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Journal

Journal of General Virology, 99(4)

ISSN

0022-1317

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Publication Date

2018-04-01

DOI

10.1099/jgv.0.001038

JOURNAL OF GENERAL VIROLOGY

RESEARCH ARTICLE
Prow et al., Journal of General Virology
DOI 10.1099/jgv.0.001038

MICROBIOLOGY SOCIETY

New genotypes of Liao ning virus (LNV) in Australia exhibit an insect-specific phenotype

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Abstract

Liao ning virus (LNV) was first isolated in 1996 from mosquitoes in China, and has been shown to replicate in selected mammalian cell lines and to cause lethal haemorrhagic disease in experimentally infected mice. The first detection of LNV in Australia was by deep sequencing of mosquito homogenates. We subsequently isolated LNV from mosquitoes of four genera (*Culex*, *Anopheles*, *Mansonia* and *Aedes*) in New South Wales, Northern Territory, Queensland and Western Australia; the earliest of these Australian isolates were obtained from mosquitoes collected in 1988, predating the first Chinese isolates. Genetic analysis revealed that the Australian LNV isolates formed two new genotypes: one including isolates from eastern and northern Australia, and the second comprising isolates from the south-western corner of the continent. In contrast to findings reported for the Chinese LNV isolates, the Australian LNV isolates did not replicate in vertebrate cells *in vitro* or *in vivo*, or produce signs of disease in wild-type or immunodeficient mice. A panel of human and animal sera collected from regions where the virus was found in high prevalence also showed no evidence of LNV-specific antibodies. Furthermore, high rates of virus detection in progeny reared from infected adult female mosquitoes, coupled with visualization of the virus within the ovarian follicles by immunohistochemistry, suggest that LNV is transmitted transovarially. Thus, despite relatively minor genomic differences between Chinese and Australian LNV strains, the latter display a characteristic insect-specific phenotype.

INTRODUCTION

The genus *Seadornavirus* comprises a newly classified group of viruses in the family *Reoviridae*, subfamily *Sedoreovirinae* [1]. The genus name refers to the 12 segments of dsRNA comprising the viral genome and the location where the original isolates were collected (Southeast Asian dodeca RNA virus). Currently the viruses classified in this group are Banna virus (BAV), Kadipiro virus and, more recently,

Received 30 November 2017; Accepted 19 February 2018
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Keywords: Liao ning virus; seadornavirus; host restriction; mosquito-borne; Australia.
Abbreviations: dsRNA, double-stranded RNA; IFA, immunofluorescence assay; IHC, immunohistochemistry; IRF, interferon regulatory factor; KRBV, Kurumba virus; LNV, Liao ning virus; MAVRIC, monoclonal antibodies to viral RNA intermediates in cells; NDiV, Nam Dinh virus; nm, nanometre; NSW, New South Wales; PaRV, Paramatta River virus; p.i., post-infection; PLC, Parry's Lagoon virus; Qld, Queensland; RRV, Ross River virus; TEM, transmission electron microscopy; TENTC, Tris-EDTA-NaCl-Casein; VP, virus protein.
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Supplementary material is available with the online version of this article.

Liao ning virus (LNV) [1–4], Balaton virus [5] and Mangshi virus [6]. The first isolation of the seadornavirus type species, BAV, occurred in 1987 from the cerebrospinal fluid and sera of human encephalitis patients in Yunnan Province, People's Republic of China [7]. Since then, additional isolates have been collected from mosquitoes, ticks, pigs and cattle in Vietnam, Indonesia and China [8–10]. Each of the viral genome segments encodes a single protein, but their exact functional attribution and location in the virion are still in dispute [3, 6].

Unlike BAV, LNV had so far only been isolated from mosquitoes [1, 4, 8, 9, 11–15]. However, Attoui *et al.* [3] reported that the Chinese isolates of LNV (LNV_{CH}) can replicate in mammalian cell lines, including MRC5 human embryonic lung cells, suggesting that LNV might be able to infect animals and humans. *In vivo* studies also suggested that LNV_{CH} can replicate in mice following primary inoculation and upon reinfection cause fatal haemorrhagic symptoms [3]. This suggested that, like BAV, LNV might be associated with disease in humans and/or animals.

Until recently, LNV had only been found in China. However, in 2014 the virus was detected by deep sequencing of cell cultures inoculated with homogenates of several mosquito pools collected in New South Wales (NSW), Australia [16]. Here we report the detection and isolation of LNV from multiple mosquito species of several genera collected from a number of different geographical regions in Australia. We also describe the phylogenetic relationships between Australian and Chinese isolates and critical phenotypic differences. In contrast to LNV_{CH}, the Australian LNV isolates were incapable of (i) replicating in commonly used vertebrate cell lines and (ii) establishing productive infection in mice. Thus, the Australian LNV isolates appear to display an insect-specific virus phenotype.

RESULTS

Isolation of Liao ning virus from mosquitoes trapped in Sydney in 2007

Using a novel virus discovery system that specifically detects viral dsRNA in inoculated cell cultures by ELISA (MAVRIC [17]), a panel of archival mosquito samples [18] were analysed to identify and isolate novel viruses. Initially, 51 out of 97 (52 %) C6/36 cultures inoculated with *Aedes vigilax* mosquito pools from Homebush Bay in Sydney (collected in 2007) tested positive for dsRNA [19]. Deep sequencing of RNA extracted from one of these cultures revealed RNA sequences that were most closely related to Chinese isolates of LNV, NE9731 and NE9712. However, the mosquito pool also contained another virus, Parramatta River virus (PaRV) [19]. The initial LNV isolate ($LNV_{NSW2007\text{-}B115745}$) was therefore plaque-purified twice to provide LNV stock for further studies. Transmission electron microscopy of this plaque- and gradient-purified virus revealed a non-enveloped virion that was approximately 80 nm in diameter with typical reoviridae morphology (Fig. 1).

Supernatants from the 51 cultures that tested positive for dsRNA after inoculation with mosquito homogenates from Sydney were inoculated onto fresh C6/36 cells and analysed by RT-PCR, using primers designed from LNV genome segments 2 and 10 [3]. All 51 samples were positive for LNV RNA, i.e. LNV was present in 52 % (51/97) of the pools tested in this mosquito population. In a follow-up study of *Ae. vigilax,* collected from a nearby location in 2014, LNV remained at a high prevalence, with 45/60 (75 %) positive pools (Table 1).

Production of polyclonal and monoclonal antibodies to LNV

To enable the rapid identification of LNV in additional mosquito samples and for further characterization of the Australian isolates of this virus, mice were immunized with a gradient-purified, formalin-inactivated virion preparation derived from a plaque-purified culture of $LNV_{NSW2007\text{-}B115745}$. An LNV-specific monoclonal antibody (6E6) was generated by standard hybridoma technology. This mAb reacted specifically with LNV antigen in fixed-cell ELISA, and neutralized virus replication in insect cells, while having no detectable cross-reactivity to a wide range of arboviruses, including other reoviruses [bluetongue virus, Corriparta virus and Parry's Lagoon virus (PLV)], flaviviruses [Murray Valley encephalitis virus, West Nile virus (WNV), PaRV, Palm Creek virus and Bamaga virus], alphaviruses [Ross River virus (RRV), Barmah Forest virus and Sindbis virus] and an Australian bunyavirus (Badu virus [20]). By immunofluorescence assay (IFA), the staining pattern was characteristically granular (Fig. 2a), suggesting reactivity with 'virus factories' [21]. In Western blots of LNV lysate, the 6E6 antibody labelled a protein of 50–55 kDa (Fig. 2b), and mass spectrometry analysis of

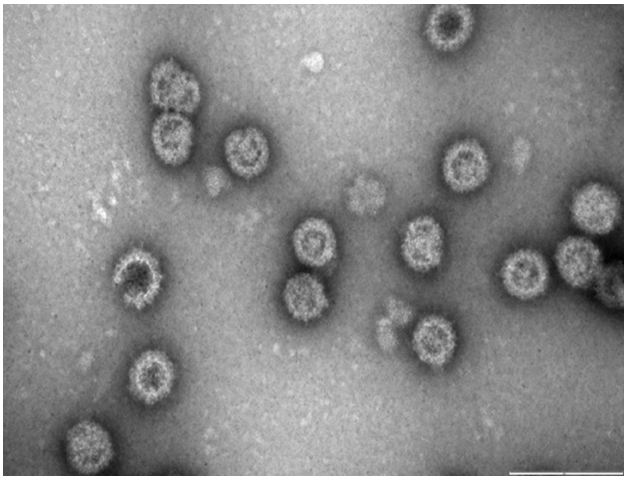

Fig. 1. Electron micrograph of gradient-purified virions from a plaque purified stock of $LNV_{NSW2007\text{-}B115745}$. A nucleocapsid is apparent in some particles, as well as regularly arranged surface capsomers. Surface projections can be distinguished in some particles. Other particles appear to be empty or incompletely assembled. Bar, 200 nm.

Table 1. Australian LNV isolates according to year of mosquito sampling, geographical location and mosquito species

Year	Species	Location*	No. of LNV-positive pools/no. of pools tested†	Co-isolation with‡
1988	*Aedes camptorhynchus*	Peel region, WA	4/6	All with CsV
1988	*Aedes alboannulatus*	Peel region, WA	1/2	NA§
1988	*Aedes alboannulatus*	Leschenault, WA	3/3	All with CsV
1990	*Aedes camptorhynchus*	Leschenault, WA	8/10	NA
1990	*Aedes camptorhynchus*	Peel region, WA	6/8	NA
1990	*Aedes clelandi*	Peel region, WA	1/1	NA
1990	*Aedes ratcliffei*	Peel region, WA	2/2	NA
2000	*Anopheles meraukensis*	Karumba, QLD	2/3	KRBV
2001	*Anopheles meraukensis*	Normanton, QLD	1/3	KRBV
2005	*Culex annulirostris*	Coopers Plains, QLD	1/60	–‖
2005	*Culex annulirostris*	Cairns, QLD	1/106	–‖
2007	*Aedes vigilax*	Homebush Bay, Sydney, NSW	51/97	PaRV
2007	*Culex annulirostris*	Homebush Bay, Sydney, NSW	1/27	–‖
2011	*Aedes vigilax*	Willie Creek, Broome, WA	1/1	–‖
2011	*Aedes vigilax*	Derby, WA	1/1	–‖
2011	*Aedes normanensis*	Fitzroy Crossing, WA	1/1	–‖
2011	*Culex annulirostris*	Geikie Gorge, Fitzroy Crossing, WA	2/2	–‖
2011	*Aedes normanensis*	Geikie Gorge, Fitzroy Crossing, WA	1/1	–‖
2011	*Culex annulirostris*	Kununurra, WA	2/25	One pool with PLV‖
2011	*Culex pullus*	Kununurra, WA	1/2	PLV‖
2011	*Anopheles meraukensis*	Kununurra, WA	1/1	–‖
2011	*Anopheles meraukensis*	Parry's Creek, Wyndham, WA	1/1	–‖
2011	*Mansonia uniformis*	Parry's Creek, Wyndham, WA	1/1	PLV‖
2011	*Aedes vigilax*	Derby, WA	1/1	–‖
2012	*Culex annulirostris*	Brisbane, QLD	1/1	NA
2013	*Aedes notoscriptus*	Ballina, NSW	1/1	NDiV‖
2013	*Aedes vigilax*	Homebush Bay, Sydney, NSW	1/1	NA
2013	*Aedes vigilax*	Bankstown, NSW	1/1	NA
2013	*Culex annulirostris*	Griffith, NSW	2/2	NA
2013	*Culex quinquefasciatus*	Murray, NSW	1/1	NA
2013	*Aedes normanensis*	Bradshaw, NT	1/2	–‖
2013	*Aedes vigilax*	Bradshaw, NT	1/1	–‖
2014	*Aedes camptorhynchus*	Peel region, WA	2/53	CsV, NDiV
2014	*Aedes vigilax*	Banyo, QLD	9/10	PaRV
2014	*Aedes vigilax*	Duck River¶, Sydney, NSW	45/60	PaRV

*WA, Western Australia; QLD, Queensland; NSW, New South Wales; NT, Northern Territory. For locations please refer to the map in Fig. 3.

†Pool sizes varied from 1 mosquito to 172 mosquitoes, with most pools comprising 20–25 mosquitoes. Nine pools of 2–25 *Ae. normanensis* collected in Kununurra and Wyndham, north-western Australia in 2014 were all negative for LNV, as were two pools of ~20 *Ae. aegypti* from Cairns, north Queensland.

‡CsV, Castlerea virus; KRBV, Karumba virus; PaRV, Parramatta River virus; PLV, Parry's Lagoon virus; NDiV, Nam Dinh virus.

§Information not available or no additional testing done.

‖Tested for PLV (specific primers), flavi- and mesonivirus (genus-generic primers) by RT-PCR.

¶Duck River is a short distance (<4 km) from Homebush Bay.

pull-downs using mAb 6E6 (Fig. 2c and Table S3) suggested that 6E6 bound to a protein in a complex comprising the core proteins, VP2, VP4, VP8 and VP9, and the outer-coat/spike protein VP10, based on the assignment of putative protein functions by Attoui *et al.* [3] and Wang *et al.* [6]. This interpretation is consistent with the strong neutralizing activity of the mAb 6E6.

Geographical distribution of LNV in Australian mosquitoes

Following the initial isolation, we subsequently tested a larger panel of mosquito samples, comprising mosquito species from four different genera (*Aedes*, *Culex*, *Mansonia* and *Anopheles*), collected from several geographical regions of Australia (Fig. 3) over a period of 26 years (Table 1)

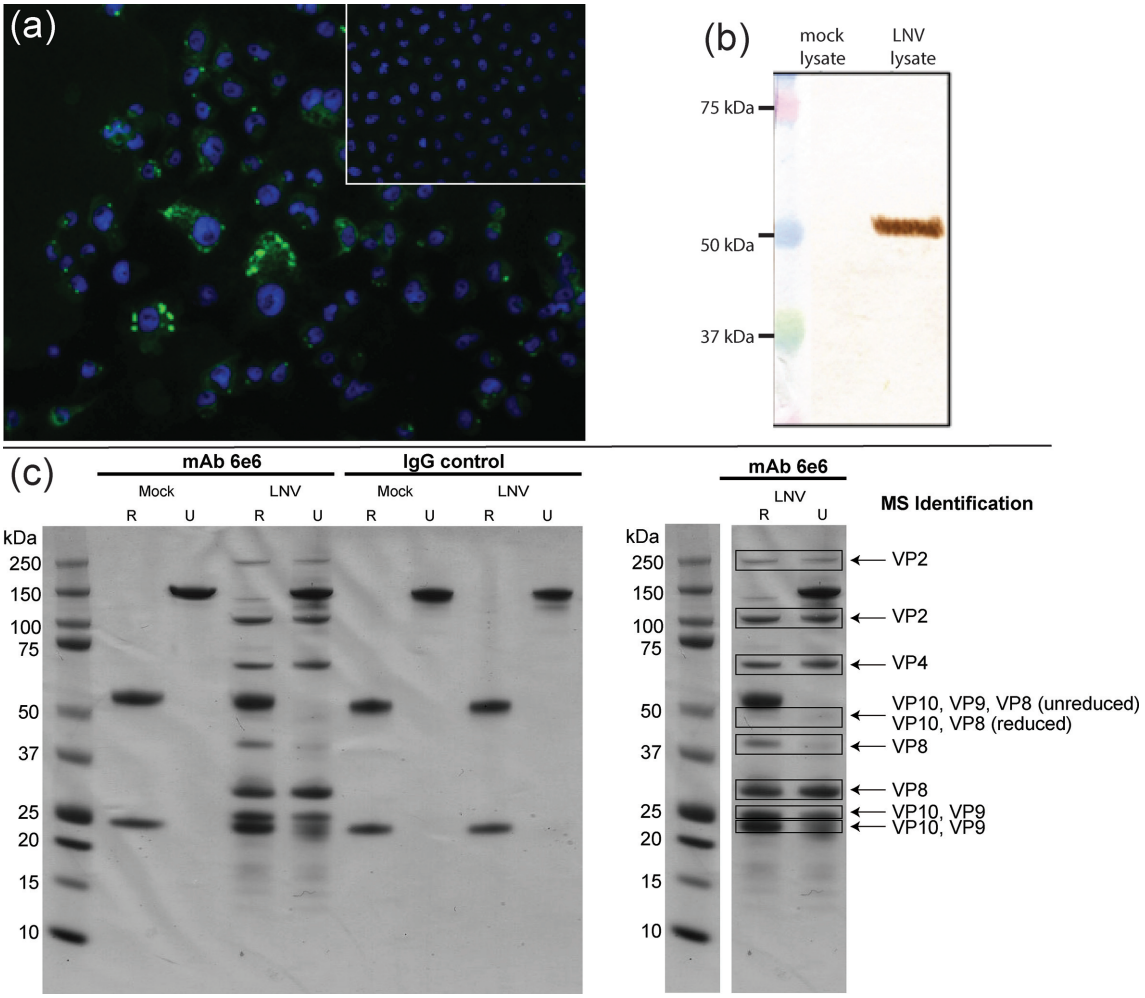

Fig. 2. (a). Immunofluorescence labelling of LNV-infected C6/36 cells using the LNV-specific mAb 6E6. The most intense labelling is typically punctate, indicating virus factories, with fainter, more diffuse, labelling elsewhere in the cytoplasm. Insert: mock-infected C6/36 cells. (b) Western blot of NP40 lysates of mock- and LNV-infected C6/36 cells using mAb 6E6, HRP-conjugated goat-anti-mouse IgG and DAB substrate. (c). Coomassie-stained gel of LNV proteins pulled down using the LNV-specific mAb 6E6. Proteins subsequently identified using mass-spectroscopy are indicated in the right panel and comprise spike (VP10) and core (VP2, 4, 8 and 9) proteins, presumably pulled down as a complex before being separated by SDS-PAGE. R, reduced; U, unreduced.

[22–24]. Screening of fixed-cell monolayers from inoculated C6/36 cultures by ELISA, using mAb 6E6, revealed a large number of additional isolates from different regions of Australia. Many of the mosquito pools that yielded LNV isolates were also positive for other mosquito-borne viruses not previously detected by virus isolation (Table 1). These included the insect-specific flaviviruses PaRV [19] and Karumba virus (KRBV [25]), a novel *Negevirus*, Castlerea virus (CsV [26]), the orbivirus PLV [27] and the mesonivirus (*Nidovirales*) Nam Dinh virus (NDiV [28, 29]).

LNV replication in insect and vertebrate cell lines

The Chinese LNV strains were reported to replicate and cause cytopathic effect (CPE) in some mammalian cell lines, including BHK-21 and MRC5 [3]. To determine whether Australian isolates of LNV could replicate in both mosquito and vertebrate cells, a range of cell lines were tested for permissiveness and productive replication of a plaque-purified LNV isolate from Sydney ($LNV_{NSW2007\text{-}B115745}$). At a multiplicity of infection (m.o.i.) of 0.1, this virus replicated in all tested mosquito cell lines (derived from *Aedes*, *Culex* and *Anopheles* mosquitoes), with the titres peaking between 3 and 7 days post-infection (p.i.). This LNV isolate grew to titres of 10^6–10^7 $TCID_{50}$ ml^{-1} in all mosquito cell lines, except HSU cells (*Culex quinquefasciatus*), which produced titres of 10^3 $TCID_{50}$ ml^{-1} (Fig. 4a). In contrast, there was no detectable replication in a range of vertebrate cell lines (human, monkey, mouse, hamster, rabbit, opossum and dog) or in murine peritoneal macrophages during a similar incubation period, whether tested by $TCID_{50}$, quantitative RT-PCR (qRT-PCR) or IFA, at an m.o.i. 0.1 or 5 (Fig. 4b, c).

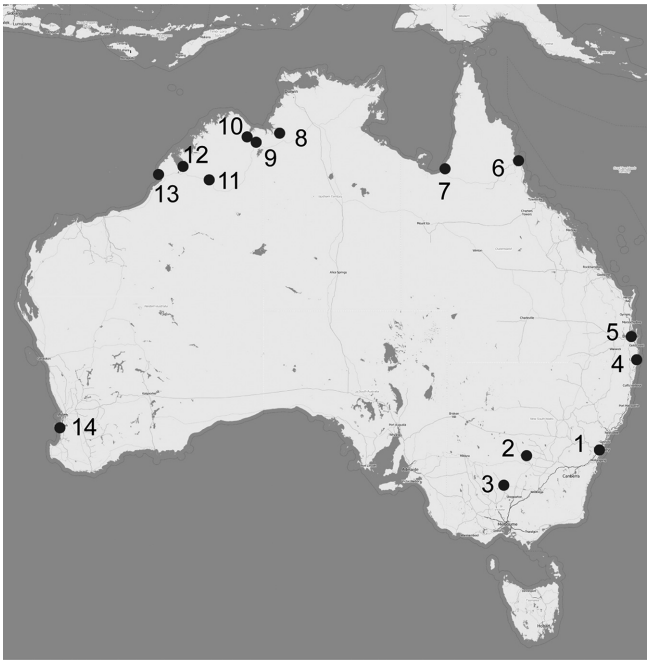

Fig. 3. Map of Australia showing the locations where LNV-infected mosquitoes were collected: (1) Sydney (including Homebush Bay, Duck River and Bankstown); (2) Griffith, NSW; (3) Murray, NSW; (4) Ballina, NSW; (5) Brisbane (including Coopers Plains), Qld; (6) Cairns, Qld.; (7) Karumba, Qld.; (8) Bradshaw, NT; (9) Kununurra, WA; (10) Wyndham, WA; (11) Fitzroy Crossing, WA; (12) Derby, WA; (13) Willie Creek, Broome, WA; (14) Peel Region, WA.

Phylogenetic analysis of Australian LNV isolates

Analysis of the nucleotide sequences of Australian LNV isolates obtained from mosquitoes collected between 2005 and 2013 from NSW, QLD and the Northern Territory showed that they shared a high degree of nucleotide (96–99 % identity) and amino acid (98–100 % identity) sequence conservation across the eight genome segments analysed (Figs 5 and S1, available in the online version of this article). Phylogenetic trees derived from these sequences grouped these isolates into a single clade that also included the LNV sequences previously detected in mosquito pools from NSW (NSW consensus), as reported by Coffey *et al.* [16] (cluster 1 in Figs 5 and S1) and a partial sequence obtained from segment 10 of an LNV isolate from north-western Australia in 2011 (data not shown). However, when two LNV isolates collected 24 years apart from south-western Australia ($LNV_{WA1990\text{-}SW10194}$ and $LNV_{WA2014\text{-}DC60042}$), were included in this analysis, their genome sequences were shown to be very similar to one another (99 % nucleotide and 100 % amino acid sequence identity), but significantly divergent from all other Australian isolates and from previously reported LNV_{CH} strains (cluster 2 in Figs 5 and S1; Table S1). These results indicate that the Australian LNV isolates form two separate genotypes that are distinct from the two Chinese genotypes previously identified by Attoui *et al* [3]. The NE9712 Chinese prototype virus appears to be the most divergent LNV strain, with as little as 79–81 % aa identity to all other strains, based on the ORF of segment 10 (core or outer-coat/cell-attachment protein), and a maximum of 97 % identity in the ORF of segment 2 (T2 layer of core/subcore).

To assess whether there was evidence of reassortment between these different genotypes of LNV, the phylogenetic trees derived from each of the eight genome segments were compared. No significant difference in the topology of these trees was observed, suggesting no clear evidence of reassortment between the four genotypes (Figs 5 and S1).

Assessment of natural exposure of humans and animals to LNV infection in Australia

To determine whether humans and/or animals are naturally infected with LNV in Australia, 408 human plasma samples, collected from blood donors from NSW and QLD, and 700 rabbit sera, collected from different regions of Australia [30], were screened for the presence of LNV-specific antibodies by ELISA and LNV neutralization tests. While 10–15 % of rabbits and 1.4 % of human plasma samples were reactive in a 'fixed-cell' ELISA, none were positive when tested in an LNV microneutralization assay. The assessment of additional human plasma samples, collected in 2015 from blood donors who donated in areas with significant RRV transmission during one peak season [31], for the presence of LNV by isolation of infectious virus on C6/36 cell cultures (n=762) and detection of viral RNA by qRT-PCR (n=806) also identified no positive samples.

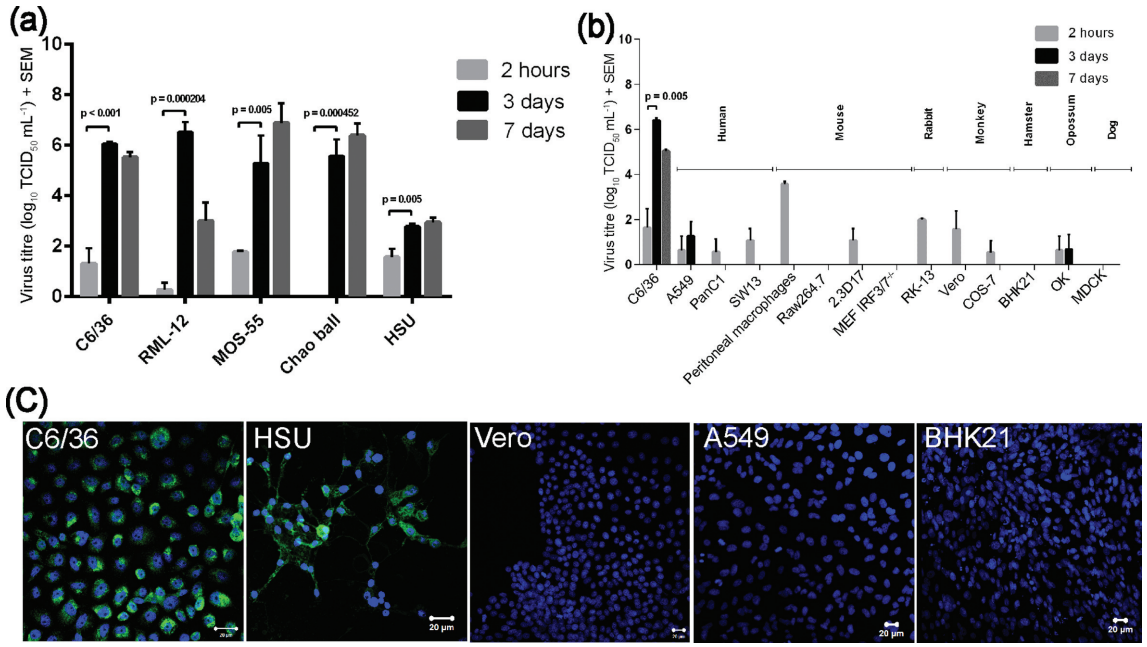

Fig. 4. Replication of $LNV_{NSW2007\text{-}B115745}$ in mosquito and vertebrate cell lines. Viral titres were quantified from replication kinetics experiments at 2 h p.i. (light grey bar), day 3 p.i. (black bar) and day 7 p.i. (dark grey bar) at an m.o.i. of 0.1 (n=6 for all mosquito cell lines; $n \geq 3$ for vertebrate cell lines; C6/36 titres were repeated across numerous independent experiments as a positive control). (a) The mosquito cell lines were derived from *Aedes albopictus* (C6/36 and RML12), *Anopheles gambiae* (Mos55), *Culex tarsalis* (Chao Ball) and *Culex quinquefasciatus* (HSU). (b) The vertebrate cell lines were derived from human (A549, PanC1 and SW13), mouse (peritoneal macrophages, Raw264.7 and MEF IRF3/7$^{-/-}$), rabbit (RK-13), African green monkey (*Chlorocebus aethiops*) (Vero and COS-7), hamster (BHK21), opossum (*Didelphis* sp.) (OK) and dog (MDCK). (c) Confocal micrographs of immunofluorescence-labelled cell cultures, using mAb 6E6 and FITC-conjugated rabbit-anti-mouse IgG, to demonstrate LNV replication in insect (C6/36, HSU) and vertebrate cells (Vero, A549, BHK21) 3 days p.i. at an m.o.i. of 0.1. No replication is evident in the vertebrate cells (no green fluorescing signal). The nuclei (blue) were stained with Hoechst reagent.

We also tested 52 serum or plasma samples from horses from south-eastern Queensland and northern NSW by LNV microneutralization assay. Despite high seroprevalence for other arboviruses, notably RRV [32] and a range of flaviviruses [33], no LNV-specific reactivity was detected in any of the samples. Thus, despite the frequent co-detection of LNV with other arboviruses, including RRV, in mosquito samples, no evidence for LNV infection was apparent in humans, rabbits or horses.

High frequency of vertical transmission of LNV between mosquito generations

The lack of LNV replication in various vertebrate cell lines suggested that Australian isolates of LNV may be insect-specific, a conclusion corroborated by the serological studies that failed to provide evidence of vertebrate infection. We then assessed whether LNV utilized vertical transmission, whereby the virus is passed from infected female mosquitoes to their progeny (see the Supplementary Material for details of the methodology). Approximately 80 % of male and female progeny, reared from eggs of wild-caught female *Ae. vigilax* from Sydney, a population with known high prevalence of LNV infection (see above and Table 1), were positive for LNV infection, as determined by virus isolation from individually homogenized mosquitoes inoculated onto C6/36 cells (Table 2). This high proportion of vertical transmission was similar to the prevalence of LNV observed in the wild-caught parental females. Interestingly, we also detected LNV RNA in the water of pans used to rear the progeny mosquitoes (data not shown), so another route of LNV infection might involve environmental water-borne infection of larvae.

To help identify the possible mechanism of vertical transmission of LNV, immunohistochemistry (IHC) was used to assess the tissue distribution of the virus in male and female *Ae. vigilax* naturally infected with LNV. Of importance for potential transmission mechanisms, viral antigen was detected in the primary and developing follicles within the ovaries of approximately 20 % of female mosquitoes examined (Fig. 6). The IHC results also revealed viral antigen in the retinula and pigment cells within the ommatidia of the compound eyes of ~50–60 % of mosquitoes examined (Fig. 6a). Notably, the labelling tended to be a diffuse, faint signal in the cytoplasm, as well as intense granular signals, similar to the patterns seen in the insect cell lines by IFA (compare to Fig. 2).

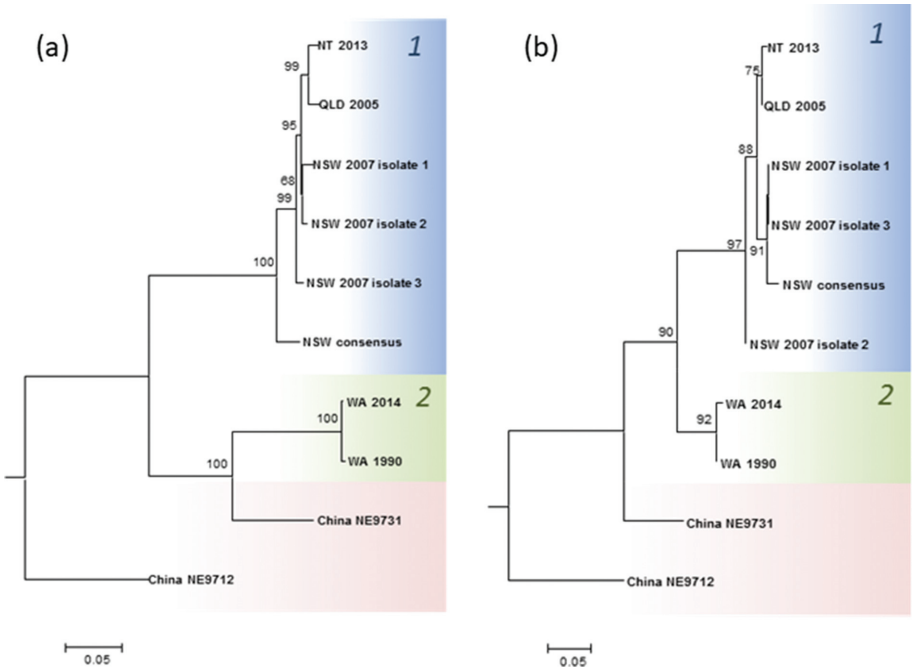

Fig. 5. Phylogenetic trees of LNV segments 1 and 2 (a and b, respectively) constructed from aligned nucleotide sequences. Two distinct lineages are shown: one from New South Wales (NSW), Queensland (QLD) and the Northern Territory (NT) (cluster 1), and the other from Western Australia (WA) (cluster 2). Branch support values (Shimodaira–Hasegawa test) are shown as a percentage next to the relevant nodes. The sequences of the Australian isolates shown are: NT 2013 (MG725838, MG725839), QLD 2005 (MG725843, MG725844), NSW 2007 isolates 1–3 (isolate 1, $LNV_{NSW2007\text{-}B115745}$: MG725848, MG725849; isolate 2, $LNV_{NSW2007\text{-}5724}$: MG725033, MG725034; isolate 3, $LNV_{NSW2007\text{-}5798}$: MG725045, MG725046), WA 2014 (isolate DC60042: MG725860, MG725861) and WA 1990 (isolate SW10194: MG725057, MG725058). A consensus sequence obtained from sequencing of the mosquito sample homogenates collected from NSW over the period 1995–2005 was designated 'NSW consensus' (MG557987, MG557988, MG557989) [16]). Two Chinese isolates are also shown: NE9712 (AY701339 and AY701340) and NE 9731 (AY317099 and AY317100).

Experimental infection in mice

Previously, the Chinese isolates of LNV were reported to cause a haemorrhagic syndrome in immunocompetent mice following reinfection 12 days after a primary infection [3]. Using young adult C57BL/6 mice (n=10), this study was replicated with the plaque-purified $LNV_{NSW2007\text{-}B115745}$. Neither clinical signs nor viraemia were detected in any of the mice after either primary or secondary infection, regardless of the challenge dose. Furthermore, the mice did not sero-convert, indicating that the virus had not replicated sufficiently to elicit an antibody response. The experiment was repeated using (i) interferon regulatory factor-3 and -7-deficient mice ($IRF3/7^{-/-}$; n=9) and (ii) B, T and NK cell-deficient $Rag2^{-/-}$ mice (n=11). These two mouse strains have previously been shown to support efficient arbovirus replication [34], but neither exhibited clinical signs or viraemia after infection with the Australian LNV strain. Thus, in contrast to the Chinese LNV strains, Australian LNV strains appear to be unable to replicate in mice.

DISCUSSION

Previous studies on two Chinese isolates of LNV reported that these viruses replicated efficiently in a range of vertebrate cells and produced a fatal haemorrhagic disease in mice following sequential inoculations with virus [3]. Thus, the initial detection of novel LNV strains in cell cultures

Table 2. Isolation of LNV from laboratory-reared progeny of wild-caught *Ae vigilax*

Collection date	Mosquito species	Location of collection of female mosquitoes	LNV detected in wild-caught female mosquitoes	LNV detected in progeny mosquitoes	LNV detected in progeny Female	LNV detected in progeny Male
2014	*Ae. vigilax*	Duck River (Sydney, NSW)	12/15* (80 %)	124/157 (79 %)	71/89 (80 %)	53/68 (77 %)

*Mosquitoes tested in pools of 10.

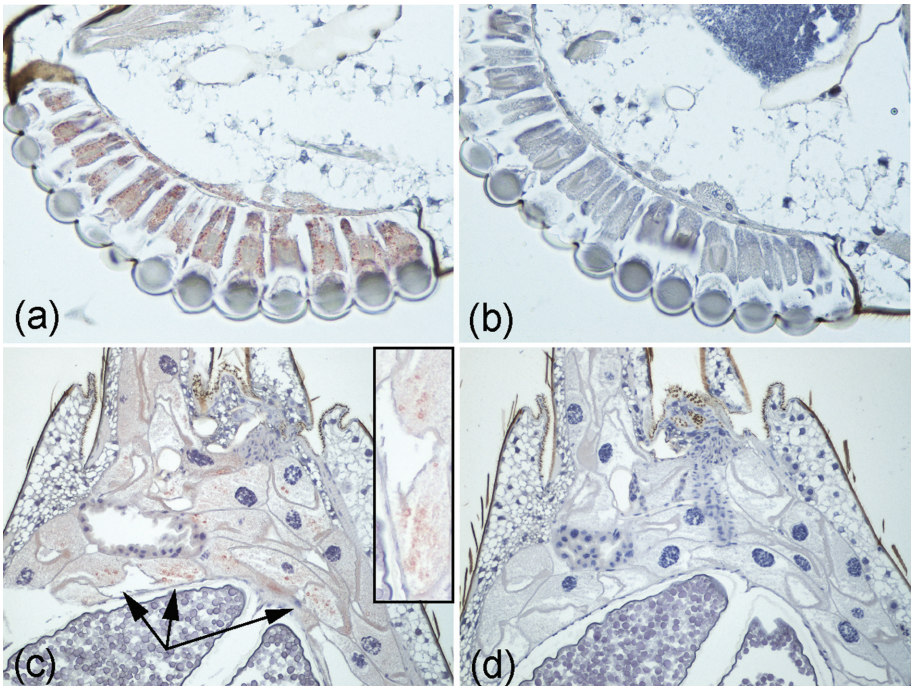

Fig. 6. Representative demonstration of LNV antigen by immunohistochemistry in the composite eye (a) and in the primary follicles [arrows and insert in (c)] of a naturally infected *Ae. vigilax* mosquito using the mAb 6E6 (punctate red signal in infected cells). (b, d) isotype controls. Counterstained with dilute haematoxylin.

inoculated with homogenized mosquito samples collected from Australia [16; this report] prompted an assessment of their prevalence and geographical distribution, their genetic relationship to the Chinese viruses and their ability to infect and/or cause disease in mammals.

While the prototype Australian isolate ($LNV_{NSW2007\text{-}B115745}$) replicated efficiently in five mosquito cell lines, the virus failed to show evidence of replication in a range of vertebrate cell lines derived from multiple different species. In addition, no virus replication or disease was evident in mice inoculated with plaque-purified LNV using the same protocol reported for the Chinese isolates [3]. Furthermore, our inability to demonstrate seroconversion to LNV in a large panel of human and animal sera, many of which contained antibodies to other mosquito-transmitted viruses, including the WNV Kunjin strain [30, 35] and RRV [32], supports a lack of LNV transmission to vertebrates. Together, these results strongly support the conclusion that Australian strains of LNV exhibit an insect-specific phenotype.

This dramatic contrast in host range between Australian and Chinese LNV strains is somewhat surprising considering the close genetic relationship determined from whole and partial genome sequencing data. Australian isolates of LNV showed between 89 and 98% amino acid sequence homology with the NE 9731 Chinese strain across the ORFs of the eight genome segments compared here (Table S4). The phylogenetic analysis of Australian LNV isolates from a range of mosquito species collected between 1990 and 2014 from different geographical locations also revealed that they clustered into two different genotypes. Most clustered with the 2007 prototype isolate ($LNV_{NSW2007\text{-}B115745}$) and the initial sequences published by Coffey *et al.* [16], showing >95 % nucleotide and 95–100 % aa sequence identity across the eight segments analysed. However, the two isolates from the south-western corner of the continent ($LNV_{WA2014\text{-}DC60042}$ and $LNV_{WA1990\text{-}SW10194}$) grouped separately and appear to form a second Australian genotype that is equidistant between the main Australian LNV cluster (92–97 % aa identity) and the NE 9731 Chinese strain (92–98 % aa identity). It is worth noting here that $LNV_{WA2014\text{-}DC60042}$ also failed to infect and replicate in vertebrate cells (data not shown), indicating that the second Australian genotype shares the insect-specific phenotype with other Australian isolates.

Our demonstration that $LNV_{NSW2007\text{-}B115745}$ fails to replicate in vertebrate cells, and the lack of evidence for natural exposure to LNV infection in humans and animals in Australia, suggest that LNV is not transmitted horizontally between mosquitoes and vertebrate hosts. This suggests that Australian strains of LNV rely on vertical transmission to maintain high prevalence in mosquito populations. This is consistent with the very high prevalence of LNV in a population of *Ae. vigilax* in Sydney and was confirmed by the detection of the virus in the progeny of these mosquitoes reared in the laboratory. The IHC analyses of infected

female mosquitoes, demonstrating viral antigen within the developing ovarian follicles, further supported this evidence that the virus is transmitted vertically. This tissue tropism within the ovaries, coupled with the high filial infection rate, indicates that vertical transmission is occurring transovarially, as has been observed for members of the genus *Orthobunyavirus* [36, 37].

Our detection of LNV RNA in the water of rearing pans that yielded LNV-infected progeny mosquitoes also suggests that larvae could potentially be infected via a water-borne route. This theory is supported by the stability of the naked LNV virion, which, like other reoviruses, can withstand a range of environmental conditions. Furthermore, the larvae of *Ae. vigilax* and some other species, shown to carry LNV in Australia, can be found in extraordinarily high density in some larval habitats, providing the ideal environment for water-borne transmission, a phenomenon that has previously been observed for seadornaviruses [5, 38]. This may also extend to the larvae of other mosquito species sharing the same habitat, either directly from contaminated water or via predation of co-habiting infected larvae. On the other hand, not all the species found to be positive tolerate brackish water and some only inhabit natural or domestic containers (e.g. some *Culex* spp). Thus, additional modes of transmission, such as shared feeding sources (nectar etc.), should be considered in light of the detection of LNV in a range of Australian mosquito species in the absence of intermediate vertebrate hosts. Alternatively, considering the stability of the infectious LNV particle, cross-contamination of pools via infected legs or wings during sorting of mosquito species should also be considered. Thus, further laboratory-based experiments are required to determine the mode or modes of transmission of LNV.

Our previous studies on insect-specific flaviviruses revealed that these viruses localize exclusively to the epithelial cells of the mosquito midgut, with no evidence of dissemination to the salivary glands and other organs [25, 39; McLean and Bielefeldt-Ohmann, unpublished data]. When we examined the tissue localization of LNV in naturally infected *Ae. vigilax* mosquitoes by IHC, we observed replication of the virus restricted to the compound eyes, ganglia of the head, and reproductive organs. While the implications of virus replication in the ovaries have been discussed, the replication of LNV in the eyes is of particular interest, as this has been observed in mosquitoes infected with WNV [39] and dengue virus type 2 [40; our unpublished data], and in *Culicoides* infected with bluetongue virus [41]. Whether infection of the eyes causes behavioural changes in mosquitoes, similar to what was observed in bluetongue virus-infected *Culicoides* [41], remains to be assessed, but it is an intriguing possibility.

The development of an LNV-specific mAb (6E6) in this study also provides a valuable research tool to further investigate the transmission dynamics and host restriction factors of LNV. Because of its strong neutralizing activity, it has also proven useful for eliminating LNV from co-infected samples where other insect-specific viruses were the main objects of interest (McLean and Bielefeldt-Ohmann, unpublished data).

The reasons for the striking difference in phenotype between the Australian LNV isolates and that reported for the Chinese LNV strains by Attoui *et al.* [3] remain to be identified. Considering the relatively high frequency with which LNV is detected in co-infected mosquito samples, one possibility is that, despite plaque purification, the Chinese stocks contained an unknown agent responsible for the disease syndrome observed in inoculated mice and the cytopathic effects observed in vertebrate cells. Without assessing the presence of viral antigen in inoculated vertebrate cells (as was the case in the present study using mAb 6E6), the detection of LNV viral RNA in inoculated vertebrate cell lines may be simply due to the persistence of viral dsRNA from the residual inoculum. In our own experiments, we showed minimal loss of infectivity of the LNV inoculum incubated on vertebrate cells for 7 days at 37 °C in the absence of replication. The stability of the naked LNV virion likely explains the persistence of viral RNA in cultures without productive infection. Alternatively, sequence differences between Chinese and Australian LNV strains are associated with variable host restriction. Indeed, we recently reported that two closely related mosquito-borne reoviruses, the orbiviruses Corriparta virus and PLV, show a similar difference in host range (the former infects vertebrates, while the latter is insect-specific) despite sharing 90 % amino acid homology based on the ORFs of their genome segments [27].

To test these hypotheses, future studies should examine thoroughly characterized stocks of LNV isolates, representing all four genotypes from Australia and China, in vertebrate infection studies. Deep sequencing of plaque-purified viral stocks should be undertaken to exclude the presence of other infectious agents and replication in inoculated cells should be confirmed by the presence of viral antigen using an LNV-specific antibody (e.g. mAb 6E6).

METHODS

Cell lines and maintenance conditions

The mosquito cell lines C6/36 and RML-12 (*Aedes albopictus*) were maintained in Rosswell Park Memorial Institute (RPMI) medium with 5 % fetal bovine serum (FBS). HSU (*Culex quinquefasciatus*) and Chao ball (*Culex tarsalis*) cells were maintained in Leibovitz-15 medium (L-15) (Thermo Fisher) supplemented with 10 % tryptose phosphate broth (Sigma-Aldrich, St Louis, Missouri, USA) [42, 43] and 15 % FBS. MOS-55 (*Anopheles gambiae*) cells were maintained with 10 % FBS Schneider's *drosophila* medium (Thermo Fisher) [44]. The mammalian vertebrate animal cell lines Vero (African green monkey), RAW264.7 (murine macrophage), MDCK (canine kidney), COS-7 (African green monkey) and MEF IRF3/7$^{-/-}$ (murine embryonic fibroblast defect in interferon signalling pathways) were cultured in RPMI with 5–10 % FBS. Additional vertebrate cell lines, including BHK-21 (baby hamster kidney), RK-13 (rabbit

kidney), 2.3D17 (murine neuronal), DF-1 (chicken fibroblast) and the human cell lines A549 (lung carcinoma), PANC1 (pancreatic carcinoma) and SW13 (adrenal gland carcinoma), were propagated in Dulbecco's modified Eagle's medium (DMEM) (Thermo Fisher) with 5–10 % FBS. OK (*Didelphis marsupialis virginiana*) cells were maintained in 10 % FBS minimum essential medium (Thermo Fisher) with 10 mM HEPES. The mosquito and vertebrate cell lines were cultured at 28 and 37 °C, respectively, with 5 % CO_2. All culture media were supplemented with 50 U ml^{-1} penicillin, 50 µg ml^{-1} streptomycin and 2 mmol l^{-1} L-glutamine, and all cell lines tested negative for mycoplasma. Peritoneal macrophages were obtained by lavage of C57BL/6 mice (QIMRB animal ethics approval A1503-692M) and immediately seeded into flasks in RPMI with 10 % FBS.

Detection and isolation of virus from mosquito pools

Adult mosquitoes were collected using CO_2-baited light traps from a number of locations throughout Australia (Fig. 3). Screening of mosquitoes for RNA viruses was performed as previously described [17, 27]. Briefly, filtered mosquito homogenates were inoculated onto monolayers of C6/36 cells in 96-well microplates and incubated at 28 °C for 5–7 days. Cells were monitored for CPE. At day 7 p.i. culture supernatant (200 µl) was collected and stored at −80 °C for further analysis and cells were fixed using 20 % acetone in PBS with 0.02 % bovine serum albumen. ELISA was performed on the fixed cells using a cocktail of the dsRNA-specific mAbs 3G1 and 2G4 (named MAVRIC) [17]. Supernatants from wells testing positive by the MAVRIC ELISA were further analysed by RT-PCR for flavi-, alpha-, mesoni- and reovirus genomic material as described [19, 27, 45]. The PCR products were sequenced by Sanger sequencing by the Australian Genome Research Facility (AGRF, Brisbane, Australia) and analysed via BLAST to published sequences in GenBank. Supernatants collected from C6/36-infected cells were used to generate LNV stocks.

Plaque assay and plaque purification

Sub-confluent C6/36 cells in 24-well plates were infected with a series of 10-fold dilutions in 2 % FBS/RPMI and incubated at 28 °C for 1 h with gentle rocking every 20 min. After 1 h, the virus inoculum was removed and cells were overlaid with 0.75 % low-melting-point agarose in 2 % FBS/RPMI and incubated at 28 °C for 3 days. The monolayers were fixed with 10 % formaldehyde and stained with 0.2 % crystal violet. For the plaque purification of LNV, the procedure above was followed, with the exception that the monolayers were not fixed but stained with 0.03 % neutral red solution (Sigma-Aldrich) for visualization of plaques. Single, clearly separated, plaques were picked and resuspended individually in 1 ml of PBS and subjected to another round of plaque purification. Virus stocks were derived from plaque-purified supernatants after a second round of purification. RNA from virus stocks were extracted and RT-PCR was performed using LNV-specific primers (see details below) to confirm the identity of the virus stocks.

Generation of virus stocks

Virus stocks were generated by infecting C6/36 monolayers with virus at an m.o.i. of 0.1. After incubation at 28 °C for 2 h, the inoculum was removed and replaced with fresh growth media (2 % FBS/RPMI). Supernatant was harvested at 3–5 days p.i. and centrifuged at 3000 r.p.m., 4 °C for 10 min. Clarified supernatant was supplemented with FBS to a final concentration of 10 % and aliquots were stored at −80 °C. The virus stock titres were determined by the 50 % tissue culture infectious dose ($TCID_{50}$) assay, as previously described [33], with a few modifications. Briefly, C6/36 were infected with 10-fold dilutions from 10^{-1} to 10^{-8} in 2 % FBS/RPMI and incubated at 28 °C for 3 days. The supernatant was then removed and the plates fixed with ice-cold 4 % formaldehyde in PBS, with 0.1 % triton X-100 for 10 min. The plates were air-dried, followed by fixed-cell ELISA using either the mAbs 3G1 and 2G4 or the LNV-specific antibody, 6E6 (see below), to determine the number of wells containing LNV-infected cells. The titres ($log_{10}TCID_{50}$ mL^{-1}) were calculated based on the method by Reed and Muench [46].

Transmission electron microscopy (TEM)

LNV particles propagated in C6/36 cells were harvested at 3 days p.i. Spent medium was clarified at 3000 r.p.m. for 15 min at 4 °C before the supernatant was added to 40 % PEG8000 solution and left to stir overnight at 4 °C before purification through a potassium tartrate gradient as previously described [19]. Purified virions were harvested and aliquots were stored at 4 °C for immediate TEM analysis, and at −80 °C for the production of mAbs and mouse antiserum (see below), respectively. Prior to TEM analysis and mAb production, purified LNV particles were inactivated in a final concentration of 0.1 % formaldehyde. Purified virions were prepared for TEM on a formvar-coated copper grid and negatively stained with 1 % uranyl acetate. All images were generated on a JEOL1010 transmission electron microscope.

Development and characterization of LNV-specific monoclonal antibody

BALB/c mice were immunized by two subcutaneous inoculations, 14 days apart, of plaque-purified, formalin-inactivated LNV virions. Mouse spleens were harvested for hybridoma production by the fusion of spleen B cells with MRX63 myeloma cells as previously described [47] 4 days after a final intravenous injection of virus and 6 weeks after the booster vaccination. Hybridomas secreting LNV-reactive antibodies were identified by fixed cell ELISA as previously described [47, 48]. The resulting mAb, 6E6, was characterized with respect to specificity by fixed-cell ELISA against a panel of arboviruses, including flaviviruses (MVEV, WNV, PaRV, PCV and Bamaga virus), alphaviruses (RRV, Barmah Forest virus and Sindbis virus), orbiviruses (PLV, Bluetongue and Corriparta virus) and a bunyavirus (Badu virus). The 6E6 mAb was applied at doubling dilutions from 1 : 10 to 1 : 1280. The dsRNA-binding mAb 3G1 [17] was used as a positive control for each virus.

Western blot analysis with 6E6 mAb, diluted 1 : 20 in TENTC (50 mM Tris, 1 mM EDTA, 0.15 M NaCl, 0.2 % casein, 0.05 % Tween 20, pH 8.0) blocking buffer, and murine LNV-specific immune serum, diluted 1 : 50 in TENTC blocking buffer, was conducted on LNV-infected and mock-infected C6/36 cell lysates using the protocol outlined in [47].

To assess the usefulness of mAb 6E6 for the localization of intracellular LNV_{AU}, an immunofluorescence assay (IFA) was performed on LNV-infected cells seeded on sterile glass coverslips in 24-well plates [17, 27]. Coverslips were mounted onto glass microscope slides with ProLong Gold anti-fade (Thermo-Fisher) and cells were visualized using confocal microscopy (Zeiss 780-NLO).

For the protein pull-down and mass spectrometry LNV- and mock-infected lysates were prepared using 1 % NP-40 lysis buffer (1 % NP-40, 50 mM Tris-Cl, 50 mM NaCl, 2 mM EDTA). Protein-G magnetic beads (Pierce) were coated with 6E6 hybridoma supernatant (or isotype control 4G2; pan-flavivirus E protein) for 1 hour at room temperature. Antibody-coated beads were washed once with NP-40 lysis buffer before the addition of mock- or LNV-infected cell lysate for 1 hour with mixing at room temperature. The beads were washed three times with PBST and the immunoprecipitated complexes were eluted by incubation in 1M glycine (pH 2.7) for 8 min at room temperature with mixing before neutralization with 1M Tris, pH 9.

The eluted fractions were assessed by SDS-PAGE analysis and bands of interest were excised from Coomassie-stained gels and destained in 50 % acetonitrile/50 mM ammonium acetate solution overnight at 37 °C. Gel slices were then dried and digested overnight with 0.5 µg trypsin in 50 mM ammonium acetate with 10 mM dithiothreitol. Digested peptides were desalted and analysed using liquid chromatography-mass spectrometry (LC-MS)/MS as previously described [49]. Proteins were identified using ProteinPilot software.

Virus genome sequencing

The following LNV_{AU} virus isolates were chosen for sequence analysis, taking into consideration geographical distribution across Australia (Figs 2, 3), mosquito collection date and mosquito genus, ensuring that virus isolates from different genera were all represented (Table 1). The final list included isolate SW10194 (LNV, WA, 1990), 5724 (LNV, NSW 2007), 150894 (LNV, QLD 2005), 248 (LNV, NT 2013) and DC60042 (LNV, WA 2014). The segments of interest were: segment 1 encoding the RNA-dependent RNA polymerase, which exhibits the most diversity between viruses and provides relevant phylogenetic analyses [1, 2, 50]; segment 2 and segment 9, both of which encode for core proteins that may provide distinction; segment 8, which encodes the outer layer of the core and is the putative binding partner to mAb 6E6; segment 10, which encodes for the cell attachment protein.

RNA from virus isolates was extracted from culture media using the QIAamp Viral RNA Extraction kit (Qiagen) without carrier RNA or the NucleoSpin RNA Extraction kit (Macherey Nagel). Contaminating host DNA was removed with Heat and Run DNase (ArcticZymes), and cDNA was generated using Protoscript II (New England Biolabs) and then converted to double-stranded DNA using an enzyme mixture of *Escherichia coli* DNA ligase, DNA polymerase I and RNase H (New England Biolabs). The DNA product was used to construct a library using the Nextera XT library kit (Illumina) with barcoded primers. The library was sequenced on a NextSeq 500 generating 2×151 bp paired reads. Virus genomes were assembled using Geneious R8 software with LNV reference sequences for segments 1–12 (AY317099–AY317110).

Additional partial sequences from various isolates were obtained via Ion-Torrent sequencing (QHFSS, Brisbane) or next-generation sequencing (AGRF, Melbourne, Australia). To obtain the complete ORF for each sequence, primers were designed (Tables S2, S3), from consensus sequences where available, to facilitate primer walking. Gel bands were extracted and purified using a gel extraction kit (Qiagen, Hilden, Germany). Extracted DNA was run through a BigDye v3.1 sequencing kit before capillary Sanger sequencing using the Prism Genetic Analyzer 3100 (Applied Biosystems, Foster City, USA).

Phylogenetic analysis

Nucleotide multiple sequence alignments were performed with MAFFT v7.017 with an algorithm selected automatically, a scoring matrix of 200PAM/k=2, a gap open penalty of 1.53 and an offset value of 0.123. FastTree 2.1.5 was then used to construct a tree using the maximum likelihood approximation method with the general time-reversible model and optimized for gamma20 likelihood. The branch support values were calculated using a Shimodaira–Hasegawa test.

Quantitative RT-PCR for the assessment of *in vitro* LNV replication

Fifty TaqMan primer/probe sets were designed based on the nucleotide sequence for segment 10 of the 2007 isolate of LNV_{AU} using the IDT PrimerQuest Tool (https://sg.idtdna.com/Primerquest/Home/Index). The sequences for segment 10 of three Australian LNV isolates (the 2007 Sydney isolate from *Ae. vigilax*, a 2013 isolate from *Cx. annulirostris* collected at Tweed Heads, NSW, and the sequence reported by Coffey *et al.* [16] derived from mosquitoes collected in NSW between 1995 and 2005) were aligned using CLC Main Workbench 7 (CLC-bio, Aarhus, Denmark). Primer/probe sets were disregarded if they did not meet the criteria of targeting locations of consensus amongst all three isolates, and having a maximum of 10 bases between the primer and probe binding regions of the same sense. The finally selected primer/probe set is shown in Table S4. RNA purified from cell lines (C6/36, MDCK, PANC1, MEF IRF3/7$^{-/-}$, DF1) infected with LNV_{AU} at an m.o.i. of 5 was subjected to cDNA synthesis and qPCR was performed using

the TaqMan Universal PCR Master kit (Thermo-Fisher) in the following cycling conditions: Uracil N-glycosylase incubation at 50 °C for 2 min (×1), PCR activation at 95 °C for 5 min (×1), then 50 cycles at 95 °C for 12 s, 55 °C for 1 min and 72 °C for 15 s, using the ViiA7 real-time PCR system (Applied Biosystems, Foster City, USA). The data were analysed using QuantStudio 6 software (Applied Biosystems). A standard curve was created based on RNA extracted from LNV_{AU} stock serially diluted from neat to 10^{-5}. Further details are provided in the Supplementary Material.

Serological screening of human and animal plasma/sera for LNV reactivity

Plasma samples from human blood donors obtained with ethical approval from the Australian Red Cross Blood Service Human Research Ethics Committee [30, 31] and serum samples from feral rabbits [30] were screened in a fixed-cell ELISA as described in Clark *et al.* [48] for reactivity to LNV proteins, with mAb 6E6 included as a positive control. Human-derived samples that tested positive in this assay were further tested for virus-neutralizing activity in a micro-neutralization assay as described in Prow *et al.* [30, 35], using C6/36 cells and a virus inoculum of 100 $TCID_{50}$/well. The mAb 6E6 was included as a positive control. Serum samples obtained from race horses bled for routine drug-testing (UQ-AEC approval no. ANRFA/318/12), from horses admitted to the UQ Equine Hospital and bled for diagnostic purposes and from horses previously enrolled in a flavivirus-screening study [35] were also tested for LNV-neutralizing activity in the micro-neutralization assay.

Screening of human plasma samples for LNV viraemia

Plasma samples from human blood donors (see above) were screened for LNV viraemia by qRT-PCR and virus isolation using C6/36 insect cells as described in detail in the Supplementary Material.

Immunohistochemistry for LNV antigen in mosquitoes

Naturally infected, field-collected mosquitoes and colony-reared mosquitoes (see Supplementary Material for details of rearing) were fixed in 10 % neutral-buffered formaldehyde and routinely processed for paraffin-embedding. Five-micron serial sections were collected on charged slides and immunolabelled using the mAb 6E6 and anti-LNV polyclonal mouse serum, following a previously described protocol [25, 39]. Immunolabelled sections were examined on a Nikon Eclipse 50i microscope and digital microphotographs were captured with a Nikon DS-Fi1 camera with a DS-U2 unit and NIS elements F software, and are presented without further manipulation.

Mouse infection studies

All animal procedures had received prior approval from the QIMR Berghofer Medical Research Institute Animal Ethics Committee (P2086, A1503-692M). Mice were kept on clean bedding and given food and water *ad libitum*. To determine whether LNV_{AU} isolates were virulent in wild-type mice, C57BL/6 mice (n=10) were infected via the intraperitoneal route with the plaque-purified 2007 isolate $LNV_{NSW2007\text{-}B115745}$ (100 p.f.u.). Infected animals were monitored daily for the onset of disease and bled daily for 5 days p.i. for the assessment of viraemia. These mice were then reinfected with $LNV_{NSW2007\text{-}B115745}$ 2 weeks after the initial infection as per the protocol described in Attoui *et al.* [3]. Surviving mice were bled by cardiac puncture at the end of the experiment (day 21 post-reinfection) and the sera were tested for LNV-specific antibodies using a fixed cell ELISA (see above). To further assess the virulence of $LNV_{NSW2007\text{-}B115745}$ in mice, genetically modified (GEM) mice, IRF3/7$^{-/-}$ and B and T cell-deficient mice (Rag2$^{-/-}$) were infected with either 1000 or 10 000 p.f.u. of $LNV_{NSW2007\text{-}B115745}$ via the intraperitoneal route. The GEM mice were bled for viraemia analysis and monitored daily as described above. Blood samples taken via the tail vein were stored on ice for 20 min before the separation of serum at 3000 r.p.m. for 5 min. The sera were stored at −80 °C until they were tested for LNV in a $TCID_{50}$ and/or plaque assay as described above.

Test for viraemia by RT-PCR on murine blood samples

To determine whether viral RNA was present in the murine blood samples in the absence of infectious virus, RNA was extracted from both the cellular components of blood samples and sera using the Macherey Nagel Nucleospin Viral RNA isolation kit. Purified RNA (2.5 µl) were then tested by RT-PCR (Superscript III One-step RT-PCR System with Platinum *Taq* DNA polymerase; Invitrogen) using LNV-specific primers targeting segment 2 or segment 10 with the cycling conditions: RT 45 °C/30 min, 94 °C/2 min, PCR; 94 °C/30 s, 45 °C/30 s, 68 °C/30 s for 40 cycles and a final extension of 68 °C/5 min. The PCR products were separated by gel electrophoresis on 1 % agarose gel.

Statistical analysis

The viral titres were calculated and plotted in GraphPad Prism 6 (San Diego, CA, USA). Statistical analyses were performed using IBM SPSS Statistics for Windows version 22 (Armonk, NY, USA). When two samples were compared, the *t*-test was used, except when the difference in variance was <4, the skewness was ≥2 and the kurtosis was >2. Otherwise, the non-parametric Kolmogorov–Smirnov test was used.

Funding information
The studies were supported by funding from the Australian Research Council (ARC DP120103994; R. A. H.); the QIMR Berghofer Seed Fund (N. A. P.); Australian Postgraduate Awards (APA) to A. C., B. M., J. J. H., C. O'B. and T. P. Australian governments fund the Australian Red Cross Blood Service to provide blood, blood products and services to the Australian community.

Acknowledgements
We thank Shuting Jin, Jessamine E. Hazlewood, Sascha K. Lopez, Brittany E. Scott-Rimington and Dr Waylon Wiseman for technical help. Dr Cassie Jansen contributed to mosquito collections in Sydney. We thank the Western Australian Department of Health for financial and

logistical support, and the laboratory staff in the Arbovirus Surveillance and Research Laboratory at The University of Western Australia for mosquito collection and sample preparation. We thank the QIMR Berghofer Animal House staff for assistance and the supply of GEM mice and the Australian Red Cross Blood Service Staff and donors for the provision of blood samples, and acknowledge the facilities and scientific and technical assistance of the Australian Microscopy and Microanalysis Research Facility at the Centre for Microscopy and Microanalysis, University of Queensland.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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