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Screening of West Nile Virus, Herpesvirus, and Parvovirus in *Rattus* spp. in Klang Valley, Malaysia

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ABSTRACT

Free-roaming and scavenging lifestyles of *Rattus* spp. in densely populated urban areas expose them to multiple pathogens that facilitate the transmission of infection to the human population more rapidly, raising public health concerns. There is limited information on the status of rat susceptibility to virus infection, particularly West Nile virus (WNV), herpesvirus, and parvovirus, to prepare for emerging zoonosis. A total of 23 (n = 23) blood samples collected from *Rattus* spp. in the wet market areas of Klang Valley, Malaysia, were subjected to molecular assay using a one-step reverse transcription-polymerase chain reaction (RT-PCR) to detect the highly conserved region of the WNV capsid and pre-membrane protein via nested polymerase chain reaction (PCR) assay targeting highly conserved amino acid motifs within the herpesviral DNA-directed DNA polymerase gene (DPOL) and polymerase chain reaction (PCR) assay targeting the parvovirus non-structural (NS) protein. As a result, 4 out of 23 (17.39%) rats were positive for herpesvirus DNA, but none were positive for WNV RNA and parvovirus DNA. The positive PCR amplicons of herpesvirus DNA were subjected to partial DNA sequencing analysis, 100% identical to *Acomys* herpesvirus SVMS 226,222 from *Betaherpesvirinae*, which is highly

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suggestive of rat cytomegalovirus (RCMV). This study has successfully demonstrated the presence of RCMV from *Rattus* spp. in the Klang Valley. The RCMV potentially crosses species barriers and establishes infection, raising public health concerns. The non-viraemic state of WNV or parvovirus infection, low sample size, and limited niche distribution emphasise the need for the expansion of this study in the future.

Keywords: Herpesvirus, Klang Valley, parvovirus, *Rattus* spp., virus, West Nile virus

INTRODUCTION

Rats, members of the rodent family Muridae, are extremely successful and dominant species worldwide due to their ability to adapt to various environments. As a result, they become the most destructive agricultural pests and vectors for several zoonotic diseases worldwide (Chakma et al., 2018). Furthermore, due to the physiological similarity between rats and humans, their huge diversity, and the fact that some species of rats have adapted to living in close contact with humans, rats play a crucial role as reservoirs and vectors for zoonotic pathogens (Belmain, 2006).

Several countries have reported the seropositivity of *Rattus* spp. to WNV with a low viraemic level. A study in Slidell, Louisiana, showed that WNV seroprevalence in *Rattus* spp. is minimal, at around 5.6% (Dietrich et al., 2005). Black rats (*Rattus rattus*) and house mice (*Mus musculus*), two peri-domestic species of wild rats in Merida, Mexico, had reactive antibodies to flaviviruses, but none was positive for WNV antibodies (Cigarroa-Toledo et al., 2016).

The *Herpesviridae* family consists of three subfamilies: *Alphabetaherpesvirinae*, *Betaherpesvirinae*, and *Gammaherpesvirinae*. *Rattus* spp. could harbour these herpesviruses before establishing lifelong latent infections in the host. However, they could be reactivated at some point, causing the infectious virus to be transmitted to the other hosts by mucosal contact or droplet infection (Maclachlan & Dubovi, 2017). The possibility of other types of herpesviruses potentially harboured by *Rattus* spp. raises public health concerns due to the potential transmission of infectious diseases to humans.

Among the parvovirus serotypes known to infect the *Rattus* spp. include Kilham's rat virus (KRV), Toolan's H-1 virus, and rat parvovirus (RPV; Maclachlan & Dubovi, 2017). Most clinical signs in rats are derived from the KRV (Kohn & Clifford, 2002). Parvovirus is species-specific, hence, the virus could only spread within the rat population, where the transmission may occur via close or direct contact with the infected rats, through contaminated fomites, and shed in the urine, faeces, milk, and nasal secretions (Macy Jr. & Compton, 2020).

Rats trapped in densely populated urban areas of Klang Valley are exposed to multiple zoonotic pathogens that could be the source of infection in humans (Mohd-Qawiem et al., 2022). It became a cause of concern to public health due to its proximity to humans. Besides, rats could harm laboratory

animal colony biosecurity risk due to inadvertent transmission, possibly causing interference in research (Ain-Fatin et al., 2020). Information on the susceptibility of rats to zoonotic or non-zoonotic virus infection, particularly in WNV, herpesvirus, and parvovirus, are scarce. Therefore, unravelling the information may aid in long-term *in vivo* biosecurity besides preventing potential zoonotic infection. This study aims to screen viruses of veterinary and medical importance in *Rattus* spp. from Klang Valley to provide a current overview of the status of the viruses in Malaysia.

MATERIALS AND METHODS

Ethical Approval

This study was approved by the UPM Institutional Animal Care and Usage Committee (IACUC), with an approval code of UPM/IACUC/AUP-U017/2019.

Sampling

The rats were trapped in selected wet market areas of Klang Valley. The trapped rats (n = 23) were brought to the Post Mortem Laboratory, Faculty of Veterinary Medicine (FPV), UPM, for further examinations. First, the animals were anaesthetised with diethyl ether (Fisher Scientific, USA) at 100 mg/mL for blood collection by intracardiac puncture, followed by terminal exsanguination for euthanasia (Mohd-Qawiem et al., 2022). Then, the blood samples were processed to obtain serum in the Laboratory of Veterinary Virology, FPV. The serum samples were centrifuged at 4,000 × *g* for 10 minutes and transferred to 1.5 mL tubes (Eppendorf, Germany) before storage in the −80°C freezer (SANYO Ultra Low, Japan) until further use. The species, age, and sex of the rats are recorded in Table 1.

Molecular Analysis

Nucleic Acid Extraction. Nucleic acid (RNA and DNA) extraction was performed from the serum samples using TRIsure™ (Bioline, United Kingdom). Extracted RNA was used for detecting WNV, while extracted DNA was used for detecting herpesvirus and parvovirus. RNA and DNA purity and concentration were determined using the BioPhotometer (Eppendorf, Germany) at the absorbance value of 260/280 (Eppendorf, Germany). A value of 1.8 to 2.0 indicates a good purity of nucleic acid.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) for Detection of WNV. Reverse transcriptase polymerase

Table 1
Number of rats captured relative to the species, sex, and age

Rat species	Sex		Age	
	Male	Female	Adult	Juvenile
Rattus rattus	8	9	9	8
Rattus norvegicus	4	2	3	3
Total	23			

chain reaction (RT-PCR) was performed using primers targeting the highly conserved region between the capsid (C) and pre-membrane (PrM) of WNV (Table 2). A synthetic plasmid was used as the positive control with the addition of a 20 µL master mix (Bioline, United Kingdom). One-step RT-PCR was performed using MyTaq™ One-Step RT-PCR (Bioline, United Kingdom) in a 25 µL reaction, as described previously (Ain-Najwa et al., 2020).

Polymerase Chain Reaction (PCR) for Detection of Herpesvirus and Parvovirus. Ready-to-use MyTaq™ Red Mix (Bioline, United Kingdom) was used by preparing a master mix reaction of 7.5 µL of ddH_2O, 12.5 µL of MyTaq™ Red Mix, and 1.0 µL each of the forward and reverse primers. Table 2 lists the primer sequences that target the conserved amino acid motifs within the herpesviral DNA-directed DNA polymerase gene and parvovirus non-structural (NS) protein. The PCR tube was then placed in the PCR machine (Eppendorf, Germany) for DNA amplification. The PCR protocols used for herpesvirus and parvovirus include the initialising step at 95 ℃ for 1 minute, denaturation step, 35 PCR cycles of 95 ℃ for 15 seconds, followed by annealing with temperatures at 46 ℃ and 50 ℃ for 15 seconds, and lastly the elongation step at 72 ℃ for 10 seconds.

Gel Electrophoresis. Gel electrophoresis was performed to separate the PCR products using 1.5% gel prepared by mixing 1.5 g of agarose gel powder (1st BASE Pte.

Table 2
Forward and reverse primers used in RT-PCR and PCR analysis for the detection of West Nile virus, herpesvirus, and parvovirus

Virus	PCR reaction	Primer	Primer sequence (5' - 3')	Product size (bp)	References
West Nile virus (WNV)		F	5'-CCAATACGTTTCGT GTTGG-3'	470	Ain-Najwa et al. (2020)
		R	5'-ATGTCTTCAGGGTC ATTTCC-3'		
Herpes virus (nested PCR)	Primary PCR Primer	F	5'-GAYTTYGCNAGYYT NTAYCC-3'	480	Moureau et al. (2007)
		F	5'-TCCTGGACAAGCAG CARNYSGCNMTNAA-3'		
		R	5'-GTCTTGCTCACCAG NTCNACNCCYTT-3'		
	Secondary PCR Primer	F	5'-TGTAACTCGGTGTA YGGNTTYACNGGNGT-3'	215 - 315	
		R	5'-CACAGAGTCCGTRT CNCCRTADAT-3'		
Parvovirus		F	5'-AGCACAGGCAGTTG GTAATGTTG-3'	1,439	
		R	5'-ATAACTCCCAGTAG AAACGCC-3'		

Note. F = Forward; R = Reverse

Ltd., Singapore) in 100 mL × TAE (Tris-acetate-EDTA) buffer solution and 5 µL of Red Safe™ (Red Safe™, South Korea). The gel electrophoresis was set at 90 V and 350 mA for 40 minutes. The DNA fragments were then observed under the UV light transilluminator (Syngene, United Kingdom).

Partial DNA Sequencing. Unpurified positive PCR products were subjected to DNA sequencing by Apical Scientific Sdn. Bhd. First, they were sequenced with the BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems, USA) and ethanol precipitation according to the manufacturer's recommendations under an ABI Prism 3130 Fluorescent DNA Analyser (Applied Biosystems, USA). The nucleotide sequences were then aligned using the BioEdit software (version 7.2). Finally, the query sequences obtained were screened against the GenBank nucleotide database using the MegaBlast search function of the National Centre for Biotechnology Information (NCBI) to compare the query sequences and obtain identical nucleotide sequences.

Phylogenetic Analysis. The phylogenetic tree was constructed by the maximum likelihood method using the Kimura2 parameter model with 1,000 bootstrap replicates. The analysis comprised 28 reference strains of herpesviruses, targeting the DNA polymerase (DPOL) gene and two outgroups from WNV and parvovirus, shown in Table 3.

Table 3
List of reference strains used in phylogenetic analysis

Herpesvirus					
No.	Accession no.	Country	Strain	Year	Isolation source
1	MF615317	Brazil	Alpha	2012	*Homo sapiens* (Human)
2	MF615329	Brazil	Alpha	2012	*Homo sapiens* (Human)
3	EF125072	Germany	Beta	2006	*Rattus tiomanicus* (Malayan wood rat)
4	HQ587046	United Kingdom	Unclassified	2010	*Acomys dimidiatus* (Eastern spiny mouse)
5	EF125070	Thailand	Beta	2006	*Rattus rattus* (House rat)
6	HQ587047	United Kingdom	Unclassified	2010	*Dipodillus dasyurus* (Wagner's gerbil)
7	AB517983	Japan	Beta	2009	*Miniopterus fuliginosus* (Bat)
8	HM060767	USA	Gamma	2010	*Loxodonta africana* (Elephant)
9	NC001826	USA	Gamma	1997	Murid
10	KY398049	Congo	Unclassified	2021	Wild rodent
11	KY398052	Congo	Unclassified	2010	Rodent
12	HQ221963	Peru	Gamma	1996	*Oligoryzomys* (Pygmy rice rat)
13	MH257598	Costa Rica	Unclassified	2011	*Oligoryzomys vegetus* (Sprightly pygmy rice rat)
14	MZ934650	United Kingdom	Unclassified	2021	*Myodes glareolus* (Bank vole)

Table 3 *(continue)*

No.	Accession no.	Country	Strain	Year	Isolation source
15	KU529537	France	Alpha	2008	*Homo sapiens* (Human)
16	KM507472	USA	Alpha	2012	*Terrapene Carolina* (Common box turtle)
17	AF031809	USA	Gamma	1997	*Alcelaphine* (Wildebeest)
18	AF031812	USA	Gamma	1999	Ovine
19	AF031811	USA	Gamma	1998	Bovine
20	AF031810	USA	Alpha	1997	Bovine
21	MH084656	Slovenia	Unclassified	2015	Ural owl
22	AF275657	United Kingdom	Gamma	2000	*Meles meles* (The Eurasian badger)
23	AF159038	USA	Gamma	2000	*Macaca fascicularis* (Long-tailed macaque)
24	GQ222415	USA	Alpha	2007	*Testudinidae* (Tortoise)
25	LC008326	Philippines	Unclassified	2012	*Ptenochirus jagori* (Greater musky fruit bat)
26	AY236869	USA	Unclassified	2003	*Iguanid* (Green iguana)
27	AF118401	Germany	Gamma	1999	Porcine
28	KF155695	Congo	Beta	2010	*Mus musculus* (House mouse)
29	From this study	Malaysia	Beta	2021	*Rattus* spp.
30	From this study	Malaysia	Beta	2021	*Rattus* spp.
31	From this study	Malaysia	Beta	2021	*Rattus* spp.
32	From this study	Malaysia	Beta	2021	*Rattus* spp.
Outgroup					
No.	Accession no.	Country	Virus	Year	Isolation Source
1	KJ131503	Senegal	WNV	1993	Rodent
2	HM989956	China	Parvovirus	2010	*Rattus norvegicus*

RESULTS

Molecular Detection of WNV, Herpesvirus, and Parvovirus

Based on the RT-PCR analysis of the wild rat sera, all 23 rats are negative for WNV RNA, as no band is observed at the 470-base pair (bp) compared to the positive control (Figure 1A). However, the nested PCR analysis of herpesvirus demonstrated that 4 (3 *R. rattus* and 1 *R. norvegicus*) out of 23 (4/23; 17.39%) rats are positive for herpesvirus DNA (Figure 1B). No parvovirus DNA is detected in the PCR analysis, as shown in Figure 1C.

Sequencing and Phylogenetic Analysis

Based on the sequence comparison with the published sequences from the GenBank database, the current isolates revealed 100% similarity to *Acomys* herpesvirus SVMS 226,222 derived from the *Betaherpesvirinae* subfamily for all positive samples of herpesvirus DNA (Figure 2). The phylogenetic analysis comprises 28 reference strains of herpesviruses targeting the DPOL gene and two outgroups from WNV and parvovirus. The sequences are highlighted based

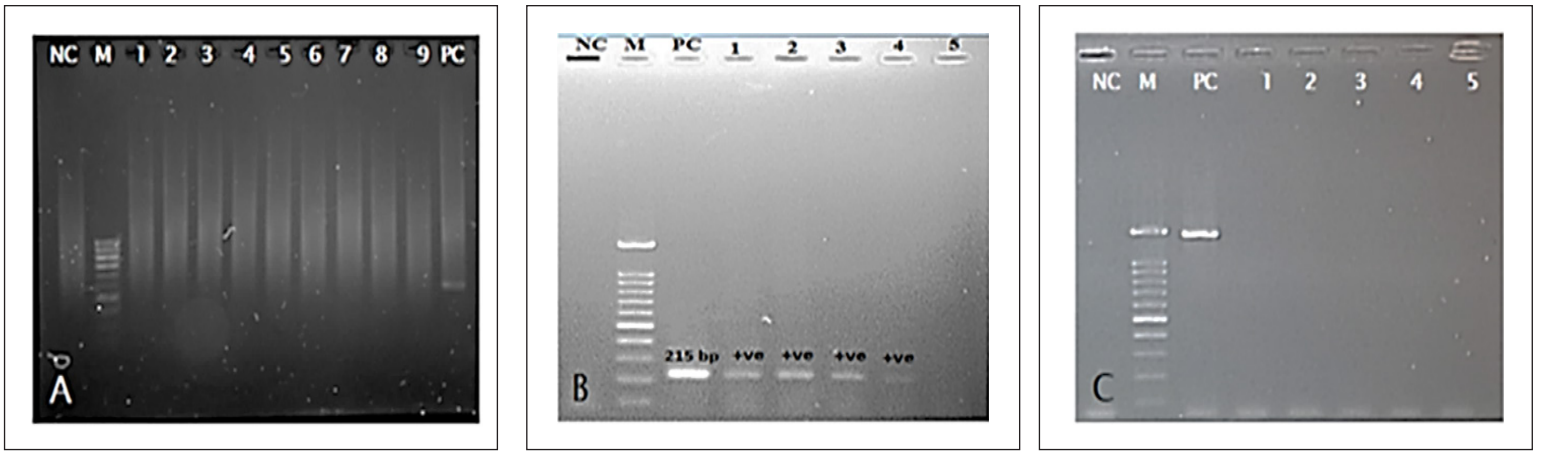

Figure 1. Gel electrophoresis analysis of WNV (A), herpesvirus (B), and parvovirus (C) nucleic acid. No bands are observed for all samples (A, C). Samples 1 to 4 showed the presence of bands at 215 bp, which is absent in sample 5 (B)
Note. NC = Negative control; M = Ladder; PC = Positive control

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Acomys herpesvirus SVMS 226.222 DNA polymerase gene, partial cds	Acomys herpesvirus SVMS 226.222	56.5	56.5	16%	0.001	100.00%	231	HQ587046.1
Turtle herpesvirus isolate 6217 DNA-directed DNA polymerase gene, partial cds	Turtle herpesvirus	54.7	54.7	16%	0.004	100.00%	181	KX374559.1
Gorilla beringei beringei lymphocryptovirus 1 isolate V-240 DNA polymerase (DPOL) gene,...	Gorilla beringei beringei lymphocrypto...	54.7	54.7	16%	0.004	100.00%	229	KU736788.1
Gorilla beringei beringei lymphocryptovirus 1 isolate B-26 DNA polymerase (DPOL) gene,...	Gorilla beringei beringei lymphocrypto...	54.7	54.7	16%	0.004	100.00%	229	KU736786.1
Columbid alphaherpesvirus 1 isolate 903/19 DNA-dependent DNA polymerase gene, parti...	Columbid alphaherpesvirus 1	54.7	54.7	16%	0.004	100.00%	234	MW625939.1
Hipposideros diadema herpesvirus gB, DPOL genes for glycoprotein B, DNA polymerase,...	Hipposideros diadema herpesvirus	54.7	54.7	16%	0.004	100.00%	3741	AB490083.2
Panulirus argus virus 1 DNA-directed DNA polymerase (pol) gene, partial cds	Panulirus argus virus 1	54.7	54.7	16%	0.004	100.00%	177	DQ465025.1

Figure 2. The BLAST analysis of herpesvirus DNA from one positive isolate. It shows 100% similarity to the subfamily *Betaherpesvirinae*

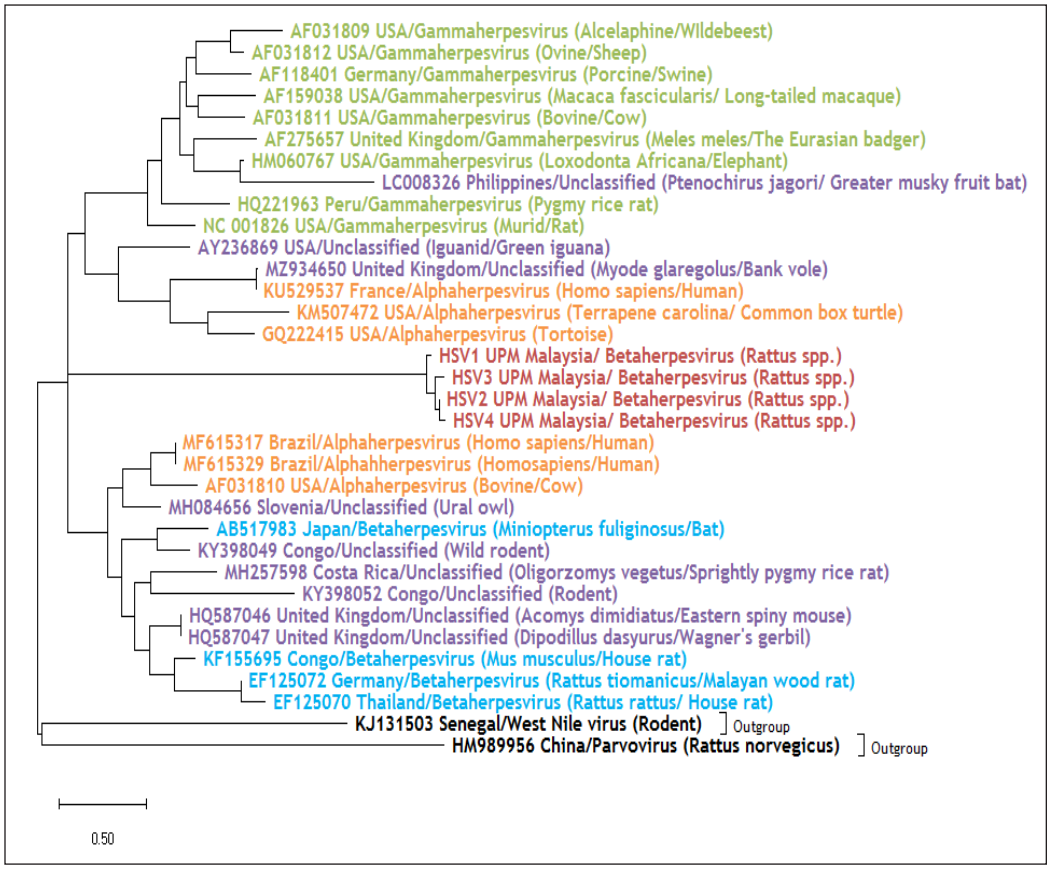

Figure 3. Phylogenetic analysis of herpesvirus in *Rattus* spp. (red) with 28 reference strains of herpesviruses targeting the DNA polymerase (DPOL) gene and two outgroups (black) from WNV and parvovirus. The strains were coloured in green gamma herpesvirus (9 strains), purple for unclassified herpesvirus (9 strains), orange for alpha herpesvirus (6 strains), and blue for beta herpesvirus (4 strains) targeting DPOL gene from multi-species. The tree was constructed using the maximum likelihood method with the General Time Reversible (GTR) model with 1,000 bootstrap replicates

on the strains of herpesvirus, i.e., alpha herpesvirus (orange), beta herpesvirus (blue/red), gamma herpesvirus (green), and unclassified herpesvirus (purple), as illustrated in Figure 3.

DISCUSSION

Understanding the risk of the virus spreading from wild rats to laboratory animals may help enhance the quality of bio-life-related research. In this study, the higher detection of herpesvirus in *Rattus* spp. suggested an increased occurrence of herpesvirus even in a small sample of the rat population. Although *R. rattus* is more predominant than *R. norvegicus*, both species were positive for herpesvirus. It is similar to the finding that nearly 50% of the rat populations in Panama were positive for herpesvirus DNA (Rabson et al., 1969). The widespread of herpesvirus implies that it is ubiquitous in *Rattus* spp. because once the rats become

infected, the virus persists for a lifetime in the host before the latent infection is established. The latency could be reactivated when the host is immunocompromised, leading to asymptomatic virus shedding or manifestation of clinical signs. The close contact transmission further provides an opportunity for the virus to be largely spread in the confined area of the rat population (Barthold et al., 2016).

Identifying the *Betaherpesvirinae* sequence that signifies the RCMV indicates that herpesvirus reflects species specificity and adaptation to their host for a long time. Although beta and gamma herpesviruses are usually considered hosts specific to the herpesvirus subfamilies, they can cross species barriers and establish endemic infections (Azab et al., 2018). Novel herpesvirus may exist in the *Rattus* spp. considering the diversity of rodent species. It could become an issue as gamma herpesviruses contribute to zoonotic infections that trigger public health concerns. It is possible because gamma herpesviruses were detected previously in other rodent species. Therefore, *Rattus* spp. is a potential host for many unknown herpesviruses and has become a threat to urban communities as a reservoir for human diseases (Ntumvi et al., 2018).

The negative results of WNV and parvovirus RT-PCR from this study could be due to the non-viraemic state of the infection, i.e., the absence of the virus in the blood during the collection of blood samples. The negative results of all samples for WNV RNA indicate the absence or low transient viremia load in *Rattus* spp. (Hirota & Shimizu, 2013). Besides, the blood serum sample used in this experiment is suitable enough to detect WNV RNA with sufficient viraemic levels. However, in a low viraemic load, WNV may persist at the site of viral shedding, especially in faeces, urine, and oropharyngeal swabs (Root et al., 2005). Meanwhile, parvovirus might be found in blood early in the disease, where viremia can persist for 2 to 10 days after infection (Kilham, 1966), rendering the infected rats to be detected afterwards. The virus is most frequently detected in spleens and mesenteric lymph nodes (Wan et al., 2006), potentially increasing the chances of virus detection than the blood sample. Although the rats may be infected with parvovirus, low viral loads implicate the negative results as the PCR assay could not detect the virus. The assay is influenced by the successful recovery of live viruses from the blood samples. However, non-viable viruses or undetectable viral loads might render unsuccessful PCR assay. Both viruses were undetectable due to the low prevalence of the pathogen in the population sampled or no exposure to the virus infection.

The small sample size decreases the chances of detecting virus infection within a population. Although negative results were obtained, these results are still in agreement with other studies. A previous study showed a 2.0% and 3.3% prevalence of parvovirus in 63,808 rats in North America and Europe (Pritchett-Corning et al., 2009). In another study, 10.4% of 162 rats were serologically positive for the KRV and Toolan's H-1

virus (Easterbrook et al., 2008). The studies demonstrated the low prevalence of rat parvovirus, similar to the current study. In addition, the low molecular prevalence could be due to the clearance of viruses by antibody responses. The antibody response limits virus replication as a critical barrier to combat infection (Murin et al., 2019). A serological assay should also be conducted to conclude whether the rats are free from these three viruses.

Nevertheless, the *Rattus* spp. within Klang Valley still carry a potential risk of causing serious health implications to the public besides biosecurity risk towards laboratory rodent colonies. The incidence of rat-associated zoonoses may rise due to the proximity of rats and communities in urban neighbourhoods. Due to impoverished urban populations, environmental factors may influence zoonotic diseases associated with rats, including poor sanitation, crowding, and homelessness. The availability of food sources in food premises may also influence the abundance of rats due to improper food storage and organic waste disposal. Therefore, the rat population still need to be controlled as they can harbour diseases that raise public health concerns.

CONCLUSION

Four rats were infected with herpesvirus, with 100% similarity to rat cytomegalovirus. Although the results suggest the circulation of herpesvirus in *Rattus* spp. in Klang Valley, they were less likely to be infected by WNV and parvovirus.

CONFLICT OF INTERESTS

The authors declare no conflict of interest regarding the publication of this paper.

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