics (ROC) graph plotting sensitivity against 100-specificity at incrementally altered positive/negative thresholds that can be used to calculate and optimize the assay threshold (cut-off) line. A positive threshold value requiring positive sera to be greater than 8.99 %

P returned DSe of 100 % (95 % CI, 83.9–100.0 %) and DSp of 98.4 % (95 % CI, 99.8–99.4%) for the tested population (Fig. 3). The area under the curve (AUC) approached 1.0, indicating almost complete separation of negative and positive populations. The test-negative population returned an average of 0.19 %P (1 SD of 3.19 %P) indicating a low and narrow distribution. A data spread plot (Fig. 4) shows that five normal horse samples were above the 8.99 %P threshold using the HeV IgM MAC ELISA with values of 14.8 %P, 14.3 %P, 14 %P, 10.4 %P and 9.3 % P, respectively. All vaccinated horses had values less than 8.99 %P except for two horses with 8.99 %P and 11.7 %P respectively. Also, one HeV positive sample at 9.2 %P was close to the assay threshold.

The twenty-one positive sera (post natural infection) from 14 different horses were examined further using additional assays (HeV iELISA, Hendra sG iELISA, VNT and HeV Luminex binding assay) to characterise the relative immune antibody levels more completely. The compiled results presented in Table 2 shows agreement that all post infection sera, except for one (blood sample #4), were highly reactive. The excepted sample, which was marginally positive at 9.2 %P in the HeV IgM MAC ELISA, also had a low virus neutralisation titre of 20. Overall, in the HeV IgM MAC ELISA, all positive sera averaged 131 %P (SD 31.6).

3.4. Duration of IgM antibody

Blood collected from two animals (Horse A and Horse B) at 8 weeks after the onset of clinical symptoms (14-OCT-2008; 11-SEP-2008) and diagnosed HeV infection were negative for detection of IgM, 1.8 %P and 1.3 %P, respectively. These two animals were both positive by HeVsG iELISA and HeV VNT due to the presence of IgG being detected in these assays.

3.5. Vaccinated horses examined using the HeV IgM MAC ELISA

One hundred and sixty-three sera from HeV vaccinated equines with confirmed HeV antibody (results not shown) were tested in the HeV IgM MAC ELISA and used to calculate the positive threshold value (Fig. 3). All 163 vaccinated horses had values <8.99 %P except for two horses with 8.99 %P and 11.7 %P (Fig. 4).
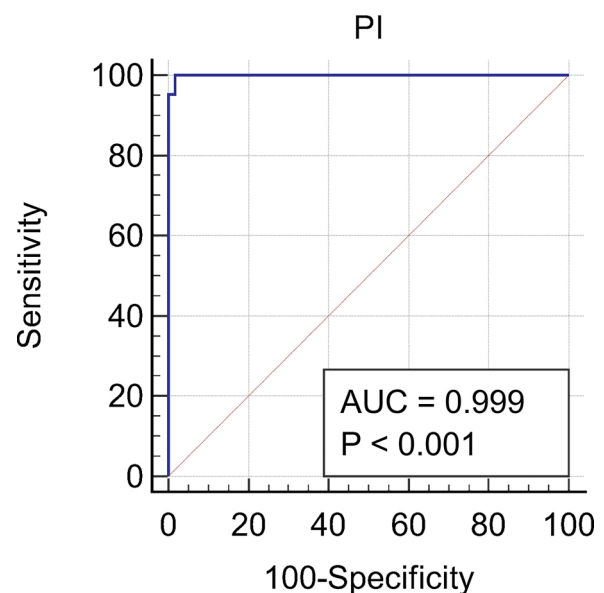

Fig. 3. ROC curve analysis of the HeV IgM MAC ELISA derived using 451 equine sera (288 normal horse sera and 163 post vaccination sera) and 21 post infection sera.
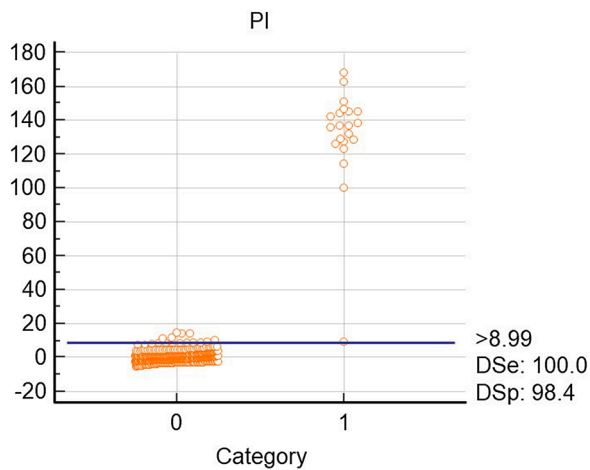
Fig. 4. HeV IgM MAC ELISA discrimination of antibody negative (0) and antibody positive (1) groups based on Hendra sG iELISA and/or Hendra VNT. Negative group - 288 normal horse serum samples and 163 post vaccination serum samples, positive group - 21 sera derived from horses following an equine HeV infection (Table 1). The solid bar represents the optimised threshold of >8.99 %P. Category 0 = unexposed, Category 1 = exposed.

Table 2

Assessment of sera from 14 different horses (21 positive sera) naturally infected with HeV using the HeV IgM MAC ELISA and other serological assays. Samples 9- 14 are six different blood samples taken on day 0, 3 (x2), 11, 14 and 16 after infection from Horse A (Table 1). Samples 15 and 16 are two different blood samples taken on day 0 and 6 after infection from Horse B (Table 1) and samples 20 and 21 are two different blood samples taken 5 days after infection from Horse C (Table 1). Assay positive detection thresholds are HeV iELISA OD > 0.2; HeV sG iELISA >0.4; HeV VNT ≥ 2; HeV sG Luminex binding >1500MFI; Hendra IgM ELISA > 8.99 %P.

Blood sample No.	HeV iELISA (OD) Positive >0.2	HeV sG iELISA (S/ P) Positive >0.4	HeV VNT (Titre) Positive ≥2	HeV sG Luminex binding (MFI) Positive >1500	Hendra IgM MAC ELISA (%P) Positive >8.99
1	1.40	1.58	640	28,904	145
2	1.63	1.66	640	28,943	145
3	1.66	1.71	1280	28,940	144
4	0.61	0.10	20	9663	9
5	1.15	1.35	640	29,036	136
6	1.11	1.40	640	27,802	146
7	1.47	1.64	640	28,755	100
8	0.55	0.02	20	1040	137
9	0.97	1.44	2048	27,143	129
10	1.8	1.71	4096	28,856	129
11	0.99	1.76	4096	28,778	163
12	1.7	1.8	2048	29,382	168
13	1.58	1.87	2048	29,429	138
14	1.8	1.83	2048	28,986	137
15	0.44	0.03	16	1594	142
16	1.79	1.63	512	28,689	127
17	0.57	1.38	128	26,706	151
18	0.86	2.18	64	8851	132
19	1.32	2.79	1024	21,658	126
20	1.38	1.81	16	28,840	123
21	1.52	2.8	>16	28,658	114

4. Discussion

Horses infected with Hendra frequently die or are euthanised in the acute stage of the disease and prior to development of an antibody response (Middleton et al., 2014). Molecular testing for HeV nucleic acid is the recommended laboratory diagnostic method for confirmation of clinical cases; serology has a limited role in confirmatory testing, being more appropriate for assessment of earlier or historical exposure (World Organisation for Animal Health (OIE), 2019). Significantly, molecular testing is most often conducted on animals with suggestive clinical presentations or histories of contact with the disease, so many cases will have no detections, the animals having unrelated aetiologies or being disease free. In such cases, and particularly prior to the introduction of HeV vaccinations, serology could be used as an additional tool for evaluation. Presently, vaccinated animals under disease investigation may return a positive HeV serology result, specifically in tests which target antibody to HeV G protein, such as the VNT. While tests are being developed which target other structural viral proteins (ACDP Serology in-house HeV DIVA ELISA), our interest was to determine whether the specific identification of IgM antibody to HeV N protein could provide further insights into the likelihood for recent HeV infection. (While we have elected to use N protein in the MAC ELISA, we note that use of the G protein in this MAC format would also be likely to provide significant bias against detection of vaccination derived antibodies in most applications).

Our validation has addressed assay characteristics of analytical and diagnostic sensitivity and specificity. The analytical components provide limited relative comparisons necessary to underpin the more general evaluation of diagnostic performances. The HeV IgM MAC ELISA exhibited satisfactory analytical performance, being unaffected by immune sera known to have reactive IgM antibody to a range of heterologous viruses such as Paramyxoviruses, Flaviviruses, Obiviruses and Alphaviruses. While other Henipaviruses have not been included in this analytical evaluation, as such sera are not readily available, published results suggest that antibody to Nipah virus can be expected to cross-react in this assay (Chua et al., 1999). Analytical sensitivity of the assay was of a comparable level relative to the VNT and sG ELISA which provides some confidence for the prospect of adequate detection.

The DSp evaluation was largely satisfactory with most sera below the optimised threshold (8.99 %P). Seven sera (1.6 %) were marginally above the threshold with values of 14.78 %P, 14.26 %P, 14.05 %P, 10.36 %P, 9.30 %P for the normal horse sera and values of 8.99 %P and 11.67 %P for the vaccinated horse sera. Another advantage of the HeV IgM MAC ELISA would be to use it to resolve the 'vaccination status' of an animal. This was proven by the testing of 163 sera from post HeV vaccinated horses which all gave results that were not significantly different from the test negative groups determined by HeV sG iELISA and HeV SNT. Currently, all serological assays are based on the HeV G protein, which is present in the Hendra virus vaccine, whereas the HeV IgM MAC ELISA ultizes the N protein and as such would not be affected by vaccination. A major advantage of this assay is the ability to determine vaccinated from infected animals which incorporates the DIVA approach. Recent advances in Hendra serology have been limited due to the development of tests involving the same HeV soluble G antigen as in use in the current Equivac HeV vaccine. Due to this consequence, and for external reporting at the Australian Centre of Disease Preparedness, in reports of results for testing for antibody against the G protein using the HeV sG iELISA, a comment is inserted "The currently available serology assays do not distinguish between antibodies due to natural infection and those due to vaccination. Any positive result must be interpreted in the context of the animal's vaccination history" (McNabb et al., 2014). Therefore, in diagnostic laboratories receiving Hendra exclusions with a lack in knowledge of previous vaccination of the infected animal, this HeV IgM ELISA would be very beneficial. Also, in the event of no history of vaccination, other N-based ELISAs are currently being developed (McNabb unpublished data) in some laboratories to help resolve the animal's vaccination status when positive results are detected using the G protein assays.

While there are no obstacles for collection of "negative" samples for DSp evaluation, antibody positive samples for the validation of HeV serology are very limited (Colling et al., 2018). From over 100 recorded equine field cases of HeV, this reference laboratory holds only 21 sera that can usefully be included in the validation process, limiting a more

complete evaluation of assay sensitivity. Furthermore, as there is a national policy in Australia to euthanise horses identified to have had HeV infection (Middleton et al., 2014), there is little prospect to define the dynamics of IgM persistence post infection. Addressing these constraints, our results on serum samples from acute infections (taken to be the period from 7 days after appearance of clinical signs to 28 days after disease onset), suggest, at least early in that period, significant levels of IgM can be detected (average 131 %P). Two horses (A and B) which recovered from HeV and were euthanised at 6 weeks after clinical disease were both negative for detectable IgM at that stage, though testing positive at 3 and 6 days after disease. Experimental infection studies with West Nile virus infections in horses demonstrated WNV specific IgM levels to peak at day 8–12 post infection and then decrease to threshold levels at 27 days post infection (Castillo-Olivares et al., 2011). Other authors have reported that detections of IgM can be more protracted, though generally less than three months (Davidson et al., 2005).

In practice, operational guidelines for use should require that all positive and indeterminate HeV IgM MAC ELISA results be supported by evidence for antibody detection in VNT or indirect ELISA and, if necessary (in HeV vaccinated animals), antibody assay to whole virus or other than G protein. Having evidence in support of specificity, the IgM result is then interpreted as evidence of recent infection, most likely to have occurred within the last month, but possibly outside of that period. If there is opportunity, a second bleed taken 1–2 weeks after the initial bleed might be collected to evaluate for declining IgM antibody levels. Results from animals in which there has been no confirmed HeV infection or history of contact should be treated with caution and subject to repeated investigations.

We consider that HeV IgM MAC ELISA serology might have situational use in series with other molecular and serology tests. Three scenarios in which the assay could support clinical disease investigations include i) as a secondary assay to confirmed positive molecular testing in investigation of late-stage equine HeV; ii) instances of conflicting laboratory results (eg. VNT antibody-positive / PCR-negative results) not otherwise readily apparent as emerging from vaccination history and iii) investigation of suspected HeV infection in which delayed sample collection has impaired effective detection by PCR. As noted above, IgM assays have found some application in investigation of other equine viral diseases. Significantly, the horse is most generally assessed and treated as an individual patient, not as herd assessment from which the sampling multiple animals, effectively providing access to temporally different instances of infection. However, for individual animals, the transience of IgM class antibodies can provide insights into the timing of infection that are otherwise not available. Furthermore, in the investigation of a novel or unusual disease outbreaks, the broadest possible range of tests are frequently applied to obtain as complete a picture as possible. To this end, we also consider it a useful contingent to maintain a validated HeV IgM MAC ELISA in our repertoire of available assays.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

Castillo-Olivares, J., Mansfield, K.L., Phipps, L.P., Johnson, N., Tearle, J., Fooks, A.R., 2011. Antibody response in horses following experimental infection with West Nile Virus lineages 1 and 2. Transbound. Emerg. Dis. 58 (3 June), 206–212. https://doi.org/10.1111/j.1865-1682.2010.01197.x.

Ching, P.K., de los Reyes, V.C., Sucaldito, M.N., Tayag, E., Columna-Vingno, A.B., Malbas, F.F.Jr., Bolo, G.C.Jr., Sejvar, J.J., Eagles, D., Playford, G., Dueger, E., Kaku, Y., Morikawa, S., Kuroda, M., Marsh, G.A., McCullough, S., Foxwell, A.R., 2015. Outbreak of henipavirus infection, Philippines. Emerging Infect. Dis. 21 (2 Febuary), 328–331. https://doi.org/10.3201/eid2102.141433.

Chua, K.B., Goh, K.J., Wong, K.T., Kamarulzaman, A., Tan, P.S., Ksiazek, T.G., Zaki, S.R., Paul, G., Lam, S.K., Tan, C.T., 1999. Fatal encephalitis due to Nipah virus among pig-farmers in Malaysia. Lancet 354 (9186 October), 1257–1259. https://doi.org/10.1016/S0140-6736(99)04299-3, 9.

Colling, A., Lunt, R., Bergfeld, J., McNabb, L., Halpin, K., Juzva, S., Newberry, K., Morrissy, C., Loomes, C., Warner, S., Diallo, I., Kirkland, P., Broder, C.C., Carlile, G., Loh, M.-H., Waugh, C., Wright, L., Watson, J., Eagles, D., Zuelke, K., McCullough, S., Daniels, P., 2018. A network approach for provisional assay recognition of a Hendra virus antibody ELISA: test validation with low sample numbers from infected horses. J. Vet. Diagn. Investig. 30 (3), 362–369. https://doi.org/10.1177/1040638718760102.

Daniels, P., Ksiazek, T., Eaton, B.T., 2001. Laboratory diagnosis of Nipah and Hendra and virus infections. Microbes Infect. 3 (4 April), 289–295. https://doi.org/10.1016/s1286-4579(01)01382-x.

Davidson, A.H., Traub-Dargatz, J.L., Rodeheaver, R.M., Ostlund, E.N., Pedersen, D.D., Moorhead, R.G., Stricklin, J.B., Dewell, R.D., Roach, S.D., Long, R.E., Albers, S.J., Callan, R.J., Salman, M.D., 2005. Immunologic responses to West Nile virus in vaccinated and clinically affected horses. J. Am. Vet. Med. Assoc. 226 (2 January), 240–245. https://doi.org/10.2460/javma.2005.226.240, 15.

Eaton, B.T., Broder, C.C., Middleton, D., Wang, L.F., 2006. Hendra and Nipah viruses: different and dangerous. Nat. Rev. Microbiol. 4 (1), 23–35. https://doi.org/10.1038/nrmicro1323.

Edson, D., Peel, A.J., Huth, L., Mayer, D.G., Vidgen, M.E., McMichael, L., Broos, A., Melville, D., Kristoffersen, J., de Jong, C., McLaughlin, A., Field, H.E., 2019. Time of year, age class and body condition predict Hendra virus infection in Australian black flying foxes (Pteropus alecto). Epidemiol. Infect. 147, 1–10. https://doi.org/10.1017/S0950268819001237 e240.

Field, H.E., 2016. Hendra virus ecology and transmission. Curr. Opin. Virol. 16, 120–125. https://doi.org/10.1016/j.coviro.2016.02.004.

Field, H., De Jong, C., Melville, D., Smith, C., Smith, I., Broos, A., Kung, Y.H., McLaughlin, A., Zeddeman, A., 2011. Hendra virus infection dynamics in Australian fruit bats. PLoS One 6 (12), e28678. https://doi.org/10.1371/journal.pone.0028678.

Juozapaitis, M., Serva, A., Zvirbliene, A., Slibinskas, R., Staniulis, J., Sasnauskas, K., Shiell, B.J., Wang, L.-F., Michalski, W.P., 2007. Generation of henipavirus nucleocapsid proteins in yeast Saccharomyces cervisiae. Virus Res. 124 (1–2), 95–102. https://doi.org/10.1016/j.virusres.2006.10.008.

Kurtz, J.B., Malic, A., 1981. Rubella-specific IgM detected by an antibody capture assay/ELISA technique. J. Clin. Pathol. 34 (12), 1392–1395. https://doi.org/10.1136/jcp.34.12.1392.

Lam, K.H., Ellis, T.M., Williams, D.T., Lunt, R.A., Daniels, P.W., Watkins, K.L., Riggs, C.M., 2005. Japanese encephalitis in a racing thoroughbred gelding in Hong Kong. Vet. Rec. 157 (6), 168–173. https://doi.org/10.1136/vr.157.6.168.

Mahalingam, S., Herrero, L.J., Playford, E.G., Spann, K., Herring, B., Rolph, M.S., Middleton, D., McCall, B., Field, H., Wang, L.-F., 2012. Hendra virus: an emerging paramyxovirus in Australia. Lancet Infect. Dis. 12 (10), 799–807. https://doi.org/10.1016/S1473-3099(12)70158-5.

Manyweathers, J., Field, H., Longnecker, N., Agho, K., Smith, C., Taylor, M., 2017. "Why won't they just vaccinate?" Horse owner risk perception and uptake of the Hendra virus vaccine. BMC Vet. Res. 13 (1), 103. https://doi.org/10.1186/s12917-017-1006-7.

McNabb, L., Barr, J., Crameri, G., Juzva, S., Riddell, S., Colling, A., Boyd, V., Broder, C., Wang, L.-F., Lunt, R., 2014. Henipavirus microsphere immuno-assays for detection of antibodies against Hendra virus. J. Virol. Methods 200, 22–28. https://doi.org/10.1016/j.jviromet.2014.01.010.

Middleton, D., 2014. Hendra virus. The veterinary clinics of North America. Equine Practice 30 (3), 579–589. https://doi.org/10.1016/j.cveq.2014.08.004.

Middleton, D., Pallister, J., Klein, R., Feng, Y.-R., Haining, J., Arkinstall, R., Frazer, L., Huang, J.-A., Edwards, N., Wareing, M., Elhay, M., Hashmi, Z., Bingham, J., Yamada, M., Johnson, D., White, J., Foord, A., Heine, H.G., Marsh, G., Broder, C.C., Wang, L.-F., 2014. Hendra virus vaccine, a one health approach to protecting horse, human, and environmental health. Emerging Infect. Dis. 20 (3), 372–379. https://doi.org/10.3201/eid2003.131159.

Queensland State Government, 2021. Summary of Hendra Virus Incidents in Horses. https://www.business.qld.gov.au/industries/service-industries-professionals/service-industries/veterinary-surgeons/guidelines-hendra/incident-summary.

Williamson, M.M., Hooper, P.T., Selleck, P.W., Gleeson, L.J., Daniels, P.W., Westbury, H.A., Murray, P.K., 1998. Transmission studies of Hendra virus (equine morbillivirus) in fruit bats, horses and cats. Aust. Vet. J. 76 (12), 813–818. https://doi.org/10.1111/j.1751-0813.1998.tb12335.x.

World Organisation for Animal Health (OIE), 2019. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Chapter 3.1.14 Nipah and Hendra Virus Diseases. OIE, Paris. https://www.oie.int/en/what-we-do/standards/codes-and-manuals/terrestrial-manual-online-access/.

Young, P.L., Halpin, K., Selleck, P.W., Field, H., Gravel, J.L., Kelly, M.A., Mackenzie, J.S., 1996. Serologic evidence for the presence in Pteropus bats of a paramyxovirus related to equine morbillivirus. Emerging Infect. Dis. 2 (3), 239–240. https://doi.org/10.3201/eid0203.960315.