

Serologic Evidence of Fruit Bat Exposure to Filoviruses, Singapore, 2011–2016

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To determine whether fruit bats in Singapore have been exposed to filoviruses, we screened 409 serum samples from bats of 3 species by using a multiplex assay that detects antibodies against filoviruses. Positive samples reacted with glycoproteins from Bundibugyo, Ebola, and Sudan viruses, indicating filovirus circulation among bats in Southeast Asia.

The genus *Ebolavirus* comprises 5 virus species: *Zaire ebolavirus* (EBOV), *Sudan ebolavirus* (SUDV), *Bundibugyo ebolavirus* (BDBV), *Tai Forest ebolavirus* (TAFV), and *Reston ebolavirus* (RESTV). The genus *Marburgvirus* comprises 1 species, *Marburg marburgvirus*, which includes 2 closely related virus strains: Marburg virus (MARV) and Ravn virus (RAVV). Viruses within the *Ebolavirus* and *Marburgvirus* genera are zoonotic; EBOV was the causative agent of the 2014–2016 Ebola virus disease epidemic in West Africa (1). *Rousettus* bats in Africa have been identified as *Marburgvirus* hosts (2), and viral nucleic acid and serologic evidence suggests that bats are also natural hosts of *Ebolavirus* spp. (3). Yet it remains unclear which species are the definitive reservoirs of filoviruses.

Ecologic models of *Ebolavirus* and *Marburgvirus* geographic distribution and habitat ranges of potential reservoir bat species suggest that both groups are distributed throughout Asia (3,4). Serologic evidence of filoviruses in frugivorous bats in Bangladesh, China, and the

Philippines has been reported (5–7), and RESTV nucleic acid was detected in an insectivorous bat in the Philippines, where RESTV is considered endemic (8). We examined pteropodid bats of 3 species: *Cynopterus brachyotis*, *Eonycteris spelaea*, and *Penthetor lucasi*, which are widely distributed across Southeast Asia and share ecologic niches (9).

The Study

During 2011–2016, we collected serum from bats of the 3 aforementioned species in Singapore and screened samples for evidence of exposure to filoviruses. Samples were collected with permission from the National University of Singapore Institutional Animal Care and Use Committee (B01/12) and the National Parks Board (NP/RP11–011–3a). We diluted venous blood 1:10 in phosphate-buffered saline and then centrifuged, recovered, and heat-inactivated the serum at 56°C for 30 minutes and stored it at –80°C.

We developed a Bio-Plex (Bio-Rad, Hercules, CA, USA) bead-based multiplex assay that simultaneously probes serum for immunoglobulins specific to the viral envelope glycoproteins (GPs) from representative strains of all described *Ebolavirus* and *Marburgvirus* species (Table 1). A human FreeStyle 293-F stable cell-line expression system was used to produce the *Ebolavirus* and *Marburgvirus* spp. GPs as a soluble GP consisting of the entire ectodomain, sGP_(1,2), which retains a native-like oligomeric conformation, as described previously with modifications (10). In brief, each GP_(1,2) coding sequence was truncated at the C-terminus to remove the predicted transmembrane domain and cytoplasmic tail, then appended with the GCN trimerization peptide sequence (10) together with a factor Xa protease cleave site and a Twin-Strep-tag sequence (IBA Lifesciences, Göttingen, Germany). The sGP_(1,2) proteins were produced in serum-free conditions and purified by Strep-Tactin XT technology (IBA Lifesciences). The Twin-Strep-tag was removed by factor Xa enzymatic cleavage; factor Xa was removed by Xarrest Agarose (Merck Millipore, Billerica, MA, USA); sGP_(1,2) was purified further by S-200 size exclusion chromatography, concentrated, and stored frozen. These sGP_(1,2)s were coupled to carboxylated beads (Bio-Rad). Screening was performed on a Bio-Rad Bio-Plex 200.

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Table 1. *Ebolavirus* and *Marburgvirus* species soluble envelope glycoproteins conjugated Bio-Plex beads used in multiplex assay to detect antibodies against filoviruses*

| Virus | Isolation host/location | Bio-Plex bead | |
|----------------------------------------------------|-------------------------|---------------|--------------------|
| | | no. | NCBI accession no. |
| Ebola virus/H.sapiens/COD/1976/Yambuku-Mayinga | Human/DRC | 33 | NC_002549.1 |
| Bundibugyo virus/H. sapiens/UGA/2007 | Human/Uganda | 64 | FJ217161.1 |
| Tai Forest virus/H. sapiens/COV/1994/Pauleoula-CI | Human/Côte d'Ivoire | 57 | NC_014372 |
| Sudan virus/H. sapiens/UGA/2000/Gulu-808892 | Human/Uganda | 77 | NC_006432.1 |
| Reston virus/M. fascicularis/USA/1989/Pennsylvania | Macaque/USA | 85 | AF522874.1 |
| Reston virus/S. domesticus/PHL/2008/Reston08-A | Swine/Philippines | 72 | FJ621583.1 |
| Marburg virus/H. sapiens/KEN/1980/Musoke | Human/Kenya | 37 | Z12132 S55429 |
| Marburg virus/H. sapiens/AGO/2005/Ang0126 | Human/Angola | 28 | DQ447656.1 |
| Ravn virus/H. sapiens/KEN/1987/Kitum cave-810040 | Human/Kenya | 49 | NC_024781.1 |

*Bio-Plex manufactured by Bio-Rad (Hercules, CA, USA). DRC, Democratic Republic of the Congo; NCBI, National Center for Biotechnology Information.

In the absence of confirmed filovirus-negative bat serum, we used methods developed by Peel et al. to establish a median fluorescence intensity (MFI) cutoff value (11). We confirmed a cutoff value of 200 MFI (online Technical Appendix, <https://wwwnc.cdc.gov/EID/article/24/1/17-0401-Techapp1.pdf>), as was previously used for *Eidolon helvum* bat serum in a Bio-Plex serologic assay (12). We screened 409 samples with our *Ebolavirus* and *Marburgvirus* spp. sGP_(1,2) Bio-Plex assay modified from that described by Bossart et al. (13). Samples were diluted 1:100 and tested in duplicate; the sGP_(1,2)-coupled beads were mixed with individual samples; and a 1:1 combination of recombinant biotinylated-protein A/protein G (1:500) (Pierce, Rockford, IL, USA) was added to the wells, followed by addition of streptavidin-phycoerythrin (1:1,000) (Bio-Rad) and determination of MFI.

Samples were positive for 17 (9.1%) of 186 *E. spelaea*, 13 (8.5%) of 153 *C. brachyotis*, and 3 (4.3%) of 70 *P. lucasi* bats (Figure 1). Positive samples reacted with EBOV, BDBV, SUDV, or TAFV sGP_(1,2). However, no samples were positive for RESTV, MARV, or RAVV sGP_(1,2). We further examined positive samples to determine cross-reactivity between the *Ebolavirus* spp. sGP_(1,2) (Table 2). Twelve (71%) samples from *E. spelaea* bats cross-reacted with ≥ 2 *Ebolavirus* spp. sGP_(1,2) (BDBV, EBOV, SUDV, or TAFV). In contrast, 8 (62%) *C. brachyotis* and 2 (66%) *P. lucasi* samples were positive for only 1 sGP_(1,2) (BDBV or SUDV).

To further determine the cross-reactivity of positive samples and to corroborate Bio-Plex assay results for a selected number of samples, we performed Western blot (WB) assays (Figure 2). The filovirus GP_(1,2) is a trimer of heterodimeric GP₁ and GP₂ subunits. The trimeric-like sGP_(1,2) is the antigen in the multiplex Bio-Plex assay, whereas linearized monomeric sGP₁ and sGP₂ subunits are the antigens in WBs. Reduced and denatured EBOV or BDBV unconjugated sGP_(1,2) was loaded on 8% sodium dodecyl sulfate–polyacrylamide electrophoresis gels, transferred to a polyvinylidene difluoride membrane, and probed with 1:100 dilutions of positive and negative bat serum, as previously determined by the Bio-Plex assay. All 3 *E. spelaea* bat samples and 2 of 3 *C. brachyotis* bat samples that were Bio-Plex positive were also positive by WB and displayed reactivity with EBOV and BDBV GP₁ and GP₂ antigens; no *P. lucasi* bat samples positive by Bio-Plex were positive by WB.

Conclusions

We present evidence of antibodies specific to filoviruses antigenically related to *Ebolavirus* spp. in 3 species of fruit bats widely distributed throughout Southeast Asia. We detected seroreactivity with *Ebolavirus* spp. but not *Marburgvirus* spp. GP. Despite the close relatedness of the viruses, we detected samples reacting with only SUDV, not RESTV, GP. This finding contrasts

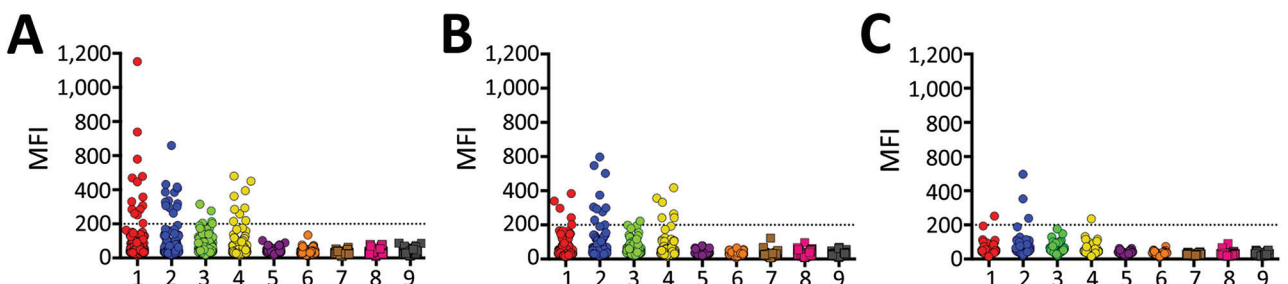


Figure 1. Mean fluorescence intensity (MFI) values obtained from Bio-Plex assay (Bio-Rad, Hercules, CA, USA) screening of individual serum samples from bats of 3 species with soluble filovirus glycoproteins. Dashed line indicates the cutoff value at 200 MFI. 1, *Zaire ebolavirus*; 2, *Bundibugyo ebolavirus*; 3, *Tai Forest ebolavirus*; 4, *Sudan ebolavirus*; 5, *Reston ebolavirus*–pig; 6, *Reston ebolavirus*–monkey; 7, *Marburg virus*–Musoke; 8, *Marburg virus*–Angola; 9, *Ravn virus*.

Table 2. Bio-Plex median fluorescence intensity values for bat serum samples positive for ≥ 1 filovirus antigen*

| Bat species, ID | EBOV | BDBV | TAFV | SUDV | RESTVm | RESTVp | MARV(Mus) | MARV(Ang) | RAVV |
|----------------------------------------|-------------|------------|------------|------------|--------|--------|-----------|-----------|------|
| <i>Eonycteris spelaea</i> , n = 186 | | | | | | | | | |
| 0805149† | 738 | | | 40 | 44 | 22 | 23 | 21 | 24 |
| 080814 | 86 | 318 | 105 | 258 | 26 | 12 | 17 | 16 | 20 |
| 082154 | 143 | 161 | 113 | 214 | 35 | 41 | 21 | 31 | 39 |
| 052313 | 284 | 408 | 177 | 285 | 89 | 72 | 29 | 23 | 30 |
| 052335 | 203 | 191 | 124 | 219 | 42 | 21 | 38 | 38 | 24 |
| 052339 | 357 | 306 | 141 | 293 | 54 | 31 | 26 | 26 | 42 |
| 071839 | 330 | 299 | 164 | 480 | 65 | 44 | 28 | 33 | 45 |
| 071842 | 446 | 327 | 202 | 362 | 65 | 49 | 42 | 38 | 57 |
| 110733 | 126 | 416 | 166 | 95 | 58 | 42 | 34 | 42 | 58 |
| 011603† | 1151 | 130 | 91 | 69 | 36 | 32 | 51 | 35 | 39 |
| 011616 | 252 | 294 | 168 | 175 | 32 | 49 | 47 | 29 | 50 |
| 011656 | 306 | 386 | 204 | 394 | 89 | 73 | 18 | 39 | 37 |
| 012309† | 579 | 659 | 315 | 69 | 35 | 31 | 27 | 33 | 35 |
| 021303 | 478 | 431 | 188 | 450 | 52 | 37 | 24 | 30 | 47 |
| 111903 | 469 | 384 | 276 | 113 | 52 | 57 | 37 | 69 | 54 |
| 111907 | 285 | 336 | 213 | 158 | 39 | 36 | 29 | 50 | 30 |
| 042722 | 260 | 262 | 174 | 167 | 75 | 31 | 54 | 24 | 42 |
| <i>Cynopterus brachyotis</i> , n = 153 | | | | | | | | | |
| 051253 | 121 | 133 | 59 | 242 | 40 | 41 | 19 | 25 | 68 |
| 0516613 | 146 | 293 | 127 | 73 | 47 | 36 | 25 | 29 | 22 |
| 0516632 | 138 | 139 | 86 | 356 | 35 | 25 | 28 | 34 | 34 |
| 0726122† | 119 | 501 | 100 | 60 | 40 | 46 | 25 | 19 | 29 |
| 1103241 | 84 | 141 | 128 | 241 | 50 | 47 | 66 | 38 | 34 |
| 100903 | 148 | 201 | 71 | 108 | 42 | 33 | 18 | 16 | 36 |
| 100914 | 74 | 228 | 70 | 55 | 39 | 38 | 30 | 27 | 26 |
| 100925 | 166 | 304 | 109 | 116 | 43 | 18 | 33 | 30 | 28 |
| 021357 | 201 | 299 | 179 | 264 | 65 | 44 | 25 | 55 | 47 |
| 050804 | 242 | 276 | 140 | 124 | 41 | 30 | 34 | 33 | 44 |
| 050818 | 383 | 374 | 198 | 332 | 60 | 55 | 29 | 26 | 68 |
| 040807† | 297 | 597 | 194 | 192 | 40 | 38 | 122 | 95 | 32 |
| 042701† | 339 | 547 | 222 | 417 | 60 | 78 | 54 | 25 | 62 |
| <i>Penthetor lucasi</i> , n = 70 | | | | | | | | | |
| 062590† | 34 | 496 | 93 | 39 | 36 | 18 | 23 | 17 | 23 |
| 070409† | 95 | 238 | 129 | 89 | 62 | 27 | 34 | 36 | 37 |
| 112112† | 251 | 352 | 148 | 235 | 51 | 29 | 23 | 23 | 29 |

*Bio-Plex manufactured by Bio-Rad (Hercules, CA, USA). Boldface indicates positive results. BDBV, Bundibugyo virus; EBOV, Ebola virus; ID, specimen identification number; MARV(Mus), Marburg virus–Musoke; MARV(Ang), Marburg virus–Angola; RESTVm, Reston virus–monkey; RESTVp, Reston virus–pig; SUDV, Sudan virus; RAVV, Ravn virus; TAFV, Tai Forest virus.

†Sample screened by Western blot and shown in Figure 2.

with previous reports of bat serum cross-reactivity with RESTV nucleoprotein (5,7,14). Possible explanations include 1) the fact that our customized Bio-Plex assay is based on conformational sGP_(1,2), which can differentiate antibody specificity better than the more sequence conserved nucleoprotein, and 2) the lack of evidence of RESTV GP positivity with *Cynopterus* and *Eonycteris* bat serum samples, which is in line with previous findings (both species were negative while only *Rousettus amplexicaudatus* bats were positive) (7). *E. spelaea* bats were previously predicted to be filovirus hosts (15), and sequences of novel filoviruses have been discovered in *E. spelaea* bat populations in Yunnan, China (14). Our data provide additional empirical evidence that populations of *C. brachyotis*, *E. spelaea*, and *P. lucasi* bats in Southeast Asia are hosts of filoviruses, which seem antigenically more closely related to EBOV, BDBV, and SUDV than to RESTV.

Examination of cross-reactivity of positive samples from *E. spelaea*, *C. brachyotis*, and *P. lucasi* bats revealed

no clear patterns of preferential reactivity with EBOV, BDBV, or SUDV GP. Factors that might contribute to the lack of *P. lucasi* positivity by WB include sensitivity differences between Bio-Plex and WB assays paired with the change in sGP_(1,2) conformation. Two Bio-Plex EBOV-positive samples (*E. spelaea* samples 0805149 and 011603) reacted with EBOV sGP₂ and BDBV sGP₁ in the WB. Bio-Plex and WB data strongly suggest the presence of yet-undetected batborne filoviruses, which are antigenically related to but distinct from BDBV, EBOV, and SUDV circulating in local bat populations. Reasons why these filoviruses have remained undetected include their inability to cross the species barrier, the rarity of spillovers into humans or domestic animals, or the fact that spillover events cause mild or no disease. We suggest that a yet-undescribed diversity of filoviruses exists in Southeast Asia bat populations, a hypothesis supported by the recent identification of filovirus sequences in *E. spelaea* and *R. leschenaulti* bats in China (14,16). Comprehensive surveillance including serology and detection of viral nucleic acid, along with virus

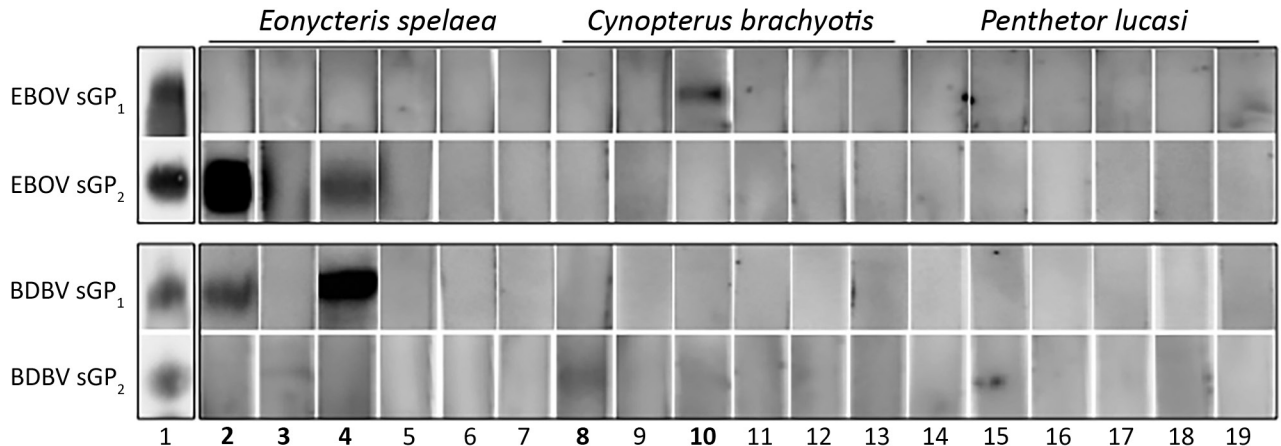


Figure 2. Western blot results of individual bat serum samples probed against *Zaire ebolavirus* and *Bundibugyo ebolavirus* glycoproteins 1 and 2 (GP₁, GP₂). Boldface indicates positivity by Western blot and underlining indicates positivity by Bio-Plex (Bio-Rad, Hercules, CA, USA). 1, soluble GP₁ and GP₂ blotted with control anti-Ebola virus nonhuman primate polyclonal serum that demonstrates cross-reactivity with *Bundibugyo ebolavirus* soluble GP. Other numbers along baseline correspond to the following sample identifiers, also used in Table 2: 2, 0805149; 3, 012309; 4, 011603; 5, 0116048; 6, 0719036; 7, 1128015; 8, 0726122; 9, 042701; 10, 040807; 11, 0512540; 12, 1009010; 13, 0408029; 14, 070409; 15, 112112; 16, 062590; 17, 0228004; 18, 0919025; 19, 0625095. BDBV, Bundibugyo virus; EBOV, Ebola virus.

isolation, will help elucidate the characteristics of filoviruses endemic to Asia and identify bat species that function as maintenance populations and reservoirs.

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Dr. Laing is a postdoctoral fellow at the Uniformed Services University and performed this work while a National Science Foundation EAPSI fellow at Duke-National University of Singapore Medical School. His research focuses on biosurveillance, batborne viruses, and antiviral immunity.

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September 2017: Zoonoses

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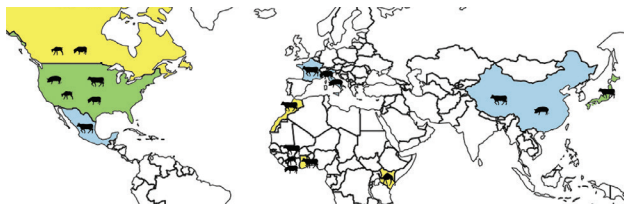
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Technical Appendix

Determination of Median Fluorescence Intensity (MFI) Cutoff Value

A Bayesian mixture model was first fitted to the data for individual glycoproteins within each bat species using Markov chain Monte Carlo. The model was implemented in R (1) with code provided by Alison Peel (2) that was adapted for use with the software packages JAGS (3) and rjags (4). The parameters of the fit were then used to calculate the MFI at which the p value of being seronegative was 0.001, as well as the p value at an MFI of 200 (Figure).

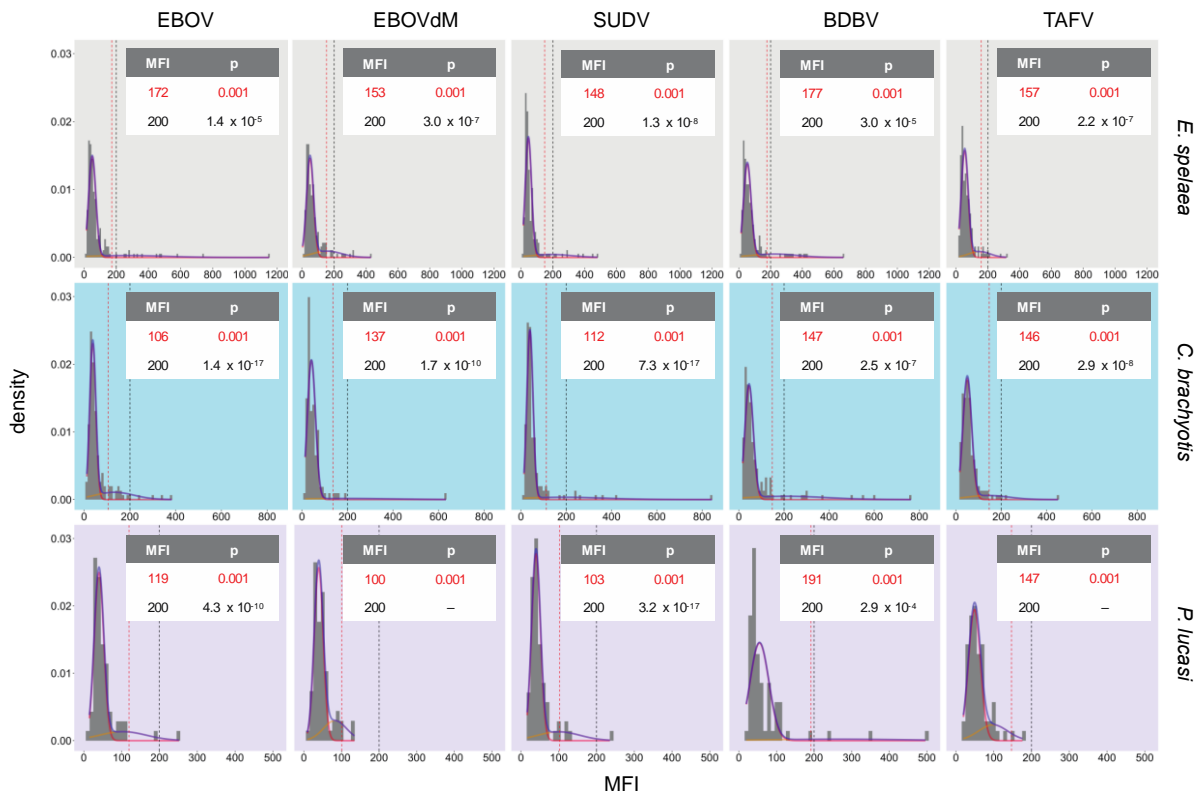


Figure. Median fluorescence intensity (MFI).

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