Characterization of the glycoproteins of bat-derived influenza viruses

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Abstract

Recently found bat-derived influenza viruses (BatIVs) have hemagglutinin (HA) and neuraminidase (NA) gene segments distinct from those of previously known influenza A viruses. However, pathogenicities of these BatIVs remain unknown since infectious virus strains have not been isolated yet. To gain insight into the biological properties of BatIVs, we generated vesicular stomatitis viruses (VSVs) pseudotyped with the BatIV HA and NA. We found that VSVs pseudotyped with BatIV HAs and NAs efficiently infected particular bat cell lines but not those derived from primates, and that proteolytic cleavage with a trypsin-like protease was necessary for HA-mediated virus entry. Treatment of the susceptible bat cells with some enzymes and inhibitors revealed that BatIV HAs might recognize some cellular glycoproteins as receptors rather than the sialic acids used for the other known influenza viruses. These data provide fundamental information on the mechanisms underlying the cellular entry and host restriction of BatIVs.

Introduction

Influenza A viruses (IAVs), which belong to the family *Orthomyxoviridae*, have 8 segmented negative sense RNA genomes. IAV is one of the most important zoonotic pathogens, with high morbidity in humans, pigs, horses, and poultry. IAVs have two envelope glycoproteins, hemagglutinin (HA) and neuraminidase (NA), and are divided into subtypes based on antigenicity. IAVs of H1-16 HA and N1-9 NA subtypes have been isolated from water birds such as migratory ducks, the natural reservoir of IAVs (Fouchier et al., 2005; Kida and Yanagawa, 1979; Webster et al., 1992).

HAs are expressed as trimers on the virion surface (Wilson et al., 1981). HA is initially synthesized as an inactive precursor HA0 and subsequently cleaved into HA1 and HA2 subunits by trypsin-like proteases of host cells (Sakai et al., 2014). The proteolytic cleavage of the HA molecule is essential for IAVs to acquire infectivity (Lazarowitz et al., 1973; Wiley and Skehel, 1987). HA1 is responsible for virus binding to sialic acid receptors on the cell surface, and HA2 mediates membrane fusion under acidic conditions in endosomes, thereby delivering the viral genomic RNA into the cytoplasm of target cells (Matlin et al., 1981; Rust et al., 2004). NAs, expressed on the virion surface as tetramers, have sialidase activity that enables mature virus particles to be released from infected cells after budding (Colman, 1994; Webster et al., 1992).

Recently, IAV-like RNA genomes were detected in succession from 2 frugivorous bat species, little yellow-shouldered bats (*Sturnira lilium*) and flat-faced fruit bats (*Artibeus planirostris*) in Guatemala and Peru, respectively. The nucleotide sequences of the HA and NA of these bat-derived influenza viruses (BatIVs) were divergent from all previously

61 known IAVs and new subtypes, H17N10 and H18N11, have been proposed (Tong et al.,
62 2012; Tong et al., 2013). However, infectious viruses have not been isolated yet. Previous
63 studies by others tried to rescue BatIVs using a reverse genetics approach, but failed to
64 generate infectious BatIVs (Juozapaitis et al., 2014; Zhou et al., 2014). Thus, the
65 information on the biological properties of BatIVs is mostly speculative and the possible
66 functions of BatIV HAs and NAs are only hypothetical, based on structural analyses (Li et
67 al., 2012; Tong et al., 2013; Zhu et al., 2012; Zhu et al., 2013).
68 In this study, we utilized a vesicular stomatitis virus (VSV) pseudotype system,
69 enabling us to directly analyze the biological functions of the BatIV glycoproteins, which
70 presumably play important roles in the replication cycle and pathogenicity. We found some
71 bat cell lines susceptible to VSVs pseudotyped with BatIV HAs and NAs. Our data suggest
72 that BatIVs do not use sialic acids as a viral receptor and may have a limited host range, at
73 least considering receptor engagement.
74

75 ## Results

76 **Generation of VSVs pseudotyped with BatIV HAs and/or NAs.** To investigate cellular
77 entry mediated by BatIV glycoproteins, VSVs pseudotyped with BatIV HAs and/or NAs
78 (VSVΔG*-H17N10, -H18N11,-H17, -H18, -N10, and -N11) were generated as described in
79 Materials and Methods. We first observed the virions of these pseudotyped VSVs using
80 transmission electron microscopy (Fig. 1). We found that the virions of all of these
81 pseudotyped VSVs showed characteristic morphology (i.e. a bullet-like shape) similar to
82 parental VSVΔG*-G. It was noted that VSVs pseudotyped with BatIV HA and NA (Fig.

1A and B), HA alone (Fig. 1D and E), and NA alone (Fig. 1G and H) all had numerous spikes on their surfaces, as was the case with VSVs pseudotyped with IAV HA (H3) and NA (N2) (Fig. 1C), H3 HA alone (Fig. 1F), and N2 NA alone (Fig. 1I). Immune electron microscopy with anti-H17 HA and anti-N10 NA antibodies revealed that both BatIV HA and NA were efficiently incorporated into VSV particles (Fig. 1K and L). No difference was found in the overall morphology among these VSV virions. These data indicated that BatIV HAs and NAs were efficiently incorporated into the VSV particles.

Cell lines susceptible to VSVs pseudotyped with BatIV glycoproteins. Since previous studies have suggested that cell lines commonly used for IAV propagation are nonpermissive for BatIVs, we screened various cell lines, including bat-derived cells, for susceptibility to pseudotyped VSVs (Table 1) (Fig. 2). VSVs pseudotyped with HAs and NAs of BatIVs and well-characterized IAV strains, A/WSN/1933 (H1N1) (WSN) and A/Aichi/2/1968 (H3N2) (Aichi), were generated and treated with trypsin before use, since BatIV HAs, like WSN and Aichi HAs, have a cleavage site potentially recognized by trypsin-like proteases (Tong et al., 2012; Tong et al., 2013). We found that VSVΔG*-WSN, -Aichi, and -VSV G infected all cell lines tested (Fig. 2A, B, and E). On the other hand, VSVΔG*-H17N10 and -H18N11 infected bat cell lines YubFKT1, IndFSPT1, and SuBK12-08, but not the other cell lines tested, except MDCK cells, which were much less susceptible than these bat cells. Since IndFSPT1 cells showed the highest susceptibility to VSVΔG*-H17N10 and -H18N11 (Fig. 2C and D), this cell line was used for the following experiments.

105

106 **Trypsin requirement for the HA function and the dispensability of NA in virus entry.**
107 IAV HAs are known to be cleaved into HA1 and HA2 subunits by trypsin-like proteases to
108 acquire the ability to mediate membrane fusion (Klenk and Rott, 1988). Western blotting
109 revealed that both H17 and H18 HAs were cleaved into HA1 and HA2 by trypsin treatment
110 (Fig. 3A). Thus, we investigated the requirement of HA cleavage for infectivity of
111 pseudotyped VSVs. As expected, VSVs pseudotyped with BatIV glycoproteins did not
112 infect IndFSPT1 cells without trypsin treatment, in a manner consistent with other IAVs
113 (data not shown), whereas trypsin-treated viruses efficiently infected this cell line (Fig. 2).
114 These data indicated that the HA cleavage was a prerequisite for BatIV infectivity. Next, to
115 clarify whether BatIV HAs was responsible for virus entry, VSVΔG*-H17N10, -H18N11,-
116 H17, -H18, -N10, and -N11 were inoculated to IndFSPT1 cells and their infectivities were
117 compared (Fig. 3B). We found that VSVΔG*-H17 and -H18 infected IndFSPT1 cells as
118 efficiently as VSVΔG*-H17N10 and -H18N11, whereas the infectivity of VSV
119 pseudotyped with WSN or Aichi HA alone was much lower than that of VSVs pseudotyped
120 with both HA and NA of the respective viruses. VSVΔG*-N10 and -N11 showed no
121 infectivity, similarly to VSVs pseudotyped with NAs of WSN and Aichi. These results
122 suggest that BatIV HA is the only glycoprotein mediating both virus attachment and
123 membrane fusion and that BatIV NA is dispensable during the entry into cells.
124

125 **Effects of chemical and enzymatic treatments of cells on susceptibility to VSVs**
126 **pseudotyped with BatIVs.** It is generally known that IAV HAs mediate membrane fusion

127 in a low pH-dependent manner (Kida et al., 1983; Rust et al., 2004). To investigate the
128 requirement of endosomal acidification for BatIV HA-mediated membrane fusion,
129 IndFSPT1 cells were treated with ammonium chloride, which is known to neutralize the pH
130 of acidic intracellular compartments, and then infected with VSVΔG*-WSN, -Aichi, -
131 H17N10, -H18N11, and VSVΔG*-G. Treatment of the cells with ammonium chloride
132 markedly reduced the infectivity of VSVΔG*-H17N10 and -H18N11, as was the case with
133 VSVΔG*-G, -WSN, and -Aichi, in a dose-dependent manner, suggesting that BatIV HAs
134 require a low pH for membrane fusion, consistent with the other IAV HAs (Fig. 4A). To
135 obtain information on the biological characteristics of cellular receptors for BatIVs,
136 IndFSPT1 cells were pretreated with tunicamycin, pronase, or neuraminidase (i.e., an N-
137 linked glycosylation inhibitor, mixture of proteases, and sialidase, respectively), and then
138 infected with pseudotyped VSVs (Fig. 4B to D). Tunicamycin treatment markedly reduced
139 the infectivities of VSVΔG*-G, -H17N10, and -H18N11, but less significantly those of
140 VSVΔG*-WSN and -Aichi (Fig. 4B). Preincubation of cells with pronase reduced the
141 infectivities of the pseudotyped VSVs, except for VSVΔG*-WSN (Fig. 4C).
142 Neuraminidase treatment reduced VSVΔG*-WSN and -Aichi infectivities, but interestingly
143 did not affect the infectivities of VSVΔG*-H17N10 and -H18N11 (Fig. 4D). We confirmed
144 that no remarkable cytotoxicity was observed during these treatments (Fig. 4E). These
145 results suggest that BatIV HAs do not recognize sialic acids which are critical components
146 of the IAV receptor and some other molecules such as glycoproteins may serve as BatIV
147 receptors.
148

Discussion

In recent years, particular attention has been paid to bat-derived viruses since some species of bats have been reported to be reservoirs of several viral zoonotic pathogens (e.g., lyssavirus, henipavirus, SARS coronavirus, and Marburgvirus) (Calisher et al., 2006; Smith and Wang, 2013; Wang et al., 2011; Wong et al., 2007). Although the zoonotic potential of BatIVs has not been fully evaluated yet, recent studies generated reassortant viruses that had HA and NA gene segments of well-characterized IAVs (i.e., H1, H3, and H7 HAs and N1, N2, and N7 NAs) and the other gene segments derived from BatIVs, and demonstrated that the reassortant viruses replicated in cultured cells and caused severe disease in mice (Juozapaitis et al., 2014; Zhou et al., 2014). However, characterization of BatIV HAs and NAs remains an open research problem, since reassortant viruses carrying the BatIV HA and NA gene segments have not been rescued due to the lack of information on cells susceptible to this novel virus. In this study, we first determined the potentially permissive bat cell lines using VSVs pseudotyped with BatIV HAs and NAs.

We demonstrated that VSVΔG*-H17N10 and -H18N11 efficiently infected the bat-derived cell lines IndFSPT1, YubFKT1, and SuBK12-08. While IndFSPT1 was derived from *Pteropus giganteus* (family *Pterodidae*), YubFKT1 and SuBK12-08 were prepared from bats belonging to the same species (*Miniopterus* sp., family *Miniopteridae*). Based on a phylogenetic study of bats (Agnarsson et al., 2011), *Miniopteridae* belongs to the same cluster as *Phyllostomidae*, from which H17N10 and H18N11 BatIVs were detected, little yellow-shouldered bats (*Sturnira lilium*) and flat-faced fruit bats (*Artibeus planirostris*), respectively (Tong et al., 2012; Tong et al., 2013). Thus, BatIV HAs appear to recognize

171 cell surface molecules shared among the bats at least in *Miniopteridae* and *Phyllostomidae*
172 families. IndFSPT1 should also have such molecules since it showed the highest
173 susceptibility to BatIV HA-pseudotyped VSVs. It was noted that VSVΔG*-H17N10 and -
174 H18N11 also infected MDCK cells, although less efficiently than these bat cell lines. This
175 result might contradict a previous report that H17 HA did not bind to the surface of MDCK
176 cells (Sun et al., 2013). However, it is conceivable that the binding affinity of BatIV HA to
177 MDCK cell surface molecules is quite low and thus below the level of detection in the
178 assay used in the previous study. In the present study, MDCK cells indeed showed much
179 lower susceptibility to BatIV HA-pseudotyped VSVs than YubFKT1, IndFSPT1, and
180 SuBK12-08. Nonetheless, it would be interesting to clarify whether MDCK cells express
181 some BatIV receptor molecules shared with the bat cell lines.
182 It is also noteworthy that VSVΔG*-H17N10 and -H18N11 did not infect Vero E6,
183 HEK293, SK-L, and QT6 cells. Previous studies show that quails can act as an intermediate
184 host in the interspecies spread of avian IAVs (Makarova et al., 2003; Perez et al., 2003;
185 Uchida et al., 2011). Furthermore, pigs are thought to serve as "mixing vessels" for the
186 production of reassortant viruses between avian and human IAVs (Chang et al., 2009;
187 Hinshaw et al., 1981; Ito et al., 1998; Kida et al., 1994; Scholtissek et al., 1985). Our results
188 suggest that BatIVs do not readily infect humans, pigs, or birds and support that notion that
189 these viruses have limited zoonotic potential (Juozapaitis et al., 2014; Zhou et al., 2014).
190 It is known that VSV G protein and IAV HA recognize ubiquitous cell surface
191 molecules for virus entry. VSV G recognizes various cell surface molecules and thus VSV
192 exhibits remarkably robust and pantropic infectivity (Finkelshtein et al., 2013;

193 Johannsdottir et al., 2009; Lichty et al., 2004; Roche et al., 2008). IAV HAs recognize
194 sialic acids typically occupying the terminal positions of glycoproteins or glycolipids
195 (Gambaryan et al., 2005; Suzuki et al., 2000). Accordingly, VSVΔG*-G, -WSN, and -Aichi
196 infected all cell lines used in this study, whereas we found that VSVΔG*-H17N10 and -
197 H18N11 infected only particular bat cell lines and that neuraminidase treatment did not
198 affect the infectivities of VSVΔG*-H17N10 and -H18N11. This result was in agreement
199 with previous results based on the crystal structure analysis and surface plasmon resonance
200 of sialylated glycans with α2,3-linkage or α2,6-linkage (Sun et al., 2013; Zhu et al., 2013).
201 Glycan microarray analyses also showed that H17 HA did not display obvious avidity to
202 any glycans (Sun et al., 2013). Interestingly, we found that the infectivities of VSVΔG*-
203 H17N10 and -H18N11 were markedly reduced by the treatment of IndFSPT1 cells with
204 tunicamycin, which inhibits N-linked glycosylation, leading to unfolding or misfolding of
205 proteins and inhibition of glycoprotein expression. Pretreatment of the cells with pronase
206 also reduced the infectivities of VSVΔG*-H17N10 and -H18N11. Taken together, our data
207 suggest that some particular glycoprotein(s) serve as receptors for BatIVs.
208 Previous studies indicated that N10 NA did not have sialidase activity (Li et al.,
209 2012; Zhu et al., 2012). It was also shown that most of the amino acid residues responsible
210 for NA activity were substituted, and proposed that N10 NA protein should be termed an
211 NA-like protein (Zhu et al., 2012). In this study, we found that VSVs pseudotyped with
212 BatIV NAs alone were not infectious, confirming that NA did not play a central role in IAV
213 entry into cells. However, it should be noted that the production efficiency of pseudotyped
214 VSVs bearing WSN and Aichi HAs alone was much lower than that of VSVs pseudotyped

215 with both HAs and NAs, suggesting that NA activity facilitated virus release from infected
216 cells and/or increased the HA function (Su et al., 2009). By contrast, no remarkable
217 difference was found in the infectivity between VSVs pseudotyped with BatIV
218 glycoproteins (i.e., HA alone vs. HA and NA). These data suggest that, unlike the other
219 IAVs, the target molecules of BatIV HAs and NAs are different and that the "HA-NA
220 balance" concept proposed for IAVs does not be applied to BatIVs.
221 Because H17N10 and H18N11 BatIVs have never been isolated, their ability to
222 infect humans and other mammals and the pathogenic potential for these hosts can only be
223 hypothesized. In this study, the replication-incompetent VSV pseudotype system enabled
224 us to investigate the cellular tropism controlled by the interaction between BatIV HA and
225 its cellular ligand, which might be some glycoproteins. Although a reverse genetics
226 approach and *in vivo* experiments for the infectious BatIV are needed to provide direct
227 evidence of its pathogenicity and host specificity, our data suggest that BatIV may
228 preferentially infect particular bat species.
229

230 ## Materials and Methods

231 **Cells.** HEK293, HEK293T, and Vero E6 cells were grown in Dulbecco's modified Eagle's
232 medium (DMEM) with 10% fetal calf serum (FCS) and penicillin-streptomycin. MDCK
233 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% calf serum,
234 L-glutamine, and penicillin-streptomycin. Bat cell lines BKT1, FBKT1, YubFKT1,
235 IndFSPT1, DemKT1, ZFBK11-97, SuBK12-08, and ZFBS13-76A were established as

236 described previously (Maeda et al., 2008; Maruyama et al., 2014). All bat cell lines were
237 grown in RPMI-1640 medium with 10% FCS, L-glutamine, and penicillin-streptomycin.
238
239 **Construction of plasmids expressing HAs and NAs.** Coding regions of the HAs and NAs
240 of BatIVs were synthesized in vector pUC19 or pUCFa, based on the nucleotide sequences
241 of GenBank (accession numbers for H17 HA, N10 NA, H18 HA, and N11 NA: CY103892,
242 CY103894, CY125945, and CY125947, respectively) (FASMAC). Each coding region of
243 the viral proteins was amplified by PCR with primers including restriction sites, the kozak
244 sequence, and the stop codon. After digestion by restriction enzymes, each gene was cloned
245 into the mammalian expression vector pCAGGS (Niwa et al., 1991). H1 HA and N1 NA of
246 A/WSN/1933 (H1N1) (WSN), and H3 HA and N2 NA of A/Aichi/2/1968 (H3N2) (Aichi)
247 were cloned into pCAGGS as described previously (Muramatsu et al., 2013).
248
249 **Vesicular stomatitis viruses (VSVs) pseudotyped with HAs and/or NAs.** Using VSV
250 containing the green fluorescent protein (GFP) gene instead of the receptor-binding VSV G
251 protein gene (VSVΔG*-G), pseudotyped viruses with HAs and/or NAs of BatIVs, WSN,
252 and Aichi were generated as described previously (Takada et al., 1997). VSVs pseudotyped
253 with IAV glycoproteins were pretreated with trypsin (final concentration 0.0005%) for 30
254 minutes at 37°C, followed by incubation with an anti-VSV G monoclonal antibody, VSV-
255 G(N)1-9, to abolish the background infectivity of parental VSVΔG*-G (Nakayama et al.,
256 2011). For virus titration, 10-fold diluted pseudotyped VSVs were inoculated into confluent
257 monolayers of each cell line on 96-well plates, and the infectious unit (IU) in each cell line

was determined 20 hours later by counting the number of GFP-expressing cells under a fluorescent microscope.

Electron microscopy. Transmission electron microscopy was carried out as described previously (Maruyama et al., 2014). Pseudotyped VSVs fixed with 0.25% glutaraldehyde were adsorbed to collodion-carbon-coated copper grids and negatively stained with 2% phosphotungstic acid solution (pH=5.8). For immune transmission electron microscopy, we used an anti-HA2 monoclonal antibody (3N12-6-4) broadly cross-reactive to group 1 HA subtypes, anti-N10 NA mouse serum (FM0137) produced by immunization with a synthetic peptide corresponding to amino acid residues 328 to 343 (AQEKGEGGIQGFILDE) of N10 NA, and an immunogold-conjugated goat anti-mouse IgG (H+L) polyclonal antibody (BB International). Samples were examined with an H-7650 electron microscope (Hitachi) at 80kV.

SDS-PAGE and western blotting. Pseudotyped VSVs were treated with or without trypsin (final concentration 0.0005%) for 30 minutes at 37°C and then mixed with SDS-PAGE sample buffer with 5% 2-mercaptoethanol and boiled for 5 minutes. After electrophoresis on 5–20% SuperSep (Wako), separated proteins were blotted on a polyvinylidene difluoride membrane (Millipore). The membrane was incubated with an anti-H3N2 chicken polyclonal antiserum or anti-HA2 monoclonal antibody 3N12-6-4, which reacts to H1, H2, H5, H6, H17, and H18 HAs, followed by incubation with peroxidase-conjugated rabbit anti-chicken IgY (H+L) or goat anti-mouse IgG (H+L)

(Jackson ImmunoResearch). The bound antibodies were visualized with Immobilon Western (Millipore).

Cell treatment with enzymes and inhibitors. IndFSPT1 cells were preincubated with the medium containing an endosomal acidification inhibitor, ammonium chloride (Wako), at 37°C for 2 hours in a CO_2 incubator, and then infected with pseudotyped VSVs appropriately diluted to yield 200–1000 IUs, followed by incubation in the presence of ammonium chloride. IndFSPT1 cells were also pretreated with pronase (a mixture of endo- and exoproteases from *Streptomyces griseus*) (Calbiochem) (Narahashi et al., 1968), for 20 minutes, an N-glycosylation inhibitor (tunicamycin from *Streptomyces* sp.) (Sigma) for 8 hours, which blocks the reaction of UDP-GlcNAc and dolichol phosphate in the first step of glycoprotein synthesis, thus inhibiting the synthesis of N-linked glycoproteins, or neuraminidase from *Vibrio cholerae* (Roche) (Uchida et al., 1977) for 1 hour at 37°C in a CO_2 incubator. Treated cells were washed with serum free RPMI-1640 medium 3 times, and then incubated with pseudotyped VSVs appropriately diluted to yield 200–1000 IUs for 1 hour. After adsorption of the virus, the inoculum was aspirated and the growth medium (10% FCS RPMI-1640 medium) was added. Cells were incubated for 20 hours, and infected cells were counted under a fluorescent microscope. Cell viabilities were assessed by the alamar blue assay. After treatments of each enzyme and inhibitor, cells were incubated with FCS-free RPMI-1640 medium containing 10% Alamar blue (Biosource) for 2 hours, and fluorescence with excitation wavelength at 530-560 nm was measured using EnVision (PerkinElmer).

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458 **Figure legends**

459 **Fig. 1 Transmission electron microscopy of pseudotyped VSVs.** VSVΔG*-H17N10 (A),
460 -H18N11 (B), -H3N2 (C) -H17 (D), -H18 (E), -H3 (F), -N10 (G), -N11 (H), -N2 (I) and
461 VSVΔG*-G (J) were fixed and stained as described in Materials and Methods. For immune
462 transmission electron microscopy of VSVΔG*-H17N10, anti-HA2 monoclonal antibody
463 (K) and anti-N10 NA mouse serum (L) were used. Scale bars represent 100 nm.
464 Arrowheads indicate gold particles.
465
466 **Fig. 2 Infectivities of pseudotyped VSVs in several cell lines.** VSVΔG*-WSN, -Aichi, -
467 H17N10, -H18N11, and VSVΔG*-G were inoculated into several cell lines (Table 1).
468 Infectious units (IUs) of each virus in different cell lines were determined by counting the
469 number of GFP-expressing cells. Each experiment was performed three times, and averages
470 and standard deviations are shown. Infectivities of VSVΔG*-H17N10 and -H18N11 in
471 some cell lines were under the limit of detection (†). Significant differences (student's t-
472 test) were found between MDCK and any of the bat cell lines ($P < 0.01$).
473
474 **Fig. 3 Infectivities of pseudotyped VSVs with BatIV HAs and/or NAs in IndFSPT1**
475 **cells.** (A) VSVΔG*-Aichi, -H17N10, and -H18N11 were treated with or without trypsin
476 (final concentration 0.0005%) for 30 minutes at 37°C and then mixed with SDS-PAGE
477 sample buffer with 5% 2-mercaptoethanol. After SDS-PAGE, separated proteins were
478 detected by western blotting with anti-H3 chicken antiserum and anti-HA2 monoclonal
479 antibody 3N12-6-4. (B) VSVs pseudotyped with HAs and/or NAs of WSN and Aichi and

VSVΔG*-H17N10, -H17, -N10, -H18N11, -H18, and -N11 were inoculated to IndFSPT1 cells. Infectious units (IUs) were determined by counting the number of GFP-expressing cells. Each experiment was performed three times, and averages and standard deviations are shown. Infectivities of VSV pseudotyped with NAs alone were under the limit of detection (†). Statistical significance was calculated using student's t-test (*P < 0.01).

Fig. 4 Effects of chemical and enzymatic modification on infectivities of pseudotyped VSVs. IndFSPT1 cells were treated with ammonium chloride (A), tunicamycin (B), pronase (C), or neuraminidase (D) as described in Materials and Methods. Treated cells were then infected with VSVΔG*-WSN, -Aichi, -H17N10, -H18N11, and VSVΔG*-G appropriately diluted to yield 200–1000 IUs. The percentages of infectivity were determined by setting the number of the untreated cells to 100%. Each experiment was performed three times, and averages and standard deviations are shown. Cell viabilities were measured by the alamar blue assay (E). The percentages of fluorescence were determined by setting the number of the untreated cells to 100%. Each experiment was performed three times, and averages and standard deviations are shown. Statistical significances compared to untreated cells were calculated using student's t-test (*P < 0.01).

Table 1 Origins of cell lines used in this study

Cell line	Species	Zoological name	Organ
Vero E6	African green monkey	*Chlorocebus* sp.	Kidney
HEK293	Human	*Homo sapiens*	Kidney
MDCK	Dog	*Canis lupus familiaris*	Kidney
SK-L	Pig	*Sus scrofa domesticus*	Kidney
QT6	Japanese quail	*Coturnix japonica*	Muscle
BKT1	Greater horseshoe bat[a]	*Rhinolophus ferrumequinum*	Kidney
FBKT1	Yaeyama flying fox[a]	*Pteropus dasymallus yayeyamae*	Kidney
YubFKT1	Eastern bent-winged bat[a]	*Miniopterus fuliginosus*	Kidney
IndFSPT1	Indian flying fox[a]	*Pteropus giganteus*	Spleen
DemKT1	Leschenault's rousette[a]	*Rousettus leschenaultii*	Kidney
ZFBK11-97	Gambian epauletted fruit bat[a]	*Epomophorus gambianus*	Kidney
SuBK12-08	Schreiber's bat[a]	*Miniopterus schreibersii*	Kidney
ZFBS13-75A	Straw-coloured fruit bat[b]	*Eidolon helvum*	Spleen

[a]Previously described (Maruyama et al., 2014).

[b]Determined by habitat and morphology.

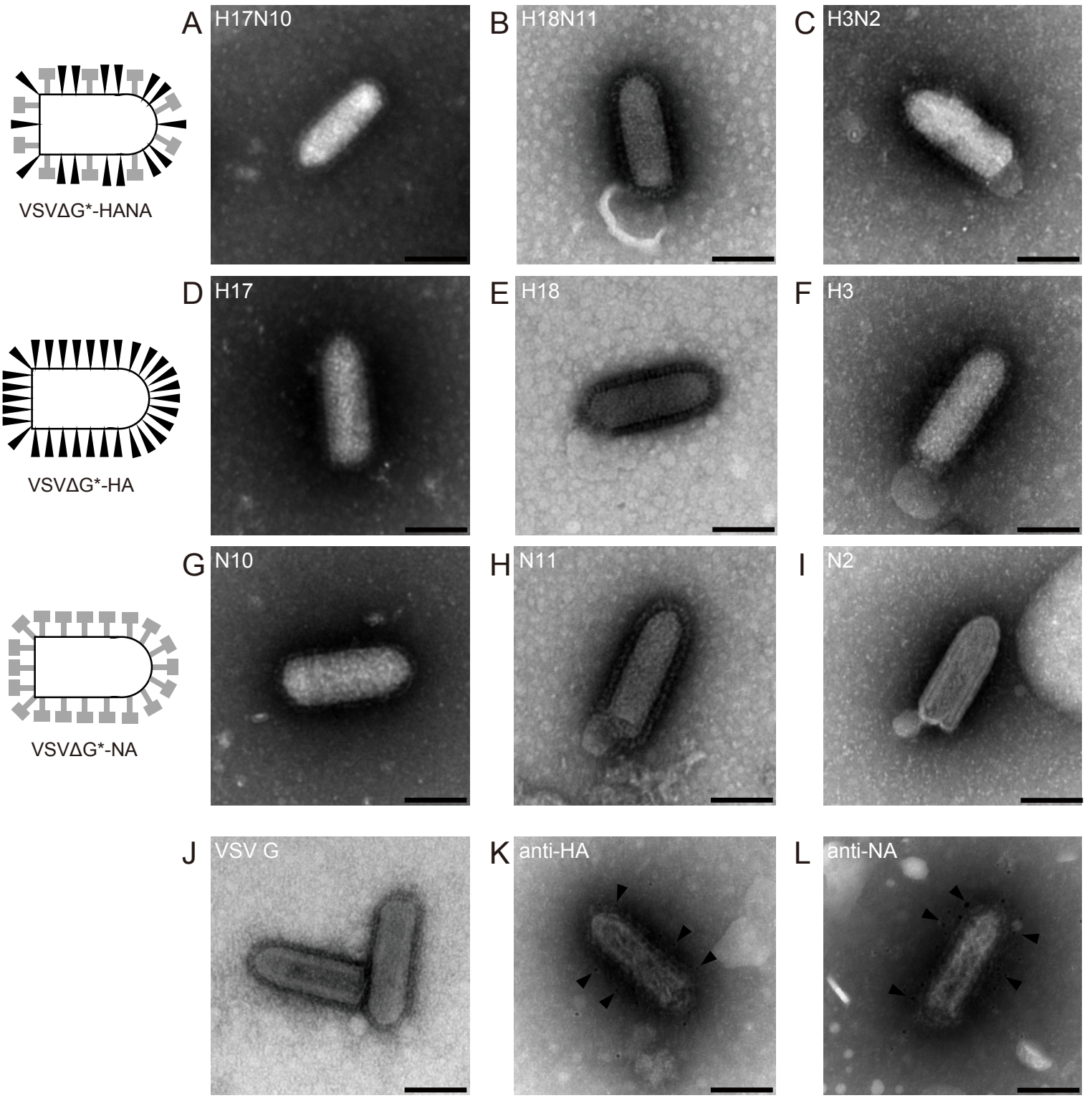


Fig. 1 Maruyama et al

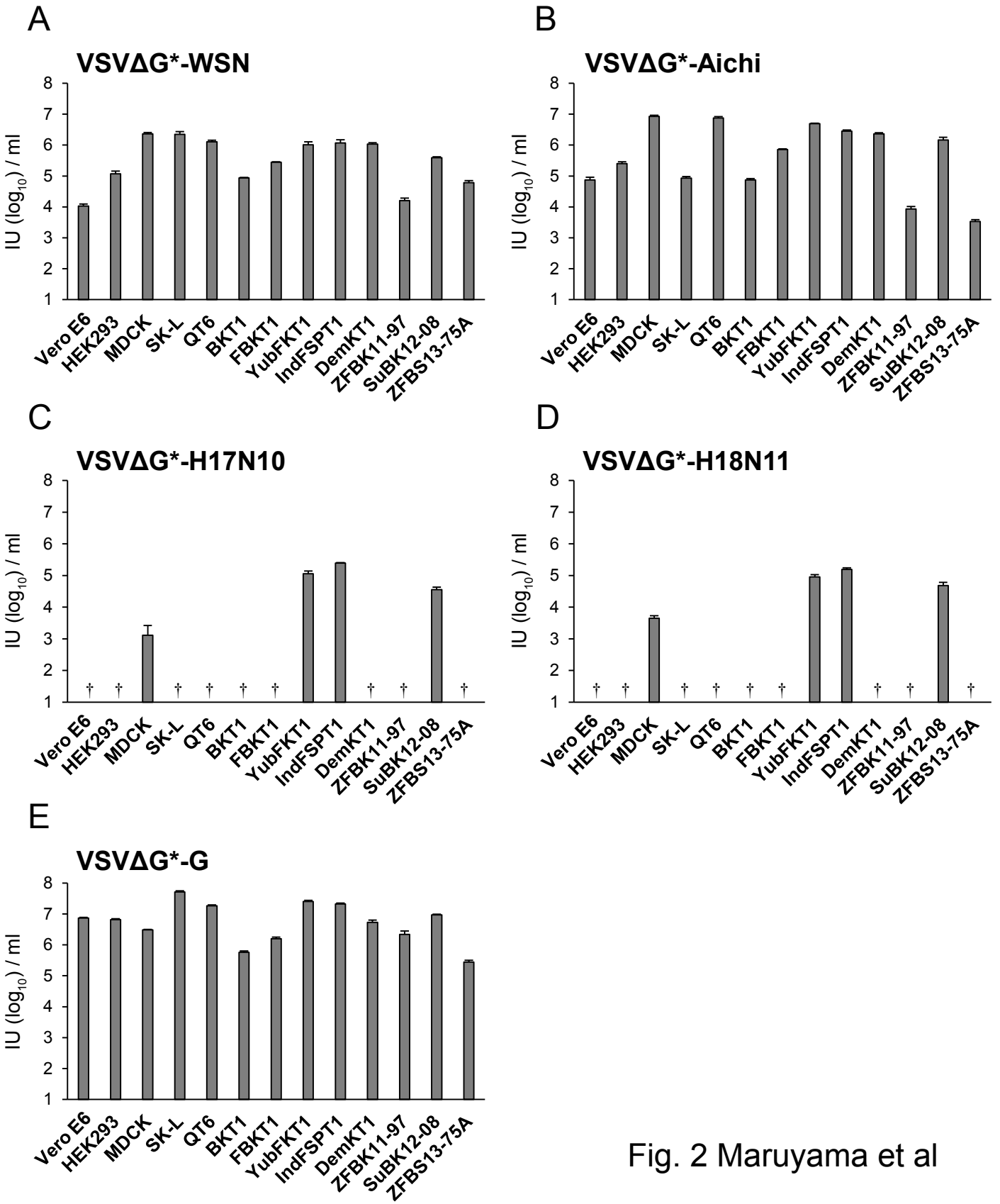


Fig. 2 Maruyama et al

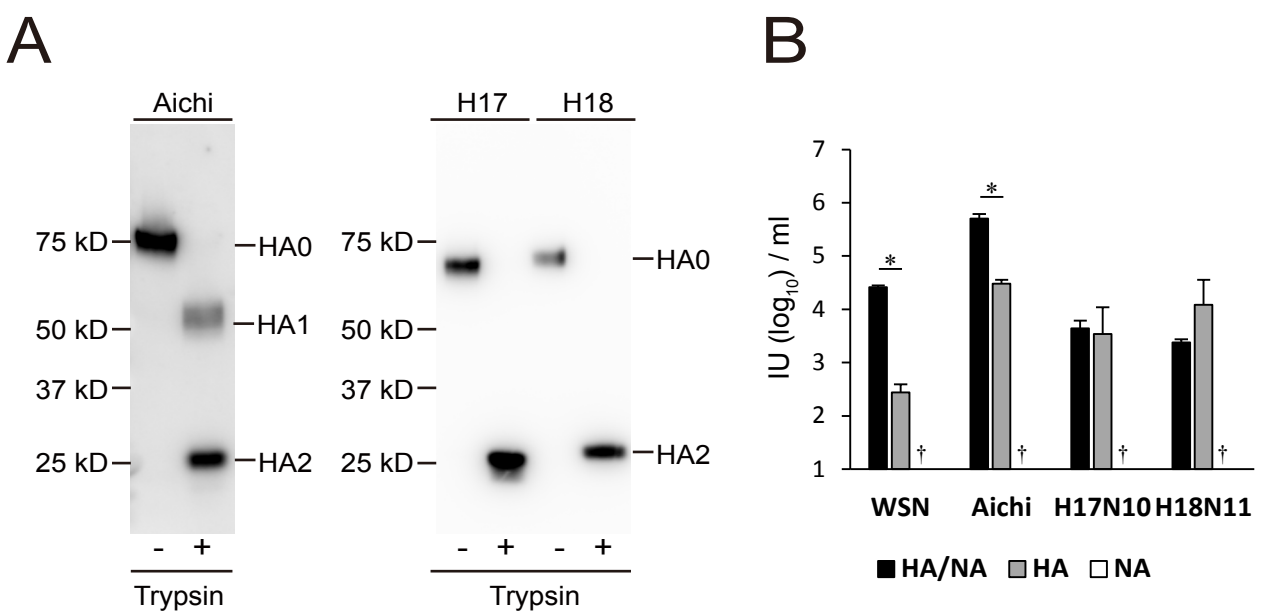
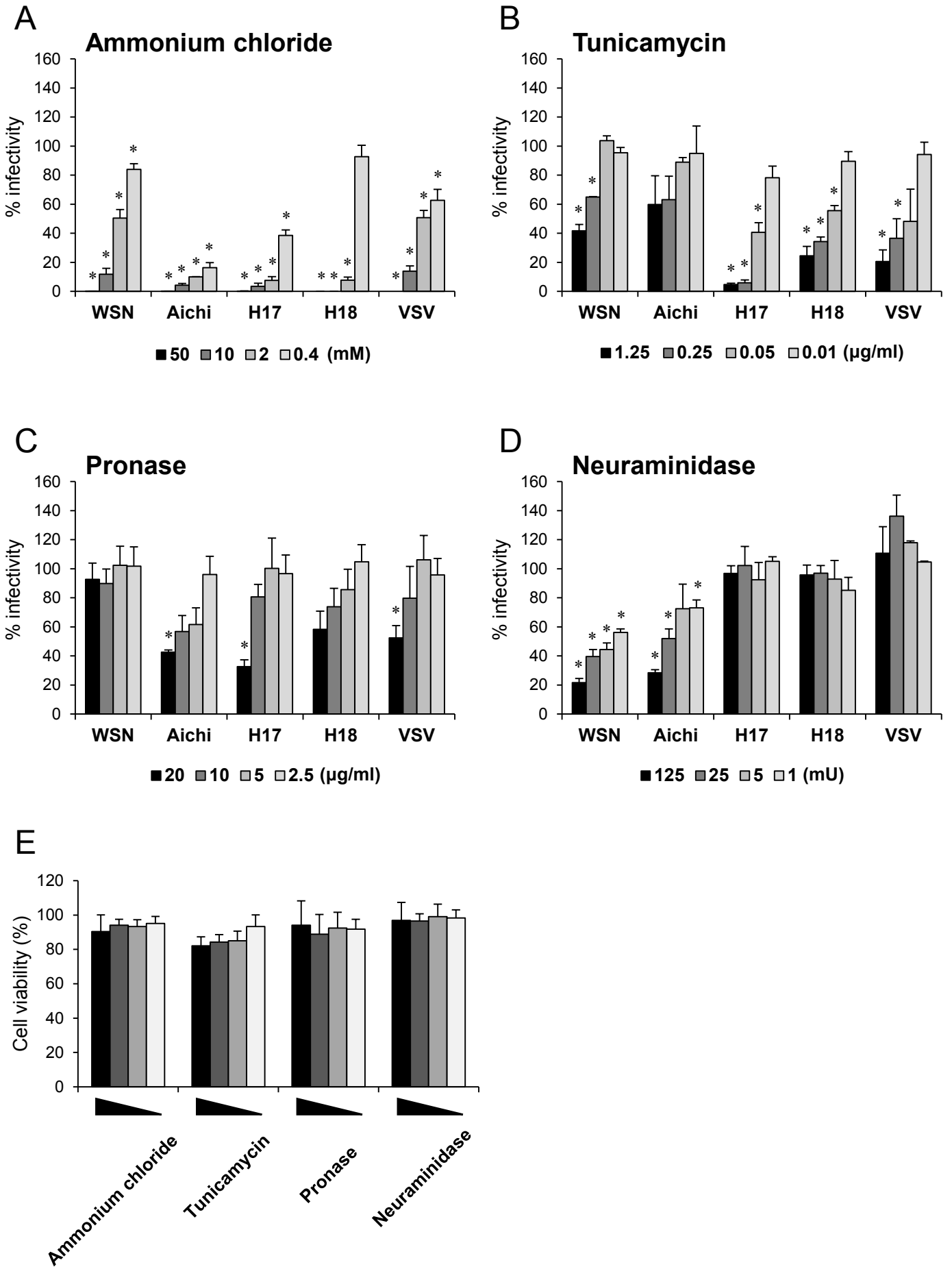


Fig. 3 Maruyama et al


A
Ammonium chloride
% infectivity
WSN
Aichi
H17
H18
VSV
50 10 2 0.4 (mM)
B
Tunicamycin
1.25 0.25 0.05 0.01 (μg/ml)
C
Pronase
20 10 5 2.5 (μg/ml)
D
Neuraminidase
125 25 5 1 (mU)
E
Cell viability (%)
Ammonium chloride
Tunicamycin
Pronase
Neuraminidase