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1 **Protein-bound sialic acid in saliva contributes directly to salivary anti-influenza**
2 **virus activity**
3
4 **Running title: Individual differences in saliva for influenza protection**
5
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13
14 Manuscript information: This manuscript contains 35 references, 4 figures.
15 Word count: Abstract = 187 words, Main text (not including abstract, Methods,
16 References and figure legends) = 2189 words, Title = 12 words
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23 **ABSTRACT**

24 The oral cavity is an entrance for respiratory viruses, such as influenza.
25 Recently, saliva has been shown to exert both antimicrobial and antiviral activities. Thus,
26 saliva may be a biological factor that contributes to the prevention of influenza infection.
27 However, the actual salivary anti-influenza A virus (IAV) activity in individuals and its
28 determinant factors are unknown. By assessing individual variations in salivary anti-IAV
29 activity in 92 people using an established new high-throughput system in this study, we
30 found that the anti-IAV activity varied widely between individuals and showed a
31 significant positive correlation with protein-bound sialic acid (BSA) level (ρ=0.473; $p <$
32 0.001). Furthermore, the anti-IAV activity of saliva with enzymatically reduced BSA
33 content was significantly lower. These results indicate that BSA is a direct regulator of
34 salivary anti-IAV activity and is a determinant of individual differences. Additionally, after
35 comparing the anti-IAV activity across the groups by age, anti-IAV activity in young
36 people (aged 5–19 years) were lower than in adults aged 20–59 years and elderly people
37 aged 60–79 years. Our study suggests that BSA levels in saliva may be important in
38 preventing influenza infection.

39

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40 **INTRODUCTION**

41 Influenza viruses are some of the most important human pathogens. Seasonal
42 influenza viruses (types A and B) cause epidemics worldwide, with the World Health
43 Organization estimating hundreds of million infections and approximately 250,000–
44 500,000 deaths per year [1, 2], leading to substantial social and economic burdens
45 worldwide [3]. Several preventive and curative strategies exist against influenza,
46 including vaccination and therapeutic agents, such as neuraminidase inhibitors and
47 adamantanes [4]. However, influenza viruses evolve rapidly, which enables them to
48 escape immunity induced by prior infections or vaccination; moreover, those viruses are
49 transmitted efficiently from human to human via respiratory droplets. Recently, there has
50 been substantial concern about the possibility of a pandemic of a novel influenza strain
51 [5], with reports of an increasing resistance of influenza A viruses to neuraminidase
52 inhibitors and adamantanes [6]. Thus, new approaches are needed to effectively prevent
53 and treat influenza infections.

54 The oral cavity is one of the entry sites for respiratory viruses. Saliva plays an
55 essential role in maintaining the integrity of human health. It provides lubrication and
56 protection and buffering action and clearance in the oral cavity, maintains tooth integrity,
57 and aids taste and digestion [7]. A previous study indicated that hyposalivation may be
58 a risk factor for acute respiratory infection [8], suggesting that saliva may contribute to
59 the role of innate immunity in the early stages of infection. Saliva induces both
60 antimicrobial and antiviral activities [9]. Additionally, multiple soluble factors in saliva
61 have potent inhibitory activity against influenza A viruses (IAVs), including sialic acid-
62 containing mucin 5 B (MUC5B), salivary scavenger receptor cysteine-rich glycoprotein-
63 340 (gp-340), alpha-2-microglobulin (A2M), and lectins (surfactant protein D [SP-D]) [10-
64 18]. Two main mechanisms for the anti-IAV activity of these factors have been proposed.
65 MUC5B, gp-340, and A2M acts as classic γ inhibitors, mediating the inhibition of IAV by

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66 presenting a sialic acid ligand that binds to the viral hemagglutinin (HA), thereby
67 preventing HA attachment to target cells [12-15]. In contrast, SP-D acts as a classic β
68 inhibitor by binding the lectin carbohydrate recognition domain (CRD) to specific
69 oligosaccharides on the head of the viral HA [16-18]. It has been shown that salivary
70 anti-IAV activity is primarily due to sialic-acid-containing proteins rather than lectin [13,
71 19]. As the first line of defense, salivary antiviral factors are likely to play an important
72 role in protection against respiratory infections. However, since previous studies on
73 salivary anti-IAV activity mentioned above have only been conducted using purifying
74 salivary proteins and small size analyses, the actual salivary anti-IAV activity of each
75 individual, and its determinant factors are unknown. To solve this problem, high-
76 throughput evaluation systems are required to evaluate multiple samples simultaneously.
77 In this study, we constructed a new high-throughput system to evaluate salivary anti-IAV
78 activity using neuraminidase activity derived from influenza virus as an index, and we
79 investigated individual variations in salivary anti-IAV activity and its determinant factors.
80

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81 **MATERIALS AND METHODS**

82 **Participants**

83 In Study 1 of the individual differences in salivary anti-IAV activity, 92 healthy
84 voluntary participants aged 20–59 years (67 men and 25 women; mean age ± S.D., 36.9
85 ± 9.9 years) were recruited using snowball sampling. This trial was registered with the
86 University Hospital Medical Information Network (UMIN; http://www.umin.ac.jp/;
87 Registration No. 000024720). In Study 2 of age-related changes in saliva, 240
88 participants aged 5–79 years (60 young people, 30 boys and 30 girls aged 5–19 years;
89 120 adults, 60 men and 60 women aged 20–59 years; and 60 elderly people, 30 men
90 and 30 women aged 60–79 years), who registered using a monitor at a contractor
91 (Research and Development, Inc.) were recruited. In these studies, the exclusion criteria
92 were as follows: 1) patients with serious disease; 2) those going to a hospital regularly
93 and under regular pharmaceutical treatment; 3) those who were pregnant or expecting
94 pregnancy; 4) those who were physically unable to complete the study-related
95 questionnaires; 5) those with scratches in the oral cavity; and 6) those considered
96 unsuitable to participate in the study by the study physician. All study protocols were
97 approved by the institutional review board of the Kao Corporation and were performed
98 in accordance with the Declaration of Helsinki. After receiving a full explanation of the
99 study, all participants and their legal guardians provided written informed consent.
100

101 **Sample collection and treatment**

102 Saliva samples were collected in a quiet room during the morning, between 09:00
103 and 10:00, and at least 60 min after eating. Subjects were asked to accumulate saliva in
104 their mouths and spit into a sterile plastic dish at the desired time or indicated time, and
105 saliva samples were collected for 5 min in Study 1 or 10 min in Study 2. The saliva
106 secretion rate (mL/min) was calculated by dividing the amount of saliva collected at the

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107 time of collection. Whole saliva samples were centrifuged at 15,000 rpm for 15 min at
108 4°C. The supernatant was collected and stored at −80°C until use. Frozen saliva samples
109 were thawed and vigorously mixed by vortexing prior to the assay.
110

111 **Cells and viral culture**

112 Madin–Darby canine kidney (MDCK) cells were cultured in minimum essential
113 medium (MEM; Sigma-Aldrich Co. LLC, St. Louis, MO) supplemented with 5% (v/v) heat-
114 inactivated fetal bovine serum (FBS; Sigma-Aldrich Co.) and 50 μg/mL gentamicin at
115 37°C in humidified air containing 5% CO_2. Influenza A virus strains [A/Puerto Rico/8/34
116 (H1N1)] and [A/Memphis/1/1971 (H3N2)] were propagated at 37°C using MDCK cells in
117 serum-free medium (SFM; Thermo Fisher Scientific Japan K.K., Kanagawa, Japan)
118 supplemented with 2 μg/mL acetylated trypsin (Sigma-Aldrich Co.) and 50 μg/mL
119 gentamicin.
120

121 **Saliva sample preparation**

122 For removal of sialic acid of saliva, 100 μL of saliva sample was mixed with 2 μL of
123 10 U/μL α2-3,6,8-Neuraminidase (Sialidase) from Arthrobacter ureafaciens (Roche) and
124 reacted at 37°C for 30 min. Heat-treated saliva sample was obtained by keeping 100 μL
125 of saliva sample at 95 °C for 30 min in a dry heat block. These sample was tested in the
126 following assays.
127

128 **Anti-IAV activity assays of saliva**

129 MDCK cells ($4×10^4$ cells/well) were cultured in a 96-well plate at 37°C for 24 h. The
130 saliva samples were diluted eight-fold in phosphate-buffered saline (PBS). Fifty
131 microliters of each saliva sample was mixed with 50 μL of the virus dilutions at a
132 multiplicity of infection (MOI) of 0.5 in PBS and 100 μL of 2× serum-free medium (SFM)

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133 at room temperature, and the 180-μL mixture was immediately applied to the MDCK cells.
134 After incubation at 37°C for 30 min, the MDCK cells were washed with PBS and
135 incubated in 100 μL of SFM at 37°C for 16–18 h for virus multiplication. Thirty microliters
136 of the MDCK cell supernatant was mixed with 20 μL of 0.25 mM 2′-(4-methylumbelliferyl)-
137 α-d-N-acetylneuraminic acid (4MU-Neu5Ac; Funakoshi) and incubated at 37°C for 30
138 min. The enzyme reaction for 4MU-Neu5Ac was stopped by adding 200 μL of 100 mM
139 sodium carbonate buffer (pH 10.7). Fluorescence from the sialidase reaction of virus NA
140 was quantified using a microplate reader (Infinite 200 multi-mode Reader, Tecan,
141 Männedorf, Switzerland) with excitation and emission wavelengths of 355 nm and 460
142 nm, respectively, for 4-metylumbelliferone (4MU). The relative sialidase activity in each
143 sample was calculated by setting the sialidase activity to 100% when PBS was used
144 instead of saliva. Finally, anti-IAV activity was defined by deducting the relative sialidase
145 activity from 100%.
146

147 **Hemagglutination inhibition (HI) assay**

148 To remove the nonspecific inhibitory activity of saliva-induced hemagglutination,
149 100-μL saliva samples were mixed with 200 μL of guinea pig erythrocytes and incubated
150 at 4°C for 2 h. The mixture was centrifuged at 2,000 *g* for 10 min, and the supernatant
151 (pretreated saliva sample) was used for the HI assay. The PR8 IAV strain was adjusted
152 to 4 hemagglutinin units (HAU) per 25 μL PBS using a pre-hemagglutinin assay. In 96-
153 well plates, a two-fold serial dilution of the pretreated saliva sample was mixed with 25
154 μL of 4-HAU PR8 IAV strain, followed by the addition of 50 μL of 0.7% guinea pig red
155 blood cells and left at 4°C for 2 h. The HI titer was calculated as the reciprocal of the
156 highest dilution that produced complete hemagglutination inhibition.
157
158

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159 **Sialic acid assay**

160 The concentrations of total sialic acid (TSA) and free sialic acid (FSA) in saliva were
161 measured using a sialic acid assay kit (Bioassay Systems). The concentration of protein-
162 bound sialic acid (BSA) was then calculated by subtracting the concentration of FSA
163 from the concentration of TSA.

164

165 **Salivary proteome analysis**

166 For ultrafiltration, 300 μL of saliva sample was mixed with 200 μL of 100 mM Tris-
167 HCl buffer (pH 8.5; Nippon Gene Co., Ltd.) in an Amicon Ultra-0.5 Centrifugal Filter Unit
168 (3 kDa; Merck Millipore Ltd), and the mixture was spun down at 14,000 *g* for 30 min at
169 4°C. The trapped saliva on the filter was filtered three times using 400 μL of Tris-HCl
170 buffer. Finally, the filter on a new tube was spun down at 1,000 *g* for 2 min at 4°C, and
171 the sample for proteome analysis was collected. The total protein concentration of each
172 sample was determined using the Pierce(TM) BCA Protein Assay Kit (Thermo Fisher
173 Scientific Inc.) and the saliva sample was prepared with equal concentrations of proteins
174 from each individual sample. The saliva sample containing 100 μg protein in Tris-HCl
175 buffer (90 μL in total) was mixed with 5 μL of 100 mM DL-dithiothreitol (MP Biomedicals,
176 LLC) for the reduction solution and heated at 56°C for 45 min. The mixture was added
177 to 5 μL of 111 mg/mL iodoacetamide (FUJIFILM Wako Pure Chemical Corporation) and
178 incubated at 37°C for 30 min in shade for alkylation. The mixture was then mixed with
179 50 μL of 0.1 μg/μL trypsin solution (Promega Corporation) and incubated at 37°C for 16
180 h. The reaction solution was added to a final concentration of 0.3% (v/v) of 10%
181 trifluoroacetic acid (FUJIFILM Wako Pure Chemical Corporation) to stop the reaction,
182 and then subjected to LC-MS/MS analysis. All reagents were diluted with Tris-HCl buffer.

183 Five μL of the purified peptides were injected into a high-performance liquid
184 chromatography system (ACQUITY UPLC: Waters) connected to a triple-quadrupole

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185 mass spectrometer (TSQ Vantage; Thermo Fisher Scientific) with an ion source of
186 electrospray ionization in multiple reaction monitoring (MRM) mode. Chromatographic
187 separation was performed on a Cadenza CD-C18 column (150 × 1 mm, 3 μm; Imtakt)
188 by binary gradient elution at a flow rate of 0.05 mL/min. Eluent A was 0.1% formic acid
189 in water, while acetonitrile containing 0.1% formic acid was used as eluent B. Ion source
190 (ESI) parameters were optimized as follows: spray voltage, 400 V; vaporizer temperature,
191 100°C; sheath gas pressure, 40 Arb; auxiliary gas pressure, 5 Arb; and capillary
192 temperature, 250°C. The peak areas for each target peptide were calculated using
193 Xcalibur (Quan Browser) software (Thermo Scientific).
194

195 **Enzyme-linked immunosorbent assay (ELISA)**

196 The concentrations of secretory IgA (sIgA), defensin alpha 1, neutrophil (DEFa1),
197 and LL-37 in saliva were measured using competitive ELISA kits (sIgA; Immundiagnostik
198 GmbH, DEFa1; CLOUD-CLONE CORP., LL-37; Hycult Biotech Inc.) in accordance with
199 the manufacturer's instructions.
200

201 **Statistical analysis**

202 Results from multiple experiments are expressed as means ± standard
203 deviation (S.D.) and all data were analyzed using IBM SPSS Statistics Version 24 (IBM
204 Japan, Ltd., Tokyo, Japan). Relationships between the two sets of data were analyzed
205 using Spearman's rank correlation. The statistical significance of differences between
206 groups was determined using one-way analysis of variance, followed by Tukey's (Fig. 3)
207 or Kruskal-Wallis (Fig. 4) multiple comparisons test. Results with $p < 0.05$ were
208 considered statistically significant.
209

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210 **RESULTS**

211 **Construction of high-throughput evaluation system for salivary anti-IAV activity**

212 To simultaneously evaluate the anti-IAV activity in many saliva samples, we
213 constructed a new high-throughput evaluation system using neuraminidase (NA)
214 derived from IAV as an index. Fig. 1A shows the schematic of the measurement flow of
215 salivary anti-IAV activity. The measurement consisted of the following steps: 1) mixing
216 the virus and saliva, 2) infection of MDCK cells using the IAV solution and saliva
217 mixture, 3) collecting the supernatant of the cells after 16–18 h of infection, and 4)
218 measurement of sialidase activities of the supernatant for multiplicated virus
219 quantification. The NA activity value when PBS was used instead of saliva was set to
220 100%, and the relative value was calculated from the NA activity value of saliva
221 samples. The value obtained by subtracting the relative NA activity value from 100 was
222 defined as the anti-IAV activity. A high correlation (ρ=0.916; $p < 0.001$) was confirmed
223 between the NA activity value in the supernatant of infected cells and the number of
224 infected cells stained for NP protein when MDCK cells were infected with IAVs mixed
225 with various saliva samples (Fig. 1B).

226

227 **Individual differences in salivary anti-IAV activity**

228 Using the established system, salivary anti-IAV activity against the PR8 IAV
229 strain (H1N1) in 92 individuals was assessed. Anti-IAV activity varied widely between
230 individuals, ranging from 2% to 97% (Fig. 2A). Furthermore, salivary anti-IAV activity
231 against the Memphis IAV strain (H3N2) was significantly positively correlated with anti-
232 IAV activity against the PR8 IAV strain (ρ=0.638; $p < 0.001$) (Fig. 2B). We then
233 performed a correlation analysis of salivary anti-IAV activity against the PR8 IAV strain
234 and the amount of various salivary components measured using proteomic analysis
235 and some kits to explore factors associated with individual differences in salivary anti-

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236 IAV activity (Fig. 2C). The results showed the highest positive correlation between
237 salivary anti-IAV activity and BSA levels (ρ=0.473; $p < 0.001$). In addition, the
238 conentration of several proteins, such as AGTN/gp-340, ZG16B, SGP28, and C6orf58,
239 significantly positively correlated with anti-IAV activity.
240
241 **Contribution of BSA to salivary anti-IAV activity**
242 To clarify whether BSA contributes directly to the anti-IAV activity of saliva, we
243 assessed saliva samples that were heat-treated or in which BSA was enzymatically
244 removed. The amount of BSA in the saliva was significantly reduced by α2-3,6,8-
245 sialidase treatment and was not altered by heat treatment (Fig. 3A). While the anti-IAV
246 activity of untreated saliva samples averaged about 50%, the anti-IAV activity of heat-
247 treated saliva samples decreased to an average of 25% (Fig. 3B). In contrast, the anti-
248 IAV activity of sialidase-treated saliva samples decreased to less than 10% on average
249 (Fig. 3B). To further elucidate the contribution of BSA to salivary anti-IAV activity, a
250 hemagglutination inhibition (HI) test was performed. Similar to the anti-IAV activity,
251 large individual differences were observed in HI titers in some saliva samples (Fig. 3C).
252 In addition, individual HI titers highly positively correlated with anti-IAV activity (Fig.
253 3D). Although no effect of heat treatment on HI titer was observed, sialidase treatment
254 significantly decreased the HI titer (Fig. 3E).
255
256 **Age-related differences in salivary anti-IAV activity**
257 We then compared salivary anti-IAV activity across a wide range of ages (5–79
258 years divided into three groups, young people (aged 5–19), adults (aged 20–59 years),
259 and elderly people (aged 60–79 years), using the high-throughput evaluation system we
260 constructed. The saliva secretion rate decreased with increasing age and was lowest in
261 elderly people (Fig. 4A). In contrast, the salivary anti-IAV activity was similar in adults

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262 and elderly people, and was significantly lower in young people (Fig. 4B). In each age
263 group, the salivary anti-IAV activity significantly positively correlated with salivary BSA
264 level (young; ρ=0.366, $p < 0.05$, middle adults; ρ=0.589, p-value < 0.001, elderly; ρ=608,
265 p-value < 0.001, total; ρ=0.511, p-value < 0.001) (Fig. 4C).
266

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267 **DISCUSSION**

268 In this study, we constructed a high-throughput system to evaluate salivary
269 anti-IAV activity. Using this system, we found that there are large individual differences
270 in salivary anti-IAV activity, and that the amount of BSA in saliva is important for these
271 individual differences.

272 In general, the main method of measuring the viral infection titer is to count the
273 number of infected cells by staining with antibodies against the virus and clumps of
274 cellular degeneration associated with viral infection. While these methods are suitable
275 for measuring the virus titer, it is difficult to evaluate multiple samples because this
276 approach requires a great deal of time and labor because of the need for operations,
277 such as multi-step dilutions and infected cells or colony couting. In our constructed
278 system, the neuraminidase activity derived from the propagated influenza virus in the
279 culture medium was measured using a multi-plate reader, which eliminates the
280 operations described above and is suitable for the analysis of multiple samples. A
281 significant positive correlation was observed between salivary anti-IAV activity against
282 the PR8 (H1N1) and Memphis (H3N2) strains (Fig. 2B), indicating that individual
283 differences in salivary anti-IAV activity are similar across influenza virus strains. It is
284 expected that this method can be used in the evaluation of many influenza virus strains
285 in the future.

286 Using the constructed system, it was clarified that the individual variations in
287 salivary anti-IAV activity most positively correlated with the amount of BSA (Fig. 2C).
288 Moreover, the HI titers of saliva were significantly reduced by treatment with α2-3,6,8-
289 sialidase (Fig. 3B). Some studies have reported that saliva contains soluble factors that
290 can exert anti-influenza activity, such as sialic acid binding protein and lectins. It has
291 been reported previously that several purified salivary proteins, such as gp-340 and

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292 A2M, can inhibit the hemagglutination of erythrocytes by presenting a sialic acid ligand
293 for the viral HA [12-15]. These reports are consistent with our results, and indicate that
294 the total amount of sialic acid possessed by these proteins determines the anti-IAV
295 activity of saliva. In other words, the amount of BSA in the saliva is expected to be an
296 indicator of frontline oral defenses against influenza viruses.

297 MUC5B has potent inhibitory activity against IAV. However, our data showed
298 that salivary MUC5B levels weakly correlated with the anti-IAV activity of saliva. White
299 et al. reported that the sialic acid of MUC5B is more easily cleaved than that of gp-340,
300 the levels of which highly correlated with anti-IAV activity. Our results suggest that the
301 sialic acid of MUC5B is cleaved by sialidase in the oral cavity [20-22], and the anti-IAV
302 activity of MUC5B in saliva may be reduced. In considering individual variations in the
303 anti-IAV activity of saliva, it is necessary to consider oral sialidase activity. In this study,
304 SGP28, C6orf58, and PIP also strongly correlated with the anti-IAV activity of saliva.
305 These molecules are known to have sugar chains, but the presence of sialic acid is not
306 clear [23-25]. They may also contribute to the anti-IAV activity of saliva via sialic acid,
307 which is less susceptible to cleavage by sialidase. It is known that sialic acids are
308 structurally diverse and that O-acetylated sialic acids are resistant to bacterial sialidase
309 [26]. In the future, it will be necessary to clarify the presence or absence of sialic acid in
310 these molecules, including their structural features.

311 Interestingly, the anti-IAV activity of saliva was reduced following excessive
312 sialidase treatment, but was not completely lost (Fig. 3A, 3 B). Moreover, salivary anti-
313 IAV activity also decreased following heating, which did not affect the concentration of
314 sialic acid. Therefore, it is possible that sialic acid-independent factors are also
315 involved in anti-IAV activity. One of the possible factors is the action of lectin-like
316 proteins, such as ZG16B, which have shown a high correlation with anti-IAV activity

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317 [27, 28]. Lectin (carbohydrate-binding proteins), including conglutinin, SP-D, MBL, and
318 SAP, bind selectively to specific carbohydrate structures (mannose- over galactose-
319 type sugars) located at the head of the influenza HA of susceptible strains, thereby
320 blocking the ability of HA to bind to sialylated cell-surface receptors [16, 17].
321 Furthermore, lectin-like proteins may also inhibit the fusion of the viral membrane with
322 the host cell by binding to sialic acid on the surface of the host cell [18]. In our
323 evaluation system, since host cells were exposed to a mixture of saliva and virus for 30
324 min, the action of lectin-like proteins in saliva that inhibit the contact between host cells
325 and virus would also contribute to the anti-IAV activity of saliva. On the other hand, in
326 our evaluation system, the activity of molecules that inhibit the later mechanisms of
327 virus propagation, such as replication and release, is not reflected in the anti-IAV
328 activity. For this reason, defensin and lactofferin, which are known to inhibit replication
329 of IAV [12, 29-33], showed only a weak correlation with anti-IAV activity in our study.

330 The current thinking regarding influenza infection morbidity is that the risk
331 factors are related to age, higher rates of infection in school-aged children relative to
332 adults, and lower rates in the elderly. Children spend a great deal of time in
333 communities where daily contact with other people is extensive; for example, in
334 schools, playgroups, and daycare centers, and it is assumed that close contact favors
335 infection [34, 35]. The results of evaluating differences in salivary properties by age
336 (Fig. 4) showed that salivary volume, which has been reported as a predictive risk
337 factor for influenza transmission [8], was higher in the younger age group. On the other
338 hand, the anti-IAV activity of saliva in the young group was significantly lower than that
339 in the middle and elderly groups. These results suggest that low salivary anti-IAV
340 activity may also be associated with a high rate of IAV transmission in school-aged
341 children. In addition, salivary anti-IAV activity was highly correlated with sialic acid in
342 each age group, indicating that the determinants of anti-IAV activity were not as

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343 sensitive to age. Thus, qualitative control of saliva in young people may be effective in
344 preventing IAV infection. Prospective intervention trials with techniques that provide
345 qualitative control of saliva are needed to gain insight into the contribution of salivary
346 anti-IAV activity to IAV infection in young people.

347 Our study revealed that there are large individual differences in the anti-IAV
348 activity of saliva, which mainly depends on the amount of BSA. BSA directly contributes
349 to salivary anti-IAV, which may contribute to influenza morbidity in young people. In
350 conclusion, the understanding of individual differences in salivary anti-IAV activity may
351 provide new insights into creating effective methods to prevent influenza infection.

352

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453 **Acknowledgments**

454 We would like to thank Editage (www.editage.com) for English language editing.

455

456 **Author contributions**

457 T.M. and N.O. designed the project; K.K., C.S., and H.K. performed the research; K.K.,
458 C.S., H.K., T.M., Y.K., and T.S. analyzed the data; K.K., C.S., and T.M. wrote the
459 paper. T.M., N.O., Y.K., and T.S. edited the manuscript.

460

461 **Competing financial interests**: The authors declare no competing financial interests
462 in this project.

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465 **Figure Legends**

466 **Figure 1. Construction of high-throughput assay of salivary anti-IAV activities.**

467 (A) Schematic of the measurement of salivary anti-IAV activities. (B) Correlation
468 between viral sialidase activity and infected cell count. The plot indicates significant
469 moderate positive correlation. ρ, Spearman's correlation coefficient. ***, $p < 0.001$

470

471 **Figure 2. Individual differences of salivary anti-IAV activities.**

472 (A) Salivary anti-IAV activities against PR8 IAV strain (n=92). Data are presented as
473 mean ± SD from three independent experiments. A bar graph shows the activity in
474 each subject. (B) Correlation between salivary anti-IAV activity against PR8 and
475 Memphis IAV strains (n=65). The plot shows significant moderate positive correlation.
476 (C) The correlation between salivary anti-IAV activity against the PR8 IAV strain and
477 amount of various salivary components (n=72–85). A bar graph shows Spearman's
478 correlation coefficient (ρ) in each subject. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$

479

480

481 **Figure 3. Contribution of protein-bound sialic acid to salivary anti-IAV activity.**

482 Effect of α2-3,6,8-sialidase or heat treatment on (A) the concentration of BSA and (B)
483 salivary anti-IAV activity against the PR8 IAV strain. (C) Hemagglutination inhibition
484 assay with saliva (n=8, A-H). The bar graph shows the HI titers of each subject. This
485 experiment was repeated three times with similar results. (D) Correlation between
486 salivary anti-IAV activity against the PR8 IAV strain and the HI titer of saliva. The plot

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487 shows a moderately positive correlation. (E) The effect of α2-3,6,8-sialidase and heat
488 treatment on the HAI titer of saliva. Data are presented as mean ± SD for each group.
489 Average plateau values were compared using one-way analysis of variance and
490 Tukey's test. ρ, Spearman's correlation coefficient. *, $p < 0.05$; ***, $p < 0.001$.

491

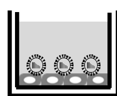
492 **Figure 4. Age-related differences in salivary anti-IAV activity.**

493 Comparison of (A) the total saliva volume (mL/min), (B) salivary anti-IAV activity
494 against the PR8 IAV strain, and (C) correlation between salivary anti-IAV activity
495 against the PR8 IAV strain and the concentration of BSA in young people (30 boys and
496 30 girls aged 5–19 years), adults (60 men and 60 women aged 20–59 years), and 60
497 elderly people (30 men and 30 women aged 60–79 years). Data are presented as
498 mean ± SD of each group. Average plateau values were compared using one-way
499 analysis of variance and the Kruskal-Wallis test. *, $p < 0.05$; ***, $p < 0.001$.

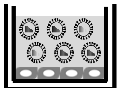
Figure. 1

(A)

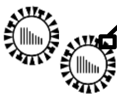
Mixing of virus and saliva



Virus infection for MDCK cells
37℃, 30 min in 96 well plate



Virus multiplication
37℃, 16-18 h in 96 well plate



Measurement sialidase activities
for virus quantification

(B)

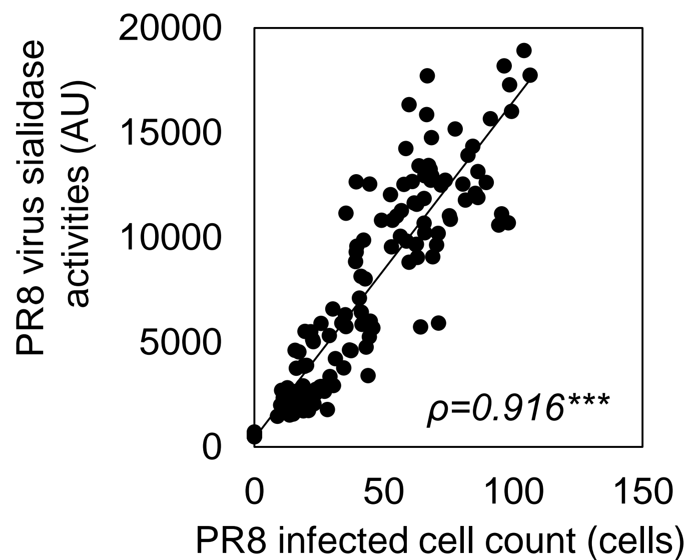
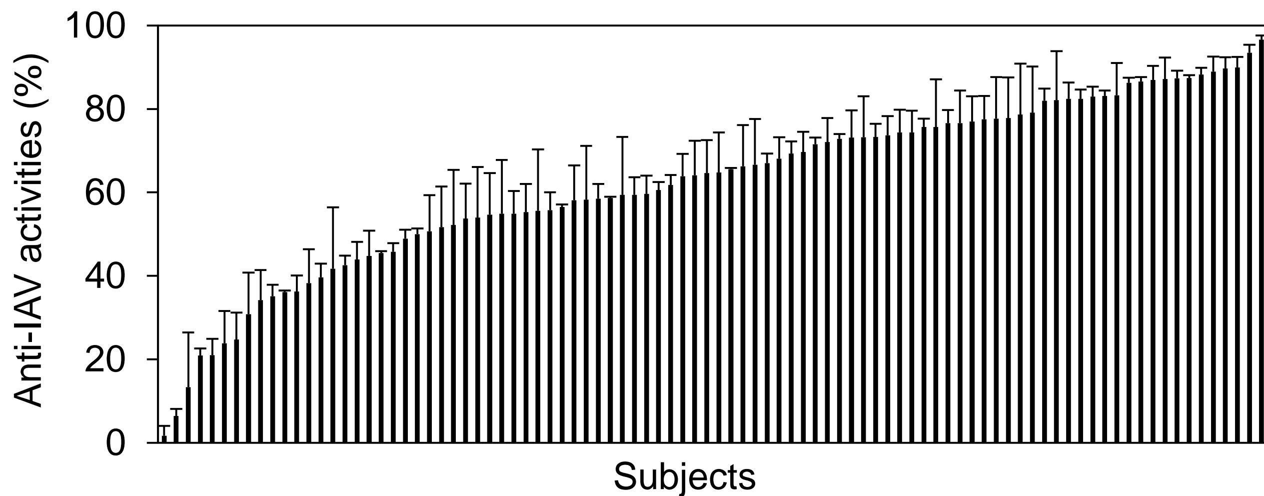


Figure. 2

(A)



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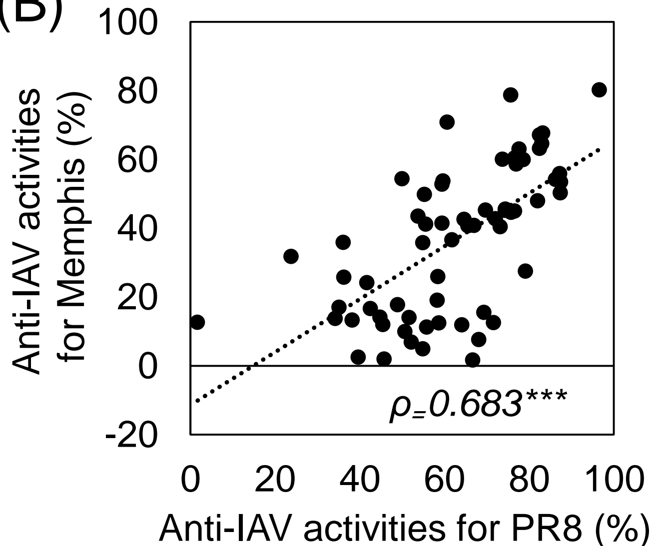


(C)

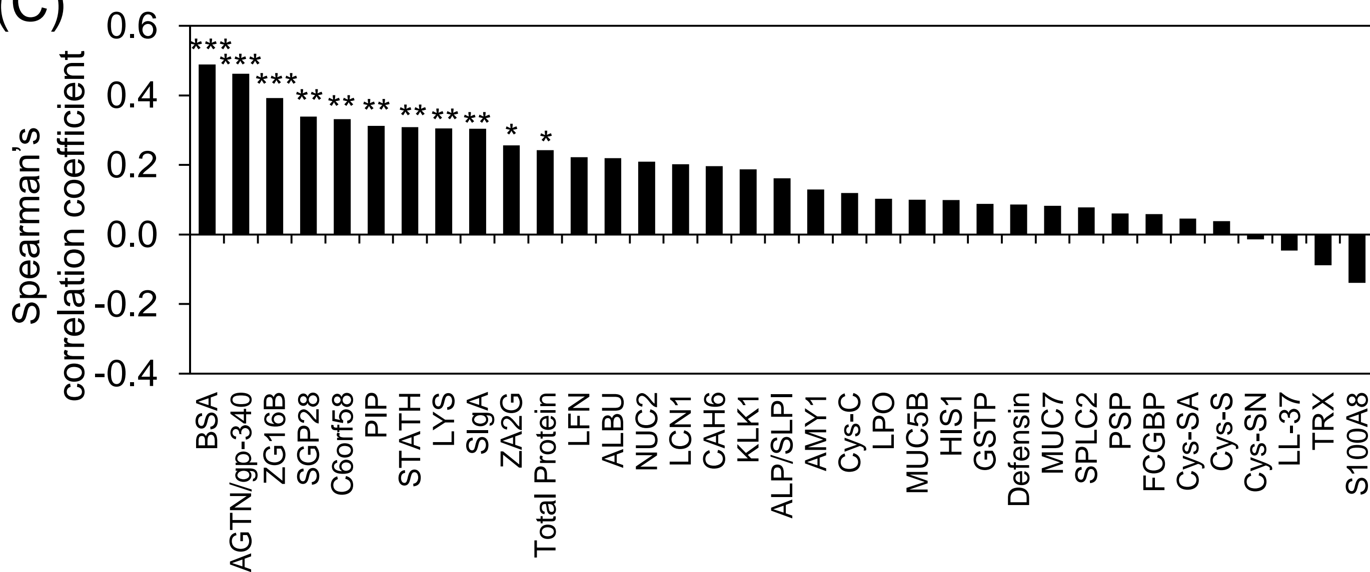
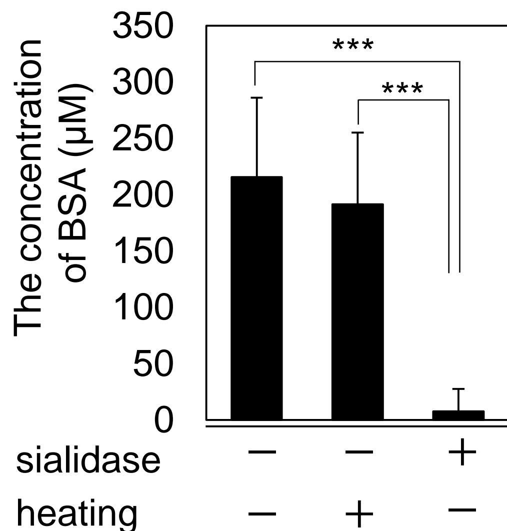


Figure. 3

(A)



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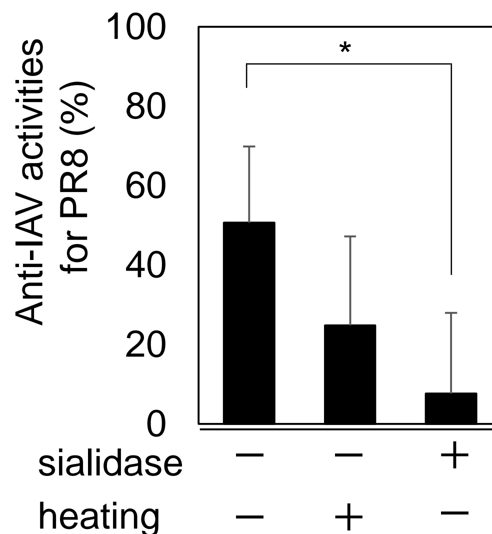
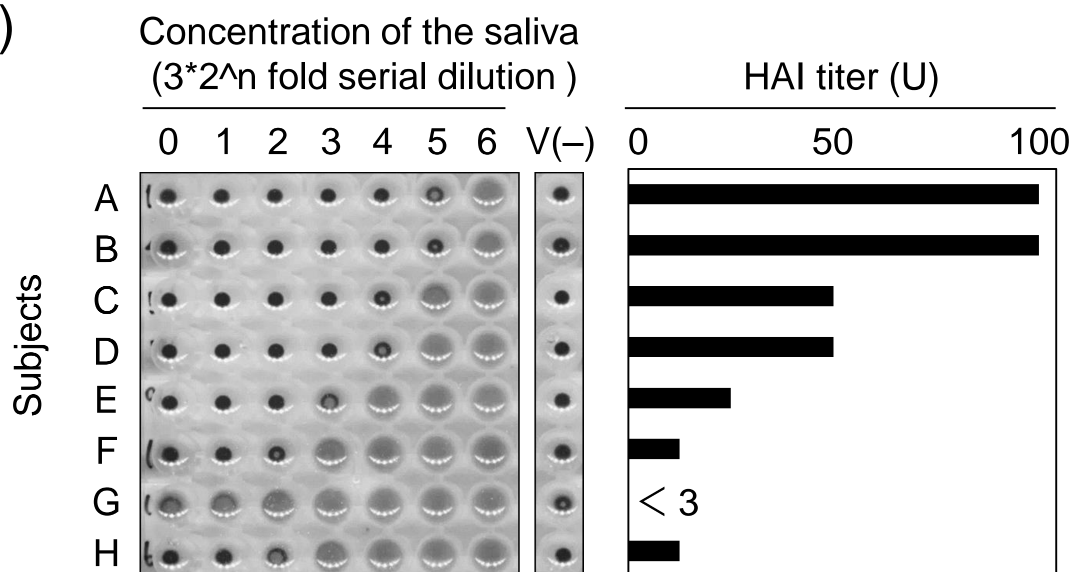
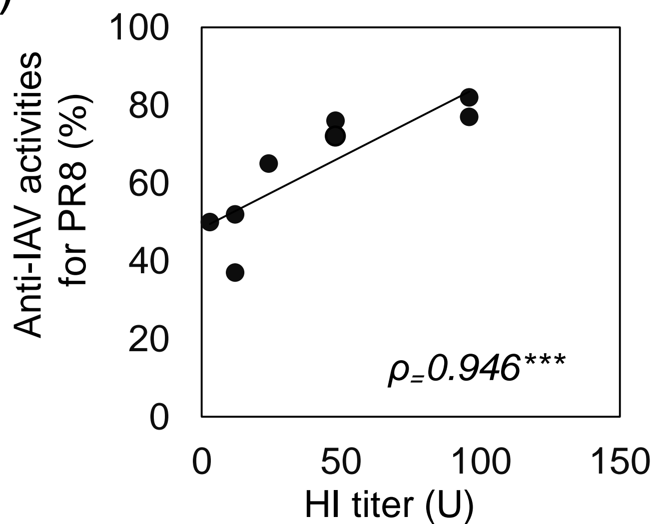


(C)



(D)



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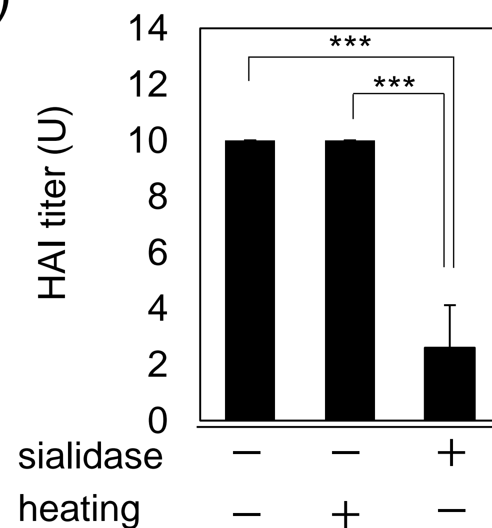
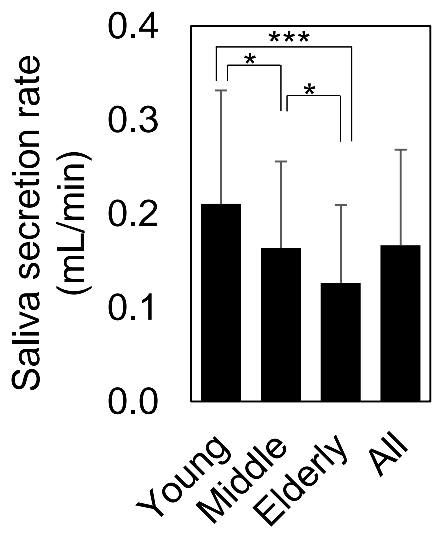


Figure. 4

(A)



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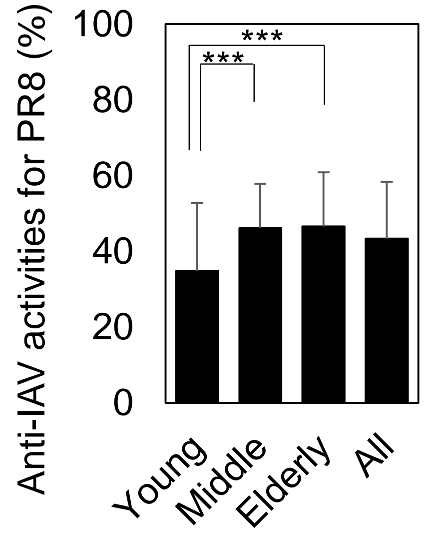

(C)