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| 1 | Isolation and Characterization of an Orthoreovirus from Indonesian Fruit Bats |
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27 Abstract

28 Nelson Bay orthoreovirus (NBV) is an emerging bat-borne virus and causes respiratory 29 tract infections in humans sporadically. Over the last two decades, several strains genetically 30 related to NBV were isolated from humans and various bat species, predominantly in Southeast Asia (SEA), suggesting a high prevalence of the NBV species in this region. In this study, an 31 32 orthoreovirus (ORV) belonging to the NBV species was isolated from Indonesian fruit bats' feces, tentatively named Paguyaman orthoreovirus (PgORV). Serological studies revealed that 33 34 81.2% (108/133) of Indonesian fruit bats sera had neutralizing antibodies against PgORV. 35 Whole-genome sequencing and phylogenetic analysis of PgORV suggested the occurrence of 36 past reassortments with other NBV strains isolated in SEA, indicating the dispersal and 37 circulation of NBV species among bats in this region. Intranasal PgORV inoculation of 38 laboratory mice caused severe pneumonia. Our study characterized PgORV's unique genetic background and highlighted the potential risk of PgORV-related diseases in Indonesia. 39

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41 Keywords: Orthoreovirus; Nelson Bay orthoreovirus; Indonesia; Fruit bats; Virus isolation;
42 Viral pathogenicity

43 Introduction

44 Nelson Bay orthoreovirus (NBV) is an emerging bat-borne virus that sporadically causes respiratory tract infection in humans [1–5]. The NBV prototype strain was isolated from 45 46 the blood of a flying fox (Pteropus poliocephalus) collected in Nelson Bay, Australia, in 1968 and was designated as Nelson Bay virus [6]. NBV belongs to the genus Orthoreovirus (ORV) 47 48 in the subfamily Spinareovirinae, the family Reoviridae. Ten species belong to this genus, including Mammalian orthoreovirus (MRV), Avian orthoreovirus (ARV), Nelson Bay 49 50 orthoreovirus (NBV), Baboon orthoreovirus (BRV), Reptilian orthoreovirus (RRV), Mahlapitsi 51 orthoreovirus (MAHLV), Piscine orthoreovirus (PRV), Broome orthoreovirus (BrRV), 52 Neoavian orthoreovirus (NARV), and Testudine orthoreovirus (TRV). The common ORV 53 characteristics include a non-enveloped, icosahedral shape consisting of ten linear double strand 54 RNA segments with three large segments (L1–L3), three medium segments (M1–M3), and four 55 small segments (S1-S4). ORV can be subdivided into fusogenic and non-fusogenic groups 56 based on the ability to form multinucleated syncytial cells. Most ORV species, except for MRV 57 and PRV, are fusogenics that encode the fusion-associated small transmembrane (FAST) 58 protein influencing syncytial formation.

59 Bats serve as important natural reservoirs of several pathogens, including ORVs. MRV, 60 NBV, and BrRV have been isolated from insectivorous and frugivorous bats [6-19]. Bat MRVs 61 were mostly detected in insectivorous bats in Europe and China [7–10], while NBV species 62 were found in fruit bats [6,11,12,14–19]. NBV species have been reported globally in Australia, Southeast Asia (SEA), China, and Africa [1–6,11,12,14–19]. Among 17 strains of NBV species 63 discovered, 12 have been isolated from humans and various fruit bat species in Malaysia, 64 65 Indonesia, and the Philippines, indicating NBV distribution in the SEA region [1–5,14,17–19]. Several NBV strains were isolated from patients with acute respiratory tract diseases who had 66 67 previous history of close contact with bats or traveled to SEA countries, suggesting interspecies transmission between bats and humans [1–5]. Additionally, NBV strains, Melaka virus (MelV)
and Kampar virus (KamV), caused human-to-human transmission in Malaysia [4,5].
Consequently, NBV infection could cause respiratory disease in humans and is an emerging
public health concern.

Indonesian fruit bats are carriers of many viruses, including herpesviruses, 72 73 parvoviruses, polyomaviruses, paramyxoviruses, and coronaviruses [20–26]. Moreover, NBV 74 infection incidence has been reported in four human cases in Indonesia [2,3]; however, 75 knowledge about the prevalence of NBV in bats and humans in the country is insufficient. 76 Previous studies established two members of NBV strains isolated from flying foxes (Pteropus 77 vampyrus) captured in Indonesia, named Indonesia/2010 and Garut-69 [17,19]. Here we report 78 a novel member of NBV species isolated from fruit bats in Indonesia, designated as Paguyaman 79 orthoreovirus (PgORV). Viral characterization in vitro and in vivo suggested the possibility of 80 genetic reassortment events with other NBV strains, and intranasal inoculation of laboratory 81 mice with the isolated virus caused severe pneumonia. The serological examination revealed a 82 high prevalence of NBV infection in Indonesian fruit bats.

83

84 Materials and Methods

Vero E6, BHK-21 (BHK), A549 cells were cultured and maintained in Dulbecco's
Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS). HEK293T (293T) cells
were grown in high glucose DMEM with 10% FBS, and Caco-2 cells were cultured in a
collagen-coated plate in DMEM supplemented with 10% FBS and 1% non-essential amino
acid solution. Human serine protease TMPRSS2- stably expressing Vero E6 cells (Vero T2)
were previously described [27]. NBV Miyazaki-Bali/2007 strain (MB) [2] was propagated in
293T cells, and the virus stock was titrated by plaque assay using Vero E6 cells.

⁸⁵ *Cells and viruses*

93

94 Bat samples and ethics statements

95 Blood, feces and organs were collected from fruit bats in eight areas of Indonesia between 2010 and 2014 (Table 1). The samples were previously used for other virus screening 96 [20,21,23–26]. The Animal Care and Use Committee of Veterinary Teaching Hospital, IPB 97 98 University approved the protocol for bat sampling (permit number 05-2010 RSHP-IPB). 99 Collection and exportation of samples from fruit bats were performed under the permission of 100 the Directorate General of Livestock and Animal Health Services, Ministry of Agriculture, 101 Republic of Indonesia. As previously described, the bat species were identified by the external 102 morphology and the nucleotide sequence analysis of mitochondrial 16S RNA and cytochrome 103 b gene [26]. The Institutional Animal Care and Use Committee of Hokkaido University 104 approved the ethics for the viral inoculation to laboratory animal experiments (approval 105 number 20-0026).

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107 Virus titration by plaque assay

Confluent monolayer of Vero E6 in a 12 well plate was inoculated with a serially diluted virus solution, and incubated for 1 hour for viral absorption. Then, the cells were overlaid with DMEM containing 2% FBS and 0.4% agar. After three days post-inoculation (dpi), the cells were fixed with 10% buffered formalin and stained with 1% crystal violet. The viral titer was estimated as plaque-forming unit (pfu) based on the number of plaques.

113

114 Plaque reduction neutralization test (PRNT)

115 Sera from a total of 133 Indonesian fruit bats were heat-inactivated at 56°C for 30 min 116 and serially diluted four-folds from 1: 10 to 1: 640. The diluted sera were mixed with an equal 117 volume of DMEM with 2% FBS containing 100–200-pfu of either MB or PgORV and 118 incubated for 1 hour at 37° C. The mixture of the virus and serum (100 µl) was subjected to a 119 plaque assay using Vero E6 cells as described above. Neutralizing antibody titers were 120 determined as the highest dilution, which achieved more than 80% reduction in plaque 121 numbers.

122

123 Screening of the ORV genome by nested RT-PCR

124 Total RNA was extracted from feces using a High Pure Viral Nucleic Acid Kit (Roche 125 Diagnostics, Mannheim, Germany). Lungs were homogenized in TRIzol reagent (Invitrogen; 126 Thermo Scientific, Waltham, USA) and subjected to RNA extraction using a Direct-Zol RNA 127 MiniPrep kit (Zymo Research, Irvine, USA). The RNA samples were subjected to ORV 128 screening by nested RT-PCR using two sets of degenerate primers targeting the ORV RNA-129 dependent RNA polymerase (RdRp) gene as described previously [28]. The first round RT-130 PCR was performed using 1607F primer (5'-CARMGNCGNSCHMGHTCHATHATGCC-3'), 2608R primer (5'-TAVAYRAAVGWCCASMHNGGRTAYTG-3') and PrimeScript OneStep 131 132 RT-PCR Kit Ver.2 (Takara Bio, Kusatsu, Japan). The second round PCR was performed with 133 2090F primer (5'-GGBTCMACNGCYACYTCBACYGAGCA-3'), 2334R primer (5'-134 CDATGTCRTAHW YCCANCCRAA-3') and TaKaRa ExTaq Hot Start Version (Takara Bio). 135 Amplicons at approximately 300 bp were subjected to sequencing using Big Dye Terminator 136 v3.1 Cycle Sequencing Kit (Applied Biosystems; Thermo Scientific).

137

138 Virus isolation

Fecal samples positive for the ORV genome were suspended in phosphate buffer saline
(PBS) at 10% w/v. The suspensions were inoculated onto Vero T2 cells. Cells were maintained
in serum-free DMEM supplemented with 2% antibiotic-antimycotic solution (Wako, Osaka,
Japan), gentamicin (25 μg/ml), and trypsin (0.5 μg/ml). The culture supernatant was passaged

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146 *Whole-genome sequencing*

Total RNA was extracted from five viral clones (ORV13-7, ORV13-8, ORV13-20, 147 148 ORV13-25, and ORV13-27) and transcribed into double-stranded cDNA using PrimeScript 149 Double Strand cDNA Synthesis Kit (Takara Bio). The sequence library was constructed using 150 Nextera XT DNA Library Preparation Kit and sequenced on an Illumina MiSeq instrument 151 (Illumina, San Diego, USA). The obtained sequence reads were assembled into contigs via de 152 novo assembly or mapped to reference genome segments of MB strain (GenBank accession; 153 AB908278-AB908287) using CLC Genomics Workbench 20.1 (Qiagen, Hilden, Germany) 154 and Geneious 2021.1.1 software (Biomatters, Newark, USA). Nucleotide sequences of 155 ORV13-27 segments were deposited in DDBJ with the accession numbers LC632072-156 LC632081.

into fresh monolayer cells after 5-dpi. The supernatants from wells showing a cytopathic effect

(CPE) were harvested and maintained at -80°C as a master stock of isolated viruses.

157

158 Phylogenetic analysis

Reference nucleotide sequences of NBV species were obtained from the GenBank database and aligned with PgORV sequences. The best fit mathematical models for phylogenetic analyses of each segment were determined using MEGA7 software [29]. Phylogenetic trees were constructed using the maximum likelihood method with 1,000 bootstrap replicates.

164

165 **Replication efficiency in different cell lines**

BHK, Vero E6, Vero T2, A549, Caco-2, and 293T cells were inoculated with PgORV
(ORV13-27) at two different multiplicities of infection per cell (MOI = 0.01 or 0.0001) and

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maintained in culture media containing 2% FBS. Cell culture supernatants containing progeny
viruses were harvested at 8, 24, 48, and 72 hours post-infection (hpi), and titrated by a plaque
assay using Vero E6 cells.

171

172 Immunofluorescence assay (IFA)

173 Cells were seeded in a 24-well plate before inoculation with PgORV (ORV13-27, MOI = 0.01 or 0.0001). Cells were fixed with 10% buffered formalin at 14-hpi and permeabilized 174 175 with 0.5% Triton X-100 in PBS. After blocking with 50% Block Ace (KAC, Kyoto, Japan), 176 cells were incubated with guinea pig anti-NBV Nachunsulwe-57 polyclonal antibody, which 177 cross-reacted with PgORV [15] (Supplementary Figure S1) and Alexa 488-conjugated goat 178 anti-guinea pig IgG (Invitrogen) as a secondary antibody. Cell nuclei were stained with 179 Hoechst 33342 (Invitrogen) and the fluorescence signal was examined using a fluorescence 180 microscope (IX73; Olympus, Tokyo, Japan).

181

182 Animal experiments

Male BALB/c mice (4–6 week-old, Japan SLC, Hamamatsu, Japan) were intranasally inoculated with PgORV (1×10⁶ pfu of ORV13-27 in 50 μ l PBS) or mock (50 μ l PBS). Body weight (BW) was monitored daily up to 14-dpi and 20% BW loss was defined as a humane endpoint. To evaluate PgORV's growth and pathogenicity in laboratory mice, the lung was harvested at 1, 3, and 5-dpi for viral load measurement (n = 5) and histopathological examination (n = 3).

189

190 Measurement of the viral RNA load and cytokine gene expression in lung tissue

191The lungs from mice experimentally infected with PgORV were homogenized in PBS192at 10% w/v using TissueRuptor (Qiagen). Total RNA was extracted from the lung homogenates

with TRIzol LS reagent and Direct-Zol RNA MiniPrep kit. qRT-PCR assay was conducted 193 194 using OneStep PrimeScript III RT-qPCR Mix (Takara Bio) with the primers and probe 195 targeting the L1 segment of NBV species as described previously [30], TaqMan mouse β-actin 196 Endogenous Control (Mm00607939 s1, Applied Biosystems) or predesigned PrimeTime qPCR Assays for TNF (Ms.PT.58.12575861), IFN-γ (Ms.PT.58.41769240), IFN-β 197 198 (Ms.PT.30132453.g), IL-6 (Ms.PT.58.10005566), CCL2 (Ms.PT.58.42151692) and CXCL10 (Ms.PT.58.43575827) (IDT, Coralville, USA). The levels of viral RNA and cytokine gene 199 expression were normalized to β -actin and calculated the relative gene expression using $2^{-\Delta Ct}$ 200 or $2^{-\Delta\Delta Ct}$ method [31]. 201

202

203 Histopathology and immunohistochemistry (IHC)

204 Lungs from the PgORV-inoculated mice were fixed in 10% buffered formalin, embedded in paraffin blocks, and sectioned at 4 µm. The sections were stained with 205 206 Hematoxylin and Eosin for histopathological examination. To detect PgORV antigen in lungs, 207 the sections were deparaffinized and heated for three minutes in citrate buffer for antigen 208 retrieval. The slides were blocked with 50% Block Ace and incubated with guinea pig anti-NBV Nachunsulwe-57 polyclonal antibody [15]. Immunostaining was visualized using 209 210 VECTASTAIN ABC guinea pig IgG Kit (Vector laboratories, Burlingame, USA) and Histofine diaminobenzidine substrate kit (Nichirei Biosciences, Tokyo, Japan). 211

212

213 Statistical analysis

Data were analyzed and presented as the mean with standard deviation (SD). Statistical analysis was performed using Prism 8 (GraphPad Software, San Diego, USA). Survival analysis was performed using Kaplan-Meier estimator. One-way ANOVA with Dunnett's test was used to determine the statistical significance of cytokine gene expression in the mouselungs.

219

220 Results

221 Screening and isolation of orthoreovirus in Indonesian fruit bats

222 To estimate NBV infection among fruit bats in Indonesia, the neutralizing activity of fruit bat serum samples to NBV MB strain by PRNT was examined. Among 133 serum 223 224 samples, 118 (89.5%) had neutralizing activity against MB (1: 20 to 1: 1,280) (Table 1). 225 Seropositive bats were identified from Pteropus vampyrus (47/47), Pteropus sp. (42/42), 226 Dobsonia moluccensis (16/16), and Acerodon celebensis (14/28) (Table 1). ORV genome in 227 feces (96) and lungs (172) from fruit bats was also examined by nested RT-PCR. The ORV 228 genome was detected in the feces from *Pteropus* sp. (3/8) and *Acerodon celebensis* (3/7)collected in 2013, but not in the lungs (Table 1). Sequence analysis and BLAST search showed 229 230 that the amplicons had high nucleotide sequence identity to the RdRp gene of NBV species.

231 Next, we attempted to isolate ORV from bat feces. Six fecal suspensions positive for 232 the ORV genome were inoculated into Vero T2 cells. Finally, five of six inocula (Sample ID: 13-7, 13-8, 13-20, 13-25, and 13-27) induced CPE and syncytium formation in cells, 233 234 characteristic of fusogenic ORV infection. After the first passage, five viral clones from sample 235 ID: 13-7, 13-8, 13-20, 13-25, and 13-27 were successfully isolated. It was confirmed that 236 isolated viruses were ORVs using RT-PCR and nucleotide sequencing of the ORV RdRp gene. 237 The information of five bat samples positive for virus isolation is shown in Supplementary Table S1. 238

239

240 Whole-genome sequencing and phylogenetic analysis

We conducted complete genome sequencing of the five isolated ORVs (designated as ORV13-7, ORV13-8, ORV13-20, ORV13-25, and ORV13-27). Sequence comparison of the five viral clones displayed more than 99% nucleotide identity, indicating that all clones were the same virus strain. The isolated virus was designated as Paguyaman orthoreovirus (PgORV) based on where the bat carrying the virus was captured (Supplementary Table S1).

246 ORV13-27 was chosen from the five clones as a representative PgORV clone for further 247 characterization. The complete PgORV genome was 23,406 bp in total length, and the lengths 248 of the ten segments are shown in Table 2. Sequence analysis demonstrated that PgORV had 249 the same genome organization as other NBV strains. All the segments except for S1 encode 250 only one protein (monocistronic), but the PgORV S1 segment encodes three viral proteins 251 (tricistronic) (Table 2). The terminal sequences of the 5'-untranslated region (5'-UTR) and the 252 3'-UTR of the segments (GCUUhh and UCAUC, respectively) were conserved among the 253 genus Orthoreovirus. BLAST search revealed that L1, L3, M1-M3, and S4 segments shared 254 the highest nucleotide sequence identities with those of MB (Table 2). Phylogenetic analysis 255 showed that S1 was the most genetically divergent among the segments (Figure 1A). The 256 PgORV-S1 segment shared the same ancestor with NBVs from human patients in Malaysia 257 (Sikamat and Melaka) and NBVs from macaques in Thailand (Lopburi 01 and Lopburi 02) 258 [1,5,32]. L1, L3, M1, M2, M3 and S4 segments of PgORV formed clusters with MB and other NBV strains originated in Indonesia, including Indonesia/2010, Garut-69, HK23629/07 259 260 (HK/07), HK46886/09 (HK/09), and HK50842/10 (HK/10) strains (Figure 1B) [2,3,17,19]. 261 Conversely, L2, S2, and S3 segments of PgORV were closely related to Talikud and Samal strains isolated from bats in the Philippines [14]. L2 and S3 displayed 89.1% and 93.6% 262 nucleotide sequence identity with Talikud-74 and Samal-24, respectively, while the nucleotide 263 264 sequence of the S2 segment was highly similar to the prototype NBV (89.6% identity).

265

266 Seroprevalence of PgORV in Indonesian fruit bats

The neutralizing activity of fruit bat serum samples to PgORV infection was evaluated using PRNT. The results showed that 81.2% (108/133) of bat sera were positive for PgORV infection neutralization, and the seropositive rate was slightly lower than that of MB (89.5%) (Table 1 and Table 3). Most bat sera showed neutralizing activity against both PgORV and MB, suggesting a possible cross-neutralization between these NBV members (Supplementary Figure S2). However, 57.9% (77/133) of bat sera exhibited a higher neutralizing titer against MB than PgORV.

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275 Growth properties of PgORV in cell lines

276 Using several cell lines, we characterized the infectivity and growth kinetics of PgORV 277 in vitro. For the immunofluorescence staining of the PgORV antigen, convalescent serum from 278 a guinea pig infected with NBV Nachunsulwe-57 strain was employed as a primary antibody 279 [15]. IFA analysis showed that all examined cell lines were susceptible to the infection with 280 PgORV at an MOI of 0.01 (Figure 2A). Morphologically, distinct syncytia formations were 281 observed in BHK, Vero E6, and Vero T2 cells, but almost all cells were infected and formed 282 huge syncytia in Caco-2 and 293T cells. In A549 cells, some cells detached from the culture 283 plates at 14-hpi. The virus titers in BHK supernatants, Vero E6 and Vero T2 cells, increased 284 over time (Figure 2B). Contrastly, the virus titers in A549, Caco-2, and 293T cells peaked at 285 24 or 48-hpi, presumably due to CPE and cell detachment by PgORV infection. To observe 286 multiple cycles of viral infection in A549, Caco-2, and 293T cells, cells were inoculated with PgORV at a MOI of 0.0001. Infected cells were scarcely observed in BHK, Vero E6, and Vero 287 288 T2 cells, but A549, 293T, and Caco-2 cells were highly sensitive to the infection even at the 289 lower MOI (Figure 2C). The virus titers in the supernatants of A549, 293T, and Caco-2 cells 290 also time-dependently increased, and the 293T cells had the highest progeny virus production 291 (Figure 2D).

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Pathogenicity of PgORV in laboratory mice

294 To investigate PgORV pathogenicity, we inoculated BALB/c mice via the intranasal route with 1×10^6 pfu virus. The mice were monitored for BW and sacrificed for viral titer and 295 histopathological changes in the lungs (Figure 3A). The mice BW continuously decreased from 296 297 1-dpi to 4-dpi PgORV and then turned to recovery (Figure 3B). Five of eight mice inoculated 298 with PgORV reached an endpoint due to severe BW loss (>20%) at 4-dpi and 6-dpi (Figure 299 3C). The other three mice recovered and survived the infection. The viral titer and viral RNA 300 level in the lung were quantified using plaque assay and qRT-PCR, respectively. The peak of 301 viral titer was observed in the lung of mice at 1-dpi PgORV, and the titer decreased time-302 dependently (Figure 3D). Similarly, the viral RNA load was also the highest at 1-dpi and 303 gradually decreased (Figure 3E).

304 Additionally, histopathological changes in the lungs after PgORV inoculation was 305 examined. Macroscopically, the lungs of mice inoculated with PgORV exhibited hyperemia at 306 3 and 5-dpi (Figure 4A). Histopathological examination revealed hemorrhage and infiltration 307 of inflammatory cells in lungs at 1, 3, and 5-dpi, and massive infiltration of inflammatory cells 308 was observed at 5-dpi (Figure 4B). Viral antigens were detected in bronchial and alveolar 309 epithelial cells at 1 and 3-dpi using IHC; however, little IHC signal was detected at 5-dpi 310 (Figure 4C). Pneumonia severity correlated conversely with decreased viral load, as indicated 311 by IHC, qRT-PCR, and plaque assay. These results imply that the host immune response to 312 the infection eliminated the virus in the lung but caused severe inflammation.

313 To investigate the host immune responses against PgORV infection, we examined the expression levels of inflammatory cytokine genes in PgORV-inoculated mice lungs using 314

315 qRT–PCR. The levels of pro-inflammatory cytokines, including TNF, IFN- β , and IL-6 as well 316 as chemoattractants (CCL2 and CXCL2), were significantly increased in PgORV-infected 317 mice lungs at 1-dpi, indicating the early host responses to viral infection. Contrastly, IFN- γ , 318 which influences viral clearance, was significantly upregulated in mice lungs at 5-dpi (Figure 319 4D), supporting the hypothesis of the viral clearance by host immune responses in mice.

320

321 Discussion

322 Since NBV discovery, several NBV strains have been identified from fruit bats 323 [6,11,12,14–19]. Approximately 75% of NBV strains were isolated in SEA countries, 324 indicating a high NBV prevalence in this region. Currently, six NBV strains, including HK/07, 325 HK/09, HK/10, MB, Indonesia/2010, and Garut-69, have been reported in Indonesia 326 [2,3,17,19]. Three HK strains and MB were isolated from the respiratory tract specimens of 327 patients who had a history of visiting Bali, Indonesia, before the onset [2,3]. Garut-69 was 328 isolated from *Pteropus vampyrus* in West Java Island [19]; however, there was no information 329 on sampling sites of bats from which Indonesia/2010 was detected [17]. In this study, we 330 detected NBV seropositive bats from West Sumatra (Lima Puluh Kota), West Java (Panjalu, 331 Magelang, and Surabaya), North Sulawesi (Popayato and Paguyaman), and South Sulawesi 332 (Soppeng and Sidrap), indicating the broad NBV infection dispersal among Indonesian fruit 333 bats.

L1, L3, M1, M2, M3, and S4 segments of PgORV showed high nucleotide sequence identities (93.4%–98.2%) with MB, suggesting that PgORV and MB evolved from a common ancestor. However, the remaining L2, S1, S2, and S3 segments of PgORV were phylogenetically distant from those of MB and related to other NBVs. This phylogenetic incongruence suggests genetic reassortments between NBVs in fruit bats, reported in several studies [12,14,16,17,19,32]. The fact that bats can fly long distances and move between islands and countries may contribute to a long-distance dispersal and genetic reassortment of ORVs
[33–35]. Although multiple NBV strains were isolated from SEA countries, we could not
recognize clear relationship between phylogeny and geographic distribution of NBVs (Figure
1).

Previous studies reported that 23, 83, and 98% of fruit bats were NBV seropositive in 344 345 China, the Philippines, and Zambia, respectively [14–16]. Similarly, this study revealed that 346 81.2-89.5% of Indonesian fruit bat sera had neutralizing antibodies against NBV. Bats are 347 highly diversified with over 1,400 species worldwide and almost 200 species of fruit bats. 348 Various fruit bat species, including Pteropus poliocephalus, Pteropus hypomelanus, Rousettus 349 leschenautia, Rousettus amplexicaudatus, Rousettus aegyptiacus, Eonycteris spelaea, 350 Macroglossus minimus, Lissonycteris angolensis ruwenzorii, and Eidolon helvum, were 351 evidenced for NBV infections [6,11,12,14–19]. Here, we showed the NBV-seroprevalence in 352 Acerodon celebensis and Dobsonia moluccensis. In addition, we have identified PgORV from 353 fruit bats belonging to two different genera (Pteropus sp. and Acerodon celebensis). Other 354 study also isolated NBVs with almost identical genome sequences from Eonycteris spelaea 355 and Rousettus amplexicaudatus [14]. Collectively, these findings suggest that at least some 356 NBVs broadly distribute among fruit bats with frequent interspecies transmissions.

357 Our PRNT assay revealed a high seropositivity rate for both NBVs, PgORV, and MB 358 in Indonesian fruit bats. Among NBV members, PgORV was phylogenetically close to MB; 359 therefore, it was supposed that the antibodies could cross-react with both NBVs. Crossneutralization has been observed in several strains of NBV species. Antisera to the prototype 360 NBV cross-reacts with Pulau (PuV), MelV, and KamV [4]. Antisera against MB has also 361 362 showed the cross-reactivity to NBVs of Samal-24 and Talikud-82 [14]. Neutralizing antibodies 363 against Nachunsulwe-57 cross-reacted with MB [15]. Additionally, our PRNT assays showed that a subset of bat sera exhibited higher neutralizing activity to MB strain than PgORV, 364

suggesting that these bats would be exposed to MB or MB-related NBV rather than PgORV.
For these reasons, we cannot exclude the possibility of cross-reactivity between PgORV and
known/unknown NBV members in our PRNT assay.

368 After replication in host cells, the virus spreads to adjacent cells via two modes; cellfree and cell-to-cell transmissions [36–38]. The multinuclear syncytium is a characteristic of 369 370 cells infected with PgORV. The IFA detected the syncytia with PgORV antigen in BHK, Vero 371 E6, and Vero T2 cells at 14-hpi. However, the progeny virus titers in the culture supernatant 372 of these cell lines were under the detection limit at 24-hpi. These results suggest that the 373 primary main PgORV spreading mode is cell-to-cell mechanism. Similar to MB, Samal-24, 374 and Garut-50 [14,39], human A549, Caco-2, and 293T cells, were highly susceptible to PgORV 375 infection. Although we employed Vero T2 for virus isolation, using A549, Caco-2, and 293T 376 cells could be a better outcome for NBV isolation.

The mortality rate of PgORV-inoculated mice (62.5%) was lower than that of MB-377 378 inoculated mice (100%), indicating that MB is more pathogenic than PgORV in mice [40,41]. 379 As with the case of MB and Samal-24 [40], PgORV infection caused severe pneumonia in mice 380 lungs. Hemorrhage and infiltration of inflammatory cells were massively present in the lungs 381 at 3 and 5-dpi, while cells positive for viral antigen were abundant at 1-dpi and subsequently 382 decreased at 3 and 5-dpi, indicating PgORV clearance from the host. The manifestation of 383 PgORV-infected mice was uncorrelated with the viral load in the lungs. This discrepancy is 384 observed in mouse models infected with influenza, respiratory syncytial, and corona viruses 385 [42–44]. The expression levels of pro-inflammatory cytokines (TNF, IFN-β1, and IL-6) and chemoattractants (CCL2 and CXCL10) were significantly upregulated at 1-dpi for the initial 386 387 responses to viral infection, and subsequent recruitment of inflammatory cells causing IFN-y expression induction. TNF and IFN- γ influence virus clearance and cause immunopathology 388 389 [45,46].

In conclusion, we isolated PgORV from fruit bats, which can cause respiratory disease in mammals. We also demonstrated a high seroprevalence of NBV in multiple species of fruit bats in different areas of Indonesia. Our study emphasizes the broad distribution and circulation of NBV among fruit bats in Indonesia. Further investigations in bats, other animals, and humans should be conducted to evaluate the current burden of NBV infections in Indonesia.

395

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403 **Declaration of interest statement**

404 K.U. is an employee of Shionogi & Co., Ltd. The authors declare no competing interests.

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 Death, Tissue Damage, and Mortality in SARS-CoV-2 Infection and Cytokine Shock Syndromes. Cell
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- 503

504 Figure legend

505 Figure 1. Phylogenetic analysis of Paguyaman orthoreovirus (PgORV).

506 (A and B) Nucleotide sequences of 10 genome segments (L1-L3, M1-M3 and S1-S4) of

507 reference orthoreoviruses and PgORV were aligned. Phylogenetic trees of S1 segment (A)

508 and other segments (B) were constructed using the maximum likelihood method. Bootstrap

509 values of 1,000 replicates are shown in each node and the scale bars represent the number of

510 nucleotide substitutions per site. The host which the virus was identified, is shown in the

511 phylogenetic tree of S1 segment (A). Each color indicates the country where the virus was

512 identified (A and B).

513

514 Figure 2. Infectivity and growth of PgORV in cell lines.

515 Detection of PgORV infection by immunofluorescence staining (A, C). Cells were inoculated 516 with the virus at multiplicities of infection (MOIs) of 0.0001 and 0.01 and fixed at 14 hours 517 post infection. The cells were stained with guinea pig anti-NBV (Nachunsulwe-57) 518 polyclonal antibody for PgORV (green) and Hoechst 33342 for cell nucleus (blue) (scale 519 bars: 500 μ m). (B, D) Cells were inoculated with PgORV at MOIs of 0.0001 and 0.01. Virus 520 titers in cell culture supernatants at the indicated time points were determined by plaque 521 assay.

522

Figure 3. Infection of laboratory mice with PgORV. (A) Scheme of experimental infection
in BALB/c mice. Mice were intranasally infected with 1×10⁶ pfu of PgORV or PBS as
control. (B) The average percentage of body weight changes of mice after PgORV infection.
(C) Survival rate of mice infected with PgORV. A humane endpoint was applied when the
mice showed more than 20% body weight loss. (D) Virus titer in the lung at the indicated
time points were determined by plaque assay. (E) Viral RNA levels in the lungs were

529 normalized to the mRNA levels of mouse β-actin (ACTB) and the relative expression of L1 530 gene was calculated by $2^{-\Delta Ct}$ method.

531

Figure 4. Pathological change and cytokine expression profile in the lung of mice infected with PgORV. BALB/c mice were infected intranasally with 1×10⁶ pfu of PgORV. (A) Macroscopic appearances of mice lungs after infection. (B) Hematoxylin and Eosin (H&E) staining of the lung sections. (C) Detection of PgORV antigen in the lungs by

536 immunohistochemistry. Lung sections were stained with guinea pig anti-NBV

537 (Nachunsulwe-57) polyclonal antibody and counterstained of cell nuclei with Hematoxylin.

538 Scale bars of the left panels (a, c, e, g): 200 µm, right panels (b, d, f, h): 50 µm. (D) Cytokine

539 expression levels in the mice lungs. Data were normalized to β -actin and the relative gene

540 expression level of each cytokine was calculated by $2^{-\Delta\Delta Ct}$ method. One-way ANOVA with

541 Dunnett's test was used to determine the statistical significance of differences between

542 uninfected control and infected mice. *, p < 0.05; **, p < 0.01, ***, p < 0.001; ****, p

543 <0.0001.

544 **Table 1.** Screening of neutralizing antibody against NBV Miyazaki-Bali/2007 strain and

545 ORV genome in Indonesian fruit bats

| | Location | Bat species | Number of positive samples | | | | | | | |
|-------|-----------------|---------------------------|----------------------------|------------|---------------|--------|----------------|-----------------|-------|--|
| Year | | | N | eutralizin | Nested RT-PCR | | | | | |
| | | | 1:20 | 1:80 | 1:320 | 1:1280 | Total (%) | Feces | Lung | |
| 2010 | Panjalu | Pteropus vampyrus | 1 | 5 | 7 | 2 | 15/15 (100) | NA ^a | 0/15 | |
| 2011 | Lima Puluh Kota | Pteropus vampyrus | 0 | 4 | 4 | 2 | 10/10 (100) | NA | 0/20 | |
| | Paguyaman | Pteropus sp. ^b | 4 | 4 | 5 | 5 | 18/18 (100) | NA | 0/23 | |
| | Popayato | Pteropus sp. | 0 | 0 | 1 | 2 | 3/3 (100) | NA | 0/4 | |
| 2012 | Paguyaman | Dobsonia moluccensis | 0 | 7 | 6 | 3 | 16/16 (100) | 0/17 | 0/17 | |
| | | Acerodon celebensis | 2 | 4 | 2 | 1 | 9/18 (50) | 0/18 | 0/18 | |
| | | Pteropus sp. | 0 | 0 | 2 | 0 | 2/2 (100) | 0/2 | 0/2 | |
| | Surabaya | Pteropus vampyrus | 0 | 1 | 0 | 2 | 3/3 (100) | 0/3 | 0/3 | |
| | Magelang | Pteropus vampyrus | 3 | 3 | 5 | 8 | 19/19 (100) | 0/19 | 0/20 | |
| 2013 | Paguyaman | Pteropus sp. | 0 | 1 | 6 | 3 | 10/10 (100) | 3/8 | 0/10 | |
| | | Acerodon celebensis | 2 | 2 | 1 | 0 | 5/10 (50) | 3/7 | 0/18 | |
| 2014 | Soppeng | Pteropus sp. | 0 | 0 | 3 | 3 | 6/6 (100) | 0/7 | 0/7 | |
| | Sidrap | Pteropus sp. | 0 | 0 | 1 | 2 | 3/3 (100) | 0/15 | 0/15 | |
| Total | | | 12 | 31 | 43 | 33 | 119/133 (89.5) | 6/96 | 0/172 | |

^aNA; sample not available, ^b*Pteropus* sp.; Bat genetically closely related to *Pteropus*

547 hypomelanus.

Table 2. Molecular characteristics of orthoreovirus isolated from Indonesian fruit bat

| 549 | (ORV13-27). |
|-----|-------------|
| | |

| Genome | Accession | Size (bp) | Terminal sequences | | Encoded | Closest strain 550 | |
|---------|-----------|-----------|--------------------|--------|--------------|---------------------------|--|
| segment | NO. | | 5' UTR | 3' UTR | - protein(s) | (% nucleotide identity) | |
| L1 | LC632072 | 3896 | GCUUUA | UCAUC | λC | Miyazaki-Bali/2007 (98-2) | |
| L2 | LC632073 | 3832 | GCUUUA | UCAUC | λΒ | Talikud-74 (89.1) | |
| L3 | LC632074 | 3954 | GCUUUA | UCAUC | λΑ | Miyazaki-Bali/2007 (93.4) | |
| M1 | LC632075 | 2295 | GCUUUA | UCAUC | μA | Miyazaki-Bali/2007 (94.7) | |
| M2 | LC632076 | 2145 | GCUUAU | UCAUC | μB | Miyazaki-Bali/2007 (96.5) | |
| М3 | LC632077 | 1984 | GCUUAU | UCAUC | μNS | Miyazaki-Bali/2007 (96.4) | |
| S1 | LC632078 | 1602 | GCUUAA | UCAUC | p10, p17, σC | Sikamat (84.5) | |
| S2 | LC632079 | 1322 | GCUUAA | UCAUC | σΑ | Nelson bay virus (89.6) | |
| S3 | LC632080 | 1192 | GCUUAU | UCAUC | σNS | Samal-24 (93.6) | |
| S4 | LC632081 | 1184 | GCUUAU | UCAUC | σΒ | Miyazaki-Bali/2007 (96.6) | |

| | | | Number of positive samples Neutralizing antibody titer (PRNT ₈₀) | | | | | | |
|-------|-----------------|----------------------|---|------|-------|--------|----------------|--|--|
| Year | Location | Bat species | | | | | | | |
| | | | 1:20 | 1:80 | 1:320 | 1:1280 | Total (%) | | |
| 2010 | Panjalu | Pteropus vampyrus | 4 | 6 | 3 | 1 | 14/15 (93.3) | | |
| 2011 | Lima Puluh Kota | Pteropus vampyrus | 1 | 6 | 3 | 0 | 10/10 (100) | | |
| | Paguyaman | Pteropus sp.ª | 6 | 5 | 2 | 3 | 16/18 (88.9) | | |
| | Popayato | Pteropus sp. | 0 | 0 | 1 | 2 | 3/3 (100) | | |
| 2012 | Paguyaman | Dobsonia moluccensis | 2 | 6 | 8 | 0 | 16/16 (100) | | |
| | | Acerodon celebensis | 6 | 2 | 0 | 0 | 8/18 (44.4) | | |
| | | Pteropus sp. | 2 | 0 | 0 | 0 | 2/2 (100) | | |
| | Surabaya | Pteropus vampyrus | 1 | 0 | 0 | 2 | 3/3 (100) | | |
| | Magelang | Pteropus vampyrus | 5 | 6 | 4 | 2 | 17/19 (89.5) | | |
| 2013 | Paguyaman | Pteropus sp. | 0 | 7 | 2 | 0 | 9/10 (90) | | |
| | | Acerodon celebensis | 1 | 1 | 0 | 0 | 2/10 (20) | | |
| 2014 | Soppeng | Pteropus sp. | 1 | 3 | 1 | 0 | 5/6 (83.3) | | |
| | Sidrap | Pteropus sp. | 2 | 1 | 0 | 0 | 3/3 (100) | | |
| Total | | | 31 | 43 | 24 | 10 | 108/133 (81.2) | | |

Table 3. Neutralizing antibody against PgORV in Indonesian fruit bats.

^a*Pteropus* sp.; Bat genetically closely related to *Pteropus hypomelanus*









