The Journal of Veterinary Medical Science

NOTE

Virology

Molecular evidence of rat bocavirus among rodents in Peninsular Malaysia

Siti Nurul Izzah MOHD-AZAMI[1,2], Shih Keng LOONG[1], Jing Jing KHOO[1], Norhidayu SAHIMIN[1], Fang Shiang LIM[1], Nurul Aini HUSIN[1], Nur Hidayana MAHFODZ[1], Farah Shafawati MOHD-TAIB[3], Siti Nabilah ISHAK[3], Benjamin L. MAKEPEACE[4] and Sazaly ABUBAKAR[1,5]*

[1]Tropical Infectious Diseases Research and Education Centre (TIDREC), Universiti Malaya, Kuala Lumpur, Malaysia
[2]Institute for Advanced Studies, Universiti Malaya, Kuala Lumpur, Malaysia
[3]School of Environmental Science and Natural Resources, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, Bangi, Malaysia
[4]Institute of Infection, Veterinary & Ecological Sciences, University of Liverpool, Liverpool, UK
[5]Department of Medical Microbiology, Faculty of Medicine, Universiti Malaya, Kuala Lumpur, Malaysia

J. Vet. Med. Sci.
84(7): 938–941, 2022
doi: 10.1292/jvms.22-0037

Received: 22 January 2022
Accepted: 29 April 2022
Advanced Epub:
18 May 2022

ABSTRACT. Rat bocavirus (RBoV) and rodent bocavirus (RoBoV) have previously been detected in *Rattus norvegicus*; however, these viruses have not been reported in rodent populations in Malaysia. We investigated the presence of RBoV and RoBoV in archived rodent specimens. DNA barcoding of the rodent cytochrome c oxidase gene identified five different species: *Rattus tanezumi* R3 mitotype, *Rattus tiomanicus, Rattus exulans*, *Rattus argentiventer*, and *Rattus tanezumi sensu stricto*. Three spleens were positive for RBoV (1.84%; 3/163), but no RoBoV was detected. Phylogenetic analyzes of the partial non-structural protein 1 gene grouped Malaysian RBoV strains with RBoV strains from China. Further studies among rats from different geographical locations are warranted for this relatively new virus.

KEYWORDS: infectious disease, Malaysia, rat bocavirus, *Rattus* sp., rodent

Human bocavirus (HBoV) was first reported in Sweden more than a decade ago [2]. Prior to this discovery, clinicians were unclear as to what caused a respiratory tract illness that had an apparently obscure etiology. Since then, HBoV has been recognized as a pathogen that can cause nosocomial infections, especially in pediatric patients [6]. Other phylogenetically distinct bocaviruses have also been identified in animals, such as rodents [16], dogs [13], cats [1], and pigs [8]. Rodents are synanthropic mammals that can easily adapt to areas close to human habitation [9]. Recently, HBoV was detected in the feces of *Rattus norvegicus* in China [15]. Researchers found that the virus was closely related to the HBoV-2 strain, which suggested that rats could potentially serve as carriers of HBoV and transmit the virus to humans. Subsequently, Lau *et al.* reported the detection of a novel rat bocavirus (RBoV) in *R. norvegicus* in China [7]. A separate study in China did not detect RBoV; however, rodent bocaviruses (RoBoV) were detected [16]. Interestingly, the RoBoV strains were found to be more closely related to porcine bocaviruses than to RBoV after phylogenetic analyzes [14]. These findings showed that bocaviruses have established a niche in rodents (RBoV and RoBoV) that is markedly different from HBoVs. Research on RBoVs and RoBoVs has been largely concentrated in China. However, there is no information regarding the prevalence of these bocaviruses in Malaysian rodent populations. Therefore, this study investigated the possible presence of RBoV and RoBoV in rodent populations on Peninsular Malaysia.

Rodents from the sampling sites in UM Plantations Sdn. Bhd., Johore, Malaysia (N2.02916, E103.87076), and Kampung Tumbuh Hangat, Perak, Malaysia (N4.313903, E100.929009) were trapped at several different times between December, 2018 and December, 2019. UM Plantations Sdn. Bhd. is an oil palm plantation located in the southern state of Johore, whereas Kampung Tumbuh Hangat is a village in central Perak that is surrounded by oil palm plantations and paddy fields. This study received animal ethics approval from the Universiti Malaya Institutional Animal Care and Use Committee (G8/23122019/11102019-01/R). Permission was obtained from the Department of Orang Asli Development (JAKOA) (JHEOA.PP.30.052 Jld. 6 (19)) to conduct this study at Kampung Tumbuh Hangat, Perak. After morphological identification, the trapped rodents were euthanized, and then selected tissues were harvested and archived. This study utilized blood clots (n=147) and spleens (n=163) from 163 rodents. Blood clots from the remaining 16 rodents were insufficient for downstream experiments; therefore, they were excluded. Genomic DNA

*Correspondence to: AbuBakar, S.: sazaly@um.edu.my, Tropical Infectious Diseases Research and Education Centre (TIDREC), Universiti Malaya, 50603 Kuala Lumpur, Malaysia

was extracted using a NucleoSpin Tissue Extraction Kit (Macherey-Nagel, Düren, Germany). A 726 bp cytochrome c oxidase (*COI*) gene fragment [4] was amplified from each rodent for species group determination (Table 1). All sequences obtained were compared with those in GenBank using the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST).

A nested polymerase chain reaction (PCR) targeting the non-structural protein 1 (*NS1*) gene was performed to detect RBoV and RoBoV [16]. For the detection of RBoV, primers RBoV-HK-F1 and RBoV-HK-R1 were used in the first round of the nested PCR, while primers RBoV-HK-F2 and RBoV-HK-R2 were used in the second round of the nested PCR, resulting in the amplification of a 248 bp amplicon (Table 1). For the detection of RoBoV, primers PBNS1-F1 and PBNS2-R1 were used in the first round of the nested PCR, while primers PBNS1-F2 and PBNS1-R2 were used in the second round of the nested PCR to amplify a 413 bp amplicon (Table 1). The purified amplicons were sequenced in both directions and the obtained sequences were compared to those available in GenBank using the BLAST tool. A phylogenetic tree was then constructed using the maximum-likelihood method to determine the genetic relationships between the bocaviruses obtained in this study and other representative bocaviruses obtained from GenBank.

Analyzes of the *COI* sequences identified five rat species. These species were *Rattus tanezumi sensu stricto, Rattus tiomanicus, Rattus tanezumi* R3 mitotype, *Rattus exulans*, and *Rattus argentiventer*. The *COI* sequences were deposited in the Barcode of Life Data System (BOLD) database using the following process IDs: UMNPA004-20–UMNPA056-20, and UMNPA058-20–UMNPA068-20 for rodents captured from Johore, and UMNPA069-20, UMNPA071-20–UMNPA076-20, UMNPA078-20–UMNPA080-20, UMNPA082-20–UMNPA083-20, UMNPA085-20, UMNPA087-20–UMNPA091-20, UMNPA093-20–UMNPA102-20, UMNPA161-20–UMNPA194-20, UMNPA196-20–UMNPA216-20, and UMNPA218-20–UMNPA223-20 for rodents captured from Perak. The predominant species was the *R. tanezumi* R3 mitotype (n=116), followed by *R. tiomanicus* (n=22), *R. exulans* (n=13), *R. argentiventer* (n=11), and *R. tanezumi s. s.* (n=1) (Table 2). RBoV was detected in three *R. tanezumi* R3 mitotype spleen samples, whereas RBoV was not detected in the blood clots from any of the studied rats. The RBoV-positive rats were obtained from Perak, RBoV was not detected in rats trapped in Johore, and RoBoV was not detected in rats trapped at either site. The phylogenetic tree generated for RBoV using the *NS1* gene showed that all the RBoV strains were grouped together and were separated from the other bocaviruses (Fig. 1). The Malaysian strains were distinctively different because they clustered into one monophyletic group next to the RBoV strains from China (Accession Nos. MG905222 and KT454514). As shown in Table 3, the pairwise genetic distance showed that the Malaysian RBoV strains were similar to each other, but markedly different from the RBoV strains from China because the interspecific distances were more than 3% (the genetic distance among the Malaysian RBoV strains).

To the best of our knowledge, only cases of HBoV [3] and porcine bocavirus [10] have been reported in Malaysia. Prior to this study, there were no published data on the prevalence of RBoV or RoBoV in Malaysia. Human infections caused by HBoV have

Table 1. Primers used to detect rat bocavirus, rodent bocavirus, and rodent DNA barcoding

Species	Target	Primer name	Oligonucleotide sequence (5′-3′)	Amplicon size (bp)	Reference
Rodent	*COI*	BatL5310[a,c]	ACTTCTGGGTGTCCAAAGAATCA	726	[4]
		R6036R[b,c]	CCTACTCRGCCATTTTACCTATG		
RoBoV	*NS1*	PBNS1-F1[a]	CCCAGTACAGGAAAGACCAACC	413	[16]
		PBNS2-R1[b]	GAAGGGCATAACTTAGCCAACG		
		PBNS1-F2[a,c]	GTAAATCTATTCGGCAATGTGA		
		PBNS1-R2[b,c]	CATGTAGTGCAGTATCCGTCCA		
RBoV	*NS1*	RBoV-HK-F1[a]	CTACTGGGCATGCGAACGTA	248	[16]
		RBoV-HK-R1[b]	CAGTTGCCTGTTGGTGTGTG		
		RBoV-HK-F2[a,c]	ACAGCAGACAAGCCAACCAA		
		RBoV-HK-R2[b,c]	TGCATTGTCTTCTGGCTGTCT		

a, forward primer; b, reverse primer; c, sequencing primer. *COI*, cytochrome c oxidase; *NS1*, non-structural protein 1.

Table 2. Numbers of rats trapped in Perak and Johore

Species	Trapping site		Total number of individuals
	Perak (n)	Johore (n)	
Rattus tanezumi R3 mitotype	64	52	116
Rattus tiomanicus	10	12	22
Rattus exulans	10	3	13
Rattus tanezumi sensu stricto	1	0	1
Rattus argentiventer	11	0	11
Total number of individuals	96	67	163

Table 3. Pairwise distances generated by comparing the partial sequences for rat bocavirus non-structural protein 1 representatives to the sequences isolated from this study using MEGA11

No.	Sequence name	1 (%)	2 (%)	3 (%)	4 (%)	5 (%)
1.	Rat bocavirus strain HK2S					
2.	Rat bocavirus strain XM.FA.63	2.03				
3.	UM-SNI01	31.08	31.92			
4.	UM-SNI02	27.85	28.62	2.99		
5.	UM-SNI03	28.64	29.45	1.97	2.00	

Analyzes based on the 207 nucleotide positions of the partial *NS1* sequences.

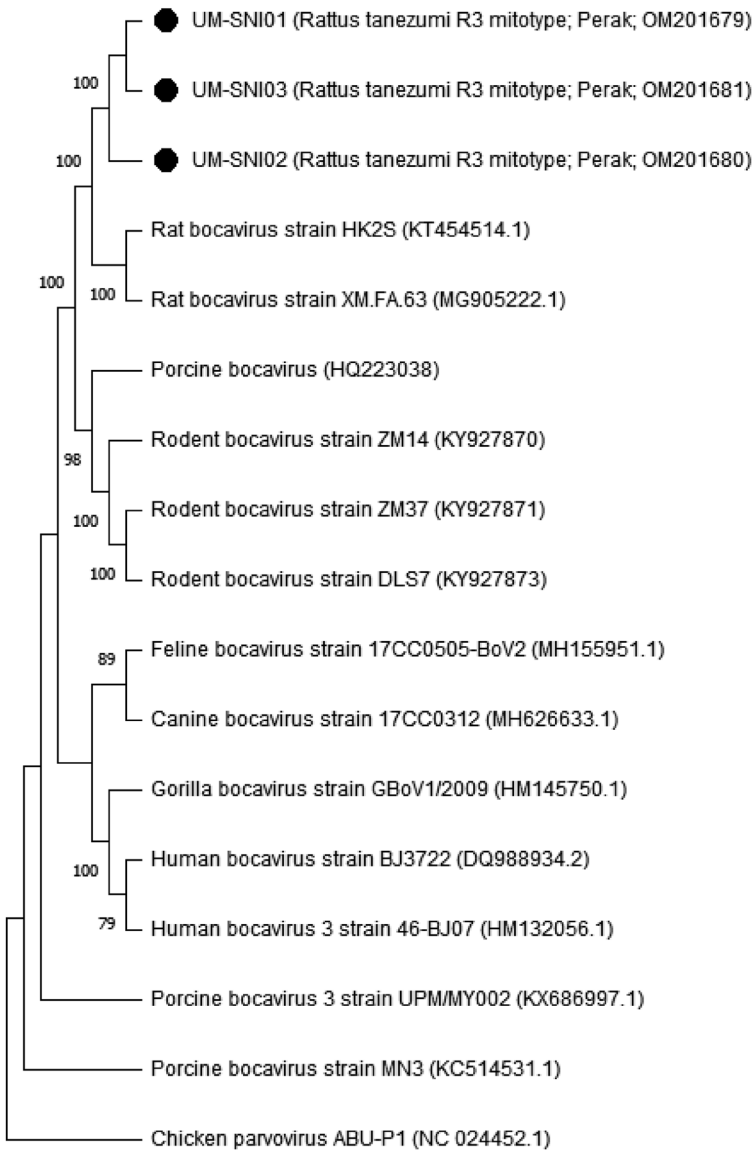

Fig. 1. Tamura-3 model with a gamma distribution maximum-likelihood phylogenetic tree of the partial *NS1* sequences for RBoV detected in this study. The tree was inferred using the maximum-likelihood method implemented in MEGA11 with 1,000 bootstrap replicates. Other bocavirus representatives were obtained from GenBank with their respective accession numbers in parentheses. The three RBoV strains discovered in the present study (UM-SNI01, UM-SNI02, and UM-SNI03) are indicated by solid black dots with their respective hosts, location where the host was captured, and accession numbers in parentheses.

attracted considerable attention in recent years due to their possible zoonotic origin [2, 10, 12]. Therefore, we sought to examine the baseline levels of RBoV and RoBoV in a cohort of archived rodent specimens.

Bocaviruses are respiratory-associated pathogens, which means that their presence is more likely to be detected in respiratory tissues. However, the rodent lung tissues in our archive were used in other studies. Therefore, spleens and blood clots were selected. HBoVs have been detected in the blood from human patients [11] and RBoVs have been found in the spleens of rats [7], making these biological samples feasible targets. Interestingly, RBoV was detected in the spleen of the *R. tanezumi* R3 mitotype in this study, but was not detected in the blood clots. RBoV has been detected in throat swabs and the feces of rodents captured from four provinces in China [14]. In addition, RBoV has also been detected in alimentary and kidney tissues [7]. These findings suggest that RBoV could reside in the alimentary and respiratory tracts, translocate into the kidney and spleen, and then are secreted in the feces. A study reported by Zhang *et al.* discovered RoBoV in the lungs of seven different rodent species: *R. norvegicus*, *Mus musculus*, *Apodemus agrarius*, *Cricetulus barabensis*, *Rattus flavipectus*, *Rattus rattus*, and *Rhombomys opimus* [16]. Further genetic analyzes revealed that RoBoV shares a relatively low amino acid similarity of approximately 51% when compared to RBoV, and is more closely related to porcine bocavirus [16]. This suggests the possibility of bocavirus spillover among rodents and pigs in China [16], but not in Malaysia because phylogenetic analyzes revealed that the detected Malaysian RBoV strains were clearly distinguishable from porcine bocavirus strains, regardless of the location in which they were detected (Fig. 1). Given that RoBoV was not found in any rodent samples in this study, this could imply that RoBoV is not present in rodents at the sampling sites.

All three RBoV strains detected in the present study were clustered in a sister clade next to the RBoVs from China (Fig. 1), indicating that Malaysian RBoV strains are genetically different from those in China. RoBoV and RBoV strains,

reported by Zhang *et al.* and Lau *et al.* were detected in urban cities [7, 16], whereas our study sites are transition areas, such as oil palm plantations and paddy fields, which have different habitat characteristics. The low RBoV detection rate of 1.84% (3/163) could be explained by the difference in rat population density between the study sites (oil palm plantation/paddy fields vs. urban cities). The study sites are spacious and uncrowded, unlike condensed urban cities [17], resulting in less contact between the rats and potentially lower transmission of RBoV. Urban rats must compete with each other for resources, and the increase in contact could contribute to the transmission of respiratory pathogens such as RBoV [5].

All the RBoV-positive strains were detected in the *R. tanezumi* R3 mitotype, which suggests that it might be a potential carrier of RBoV. However, we cannot conclude that RBoV is exclusively found in *R. tanezumi* R3 mitotype from just one relatively small-scale study. Furthermore, there is currently a lack of data on bocaviruses among rodents, especially in southeast Asia. Thus, more studies that include a larger sample size covering various locations must be conducted to gain better insights into the prevalence of RBoV in Malaysia. In many instances, we found non-specific binding in the nucleic acid amplification of RBoV. This suggests that the primers might not be specific enough to target Malaysian RBoV strains, which was somewhat expected because the primers were designed based on RBoV strains from China [16]. Consequently, this implies that we could have underestimated the RBoV detection rate in this study. Due to research limitations, such as the small sample size (n=163) and spatio-temporal factors, we were only able to detect three positive hosts from Perak and none from Johore. In the future, studies to determine the prevalence of RBoV and RoBoV in Malaysia should be expanded to include other states so that there is a more favorable representation of the population.

In conclusion, we report the detection of RBoV in the *R. tanezumi* R3 mitotype captured in Malaysia. Further surveillance of RBoVs in Malaysia and other countries in southeast Asia is warranted because very little information is available on the biology, pathology, and transmission of this relatively new virus.

CONFLICT OF INTEREST. The authors declare that they have no conflict of interest.

ACKNOWLEDGMENTS. We acknowledge funding from the Ministry of Higher Education, Malaysia, for niche area research under the Higher Institution Center of Excellence (HICoE) program (MO002-2019). This research was also supported by an institutional links grant (ID 332192305) under the Newton-Ungku Omar Fund partnership. This grant was funded by the UK Department of Business, Energy, and Industrial Strategy (BEIS) and the Malaysian Industry-Government Group for High Technology (MIGHT), and was delivered by the British Council.

REFERENCES

1. Abayli, H. and Can-Sahna, K. 2021. First detection of feline bocaparvovirus 2 and feline chaphamaparvovirus in healthy cats in Turkey. *Vet. Res. Commun.* **46**: 1–10. [Medline]
2. Allander, T., Tammi, M. T., Eriksson, M., Bjerkner, A., Tiveljung-Lindell, A. and Andersson, B. 2005. Cloning of a human parvovirus by molecular screening of respiratory tract samples. *Proc. Natl. Acad. Sci. USA* **102**: 12891–12896. [Medline] [CrossRef]
3. Etemadi, M. R., Jalilian, F. A., Abd Wahab, N., Jahanshiri, F., Amini, R., Othman, N. and Sekawi, Z. 2012. First detected human bocavirus in a Malaysian child with pneumonia and pre-existing asthma: a case report. *Med. J. Malaysia* **67**: 433–434. [Medline]
4. Herbreteau, V., Jittapalapong, S., Rerkamnuaychoke, W., Chaval, Y., Cosson, J. F. and Morand, S. 2011. Protocols for field and laboratory rodent studies. http://www.ceropath.org/FichiersComplementaires/Herbreteau_Rodents_protocols_2011.pdf [accessed on December 31, 2021].
5. Judson, S. D. and Munster, V. J. 2019. Nosocomial transmission of emerging viruses via aerosol-generating medical procedures. *Viruses* **11**: 940. [Medline] [CrossRef]
6. Kobayashi, H., Shinjoh, M., Sudo, K., Kato, S., Morozumi, M., Koinuma, G., Takahashi, T., Takano, Y., Tamura, Y. and Hasegawa, N. 2019. Nosocomial infection by human bocavirus and human rhinovirus among paediatric patients with respiratory risks. *J. Hosp. Infect.* **103**: 341–348. [Medline] [CrossRef]
7. Lau, S. K. P., Yeung, H. C., Li, K. S. M., Lam, C. S. F., Cai, J. P., Yuen, M. C., Wang, M., Zheng, B. J., Woo, P. C. Y. and Yuen, K. Y. 2017. Identification and genomic characterization of a novel rat bocavirus from brown rats in China. *Infect. Genet. Evol.* **47**: 68–76. [Medline] [CrossRef]
8. Lee, C., Tan, C., Tan, Y., Wong, J., Jacob, D., Lim, J., Ananthan, G., Arshad, S., Selvarajah, G. and Ooi, P. 2019. First nearly complete genome sequence of porcine bocavirus strain from Malaysia. *Adv. Anim. Vet. Sci.* **7**: 776–781. [CrossRef]
9. Loong, S. K., Che-Mat-Seri, N. A. A., Tan, K. K., Azizan, N. S., Ahmad-Nasrah, S., Mohd-Zain, S. and AbuBakar, S. 2021. Isolation of Kytococcus schroeteri from the brown rat Rattus norvegicus. *Asian Pac. J. Trop. Med.* **14**: 191–192. [CrossRef]
10. Mohan Jacob, D., Lee, C. Y., Arshad, S. S., Selvarajah, G. T., Bande, F., Ong, B. L. and Ooi, P. T. 2018. First molecular detection of porcine bocavirus in Malaysia. *Trop. Anim. Health Prod.* **50**: 733–739. [Medline] [CrossRef]
11. Nora-Krukle, Z., Vilmane, A., Xu, M., Rasa, S., Ziemele, I., Silina, E., Söderlund-Venermo, M., Gardovska, D. and Murovska, M. 2018. Human bocavirus infection markers in peripheral blood and stool samples of children with acute gastroenteritis. *Viruses* **10**: 639. [Medline] [CrossRef]
12. Schildgen, O. 2013. Human bocavirus: lessons learned to date. *Pathogens* **2**: 1–12. [Medline] [CrossRef]
13. Wang, Y., Li, W., Guo, X., Zhang, D., Sun, J., Fu, Z., Liu, G., Li, Y. and Jiang, S. 2021. Development of SYBR green I-based polymerase chain reaction for feline bocavirus 1 detection 3. *Biotech.* **11**: 1–6.
14. Xiong, Y. Q., Zhou, J. H., Zhang, M. Y., You, F. F., Li, D. L. and Chen, Q. 2018. Presence of rat bocavirus in oropharyngeal and fecal samples from murine rodents in China. *Arch. Virol.* **163**: 3099–3103. [Medline] [CrossRef]
15. You, F. F., Zhang, M. Y., Wu, F., Li, Q. S. and Chen, Q. 2022. Human bocavirus 2 detected in Rattus norvegicus feces in China. *Arch. Virol.* **167**: 171–175. [Medline] [CrossRef]
16. Zhang, C., Song, F., Xiu, L., Liu, Y., Yang, J., Yao, L. and Peng, J. 2018. Identification and characterization of a novel rodent bocavirus from different rodent species in China. *Emerg. Microbes Infect.* **7**: 48. [Medline] [CrossRef]
17. Zünd, D. and Bettencourt, L. M. A. 2019. Growth and development in prefecture-level cities in China. *PLoS One* **14**: e0221017. [Medline] [CrossRef]