

1 **The Consequences of Egg Adaptation in the H3N2 Component to the Immunogenicity of**
2 **Live Attenuated Influenza Vaccine**

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23 **Keywords:** Live Attenuated Influenza Vaccine, H3N2, Egg-adaptation, Primer ID

24 **Running Title:** H3N2 egg adaptation consequences in LAIV

25 Word count: 1999/2000. Brief Report.

26 **Footnote Page**

27 **Conflicts of Interest**

28 The authors have no conflicts of interest.

29 **Funding**

30 This work was supported by a Wellcome Trust Intermediate Clinical Fellowship award to TdS
31 (110058/Z/15/Z) and by a Biotechnology and Biological Sciences Research Council grant
32 (BB/K002465/1) and a Wellcome grant (205100/Z/16/Z) to WSB. RK was supported by
33 Wellcome fellowship (216353/Z/19/Z).

34

35 Portions of this work were previously presented as a poster at Options X in Singapore in 2019.

36 Abstract Number: 11111.

37

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43

44 **Abstract**

45 Adaptation in egg-passaged vaccine strains may cause reduced vaccine effectiveness due to
46 altered antigenicity of the influenza haemagglutinin. We tested whether egg adaptation
47 modified serum and mucosal antibody responses to the A(H3N2) component in the Live
48 Attenuated Influenza Vaccine (LAIV). Twice as many children seroconverted to an egg-
49 adapted H3N2 than the equivalent wildtype strain. Seroconversion to the wildtype strain
50 was greater in children seronegative pre-LAIV, whereas higher mucosal IgA responses to
51 wildtype antigen were observed if seropositive prior to vaccination. Sequencing of virus
52 from nasopharyngeal swabs from 7 days post-LAIV showed low sequence diversity and no
53 reversion of egg-adaptive mutations.

54 Word count: 100/100

55

56 **Background**

57

58 Vaccines offer the best protection against morbidity and mortality caused by influenza. Low
59 vaccine effectiveness (VE) is often attributed to an antigenic mismatch between the vaccine
60 and circulating strains caused by evolution of the influenza haemagglutinin (HA) [1].

61 Recently, despite a well-matched vaccine strain selection, low VE of the A(H3N2)
62 component was reported, potentially due to adaptive mutations caused by the production
63 of the influenza vaccine in eggs [1]. These egg-adaptive mutations can alter vaccine
64 antigenicity and lead to immune responses mismatched to circulating strains.

65

66 Of the four components of the current quadrivalent seasonal influenza vaccine, the issue of
67 egg adaptation resulting in antigenic mismatch is most problematic for H3N2 subtype
68 influenza A viruses. In 2014, the H3N2 3C.2a clade arose with a K160T mutation, giving an
69 additional putative glycosylation site in immunodominant antigenic site B on the HA head
70 [2]. In order to grow efficiently, 3C.2a strains revert from T160 to K160 when cultured in
71 eggs and this adaptive mutation has been suggested as a key reason for the recent loss of
72 H3N2 VE [1]. The reduction in immunogenicity against circulating strains caused by this
73 reversion has so far been described in the context of serum antibody responses to
74 inactivated influenza vaccine (IIV) [1]. Here, we examine the consequence of egg-
75 adaptations in the Live Attenuated Influenza Vaccine (LAIV) on serum and mucosal antibody
76 responses in young children to circulating T160-containing H3N2. We also sequence virus
77 recovered from the nasopharynx after LAIV immunisation to test whether there is reversion
78 to a human-adaptive form.

79

80 **Methods**

81

82 **Study design and sample collection**

83 The samples used in this study were generated during a larger randomised controlled trial
84 (ClinicalTrials.gov NCT02972957) comparing the impact of LAIV on the nasopharyngeal
85 microbiome. The trial was conducted in Sukuta, a periurban area in The Gambia during 2017
86 and 2018. A detailed description of the cohort and sampling is described elsewhere [3].

87 Influenza vaccine-naïve children aged 24-59 months received a single intranasal dose of the
88 trivalent Russian-backbone LAIV (Nasovac-S, Serum Institute of India Pvt Ltd) containing the
89 World Health Organisation recommended viruses for the Northern Hemisphere for either
90 2016-2017 or 2017-2018, dependent on the year of enrolment. For both vaccines, the H3N2
91 component was an A/Hong Kong/4801/2014 (H3N2)-like virus. H3N2 vaccine titres per dose
92 (50% Egg Infectious Doses (EID50)/ml) were $1 \times 10^{7.5}$ in 2017 and $1 \times 10^{7.6}$ in 2018.

93 Nasopharyngeal swabs (FLOQSwabs, Copan, Murrieta, CA, USA) were collected into
94 RNAProtect cell reagent (Qiagen) on day 2 (D2) and 7 (D7) post-vaccination. Serum and oral
95 fluid swab (Oracol Plus; Malvern Medical Development, Worcester, UK) samples were taken
96 on D0 and day 21 (D21). All samples were stored at -70°C before further processing. The
97 study was approved by The Gambia Government/MRC Joint Ethics Committee and the
98 Medicines Control Agency of The Gambia. A parent provided written or thumb-printed
99 informed consent for their children to participate.

100

101 **HAI and IgA assays**

102 Haemagglutinin inhibition (HAI) assays were carried out on serum samples using guinea pig
103 red blood cells (0.5% in PBS) in the absence of oseltamivir and with standard methods [4].

104 HAI titres pre- and D21 post-LAIV were determined to cell-cultured (in MDCK-SIAT cells) and
105 egg-cultured A/Hong Kong/4801/2014 (HK14) strains. Sequencing of viral stocks confirmed
106 the presence of T160 in cell-cultured and K160 in egg-cultured HK14. Egg-cultured virus also
107 contained further egg-adaptive mutations L194P, T203I and I260L. Seroconversion was
108 defined as a 4-fold rise in HAI titre to $\geq 1:40$, irrespective of baseline HAI titre, using a 2-fold
109 dilution series of serum starting from 1:10 dilution. Mucosal influenza-specific IgA was
110 measured in oral fluid samples at baseline and D21 post-LAIV using a protein microarray as
111 previously described [5, 6] with the percentage Surfact-Amps-20 in the blocking, washing
112 and incubation buffer increased from 0.05% to 5% to prevent background staining with oral
113 specimens. Microarrays were coated with recombinant HA1 protein expressed in human
114 cells (Sino Biological, Beijing, China) reflecting sequences of egg-adapted and cell-cultured
115 HK14. Total IgA was quantified using an ELISA and influenza-specific IgA expressed as a ratio
116 of influenza HA1-specific IgA/total IgA as previously described [6].

117

118 **RNA Extraction, Primer ID and Sequencing**

119 RNA was extracted from D2 and D7 nasopharyngeal samples previously identified as
120 positive for H3N2 shedding by RT-PCR [3], using QIAamp Viral RNA Mini Kit (Qiagen) with
121 carrier RNA. RNA was also extracted from vaccine aliquots diluted 1000-fold. RNA was
122 reverse transcribed using Superscript III (Invitrogen) and a barcoded primer specific to each
123 500bp sub-amplicon in the HA (Primer ID). Primer ID attaches a unique barcode to each
124 cDNA molecule during reverse transcription and allows for PCR and sequencing error
125 correction [7, 8]. PCR was performed using KOD polymerase (Merck). Samples were pooled
126 across sub-amplicons and prepared for sequencing using NebNext Ultra II (NEB), then
127 sequenced on an Illumina MiSeq with 300bp paired-end reads. Sequences were analysed in

128 Geneious (v11) and a pipeline in R. Forward and reverse reads were paired using FLASH
129 (<https://ccb.jhu.edu/software/FLASH>) before being mapped to a reference sequence and
130 consensus sequences made for each barcode. Degenerate barcodes were removed (see
131 Supplementary material and Figure S1) and a minimum cut-off of 5 reads per barcode was
132 chosen. Raw sequences were deposited at <https://www.ebi.ac.uk/ena> (project number
133 PRJEB34129.) The analysis pipeline can be found at
134 <https://www.github.com/Flu1/GambiaLAIV>.

135

136 **Statistical analysis**

137 Paired and unpaired proportions were compared using McNemar's and Chi² tests
138 respectively. HAI geometric mean fold rise (GMFR) within and between individuals was
139 compared using the Wilcoxon signed-rank and Mann-Whitney tests. Log₁₀-transformed IgA
140 fluorescence ratio fold-change was compared using paired and unpaired t-tests. Pairwise
141 correlations were assessed using Spearman's rank-order (GMFR) and Pearson's (log₁₀-
142 transformed IgA fold-change) test. Shannon Entropy was used to calculate the diversity of
143 mutations within each sequenced sample (Supplementary material). Genetic distance
144 between samples was calculated as described in Supplementary material. Statistical
145 analyses were performed using R version 3.5.0 and GraphPad Prism 8.0.2.

146

147 **Results**

148 Samples from 244 children were included in the HAI analysis (n=118 from 2017 and n=126
149 from 2018 [3]). Influenza-specific oral fluid IgA data were available from 214 children (n=100
150 from 2017 and n=114 from 2018). D7 nasopharyngeal swab samples from 30 children with
151 H3N2 detected by RT-PCR along with D2 samples from 22/30 were available for sequencing.

152

153 No significant differences were seen between pre-LAIV HAI titres to egg-cultured and cell-
154 cultured HK14 ($p=0.84$, Figure S2). The proportion of children who seroconverted to egg-
155 cultured HK14 virus was 25.0% (61/244, 95% confidence interval (CI) 19.6-30.4) compared
156 to 12.3% (30/244, 95% CI 8.2-16.4) to cell-cultured HK14 ($p<0.0001$). D0 to D21 GMFR to
157 egg-cultured HK14 was greater than to cell-cultured HK14 ($p<0.0001$, Figure 1A). A
158 significant correlation was present between GMFR to egg-cultured and cell-cultured HK14
159 ($r_s=0.58$, $p<0.0001$, Figure S3), although discrepant samples were observed with
160 seroconversion to only one virus (Figure 1B). In contrast, the increase in mucosal influenza
161 HA-specific IgA from D0 to D21 post-LAIV was greater to cell-culture matched HK14 HA
162 compared to egg-culture matched HK14 HA ($p=0.0009$, Figure 1C). A significant correlation
163 was observed between IgA fold-change to egg- and cell-cultured HK14 HA ($r=0.69$,
164 $p<0.0001$, Figure S4).

165

166 To explore the impact of prior H3N2 infection on serum and mucosal antibody responses to
167 HK14 in LAIV, children were stratified based on seropositivity to cell-cultured HK14 (pre-
168 LAIV HAI titre $\geq 1:10$). In seronegative children, seroconversion to egg-cultured HK14 (50.7%,
169 37/73, 95% CI 39.2-62.2%) and cell-cultured HK14 (27.4%, 20/73, 95% CI 17.2-37.6%) was
170 greater than in seropositive children (14.0% seroconversion to egg-cultured, 24/171, 95% CI
171 8.8-19.4%, $p=0.00048$ and 5.8% seroconversion to cell-cultured, 10/171, 95% CI 2.3-9.4%,
172 $p<0.0001$). This pattern was reflected in GMFR values (Figure 2A), but not in IgA responses.
173 D0 to D21 post-LAIV IgA fold change, to HK14 HA proteins representative of both egg-
174 cultured and cell-cultured HK14, was modestly higher in seropositive children compared to
175 seronegative children (Figure 2A).

176

177 To explore whether reversion of egg-adapted mutations in LAIV HK14 during
178 nasopharyngeal replication could drive responses to cell-cultured HK14, we sequenced two
179 sub-amplicons containing HA amino-acids 1-276. 20 samples with low H3N2 cycle threshold
180 (Ct) values (five D2 and fifteen D7 from sixteen individuals) were successfully amplified
181 (Supplementary material, Figure S5). No significant reversion of egg-adaptive mutations was
182 seen in any samples (Figure 2C). This included D7 samples from seven seroconverters to cell-
183 cultured HK14. Two samples showed a single sequence with P194L (<0.2% frequency) and
184 three samples showed one or two sequences with I203T (<0.2% frequency). Along with
185 position 160, these three sites were >99.9% identical to the vaccine across all samples. Few
186 mutations rose to high frequency with only five mutations occurring above 5%. Of these,
187 I23L was a pre-existing polymorphism present at 1% in the vaccine strain and the other four
188 mutations were synonymous. There was no significant difference between Shannon entropy
189 for the samples and vaccine strains (Z-test, $p=0.41$). In individuals with matched samples,
190 mutations present at higher frequencies on D2 had been lost by D7 (Figure 2C). Using the
191 relative L1-norm as a measure of genetic similarity, there was no significant difference
192 between samples taken from the same individual and other samples (Z-test, $p=0.54$, Figure
193 S6).

194

195 **Discussion**

196 We describe, to our knowledge for the first time, the impact of egg-adaptations in a recent
197 H3N2 3C.2a strain vaccine antigen on serum and mucosal antibody responses induced by
198 LAIV to the equivalent human-adapted strain reflective of circulating viruses. In keeping
199 with observations with IIV [2], serum antibody responses to cell-cultured HK14 were

200 significantly lower than to the vaccine-matched egg-cultured HK14. However, a proportion
201 of children did seroconvert to cell-cultured HK14, which was most evident in children
202 seronegative to cell-cultured HK14 prior to receiving LAIV. In the absence of prior HK14
203 exposure, the serum antibody response in these children may be broader and directed to
204 antigens outside antigenic site B [2].

205

206 In contrast to serum HAI induction, IgA responses to proteins representing cell-cultured
207 HK14 HA were equivalent or higher than those representing egg-cultured HK14 HA. IgA
208 responses were also modestly higher in children who were seropositive to HK14 prior to
209 LAIV compared to seronegative children. Therefore, in our cohort, unlike serum antibodies
210 induced by LAIV, mucosal IgA responses may largely reflect boosting of prior responses
211 acquired through natural infection. However, the lack of a significant IgA correlate of
212 protection following LAIV vaccination means that the clinical relevance of this finding is
213 uncertain. Compared to the serum HAI response, little is known about the antigen-
214 specificity of LAIV-induced mucosal IgA responses, although some studies have suggested
215 influenza-specific IgA responses are more cross-reactive than IgG responses [9, 10]. It is
216 important to note that the IgA responses measured constitute binding antibody, rather than
217 functional responses, which are challenging to measure in mucosal samples. Future work
218 could explore functional mucosal IgA responses, as well as anti-HA stalk responses which we
219 did not assess and may provide cross-reactive responses.

220

221 A previous influenza human challenge study in adults has demonstrated the reversion of an
222 egg-adapted mutation during replication in the upper respiratory tract [11]. We
223 hypothesized that a similar phenomenon could occur with LAIV replication of HK14,

224 providing a potential explanation for cell-cultured HK14-specific antibody responses after
225 vaccination with an egg-adapted antigen. Sequencing of the shed virus, however, revealed
226 no changes at sites of egg adaptation and very few significant changes in the HA. The lack of
227 a K160 fitness cost in humans is perhaps unsurprising given the majority of H3N2 isolates
228 prior to 2014 contained K160. Recent studies have found low within-host diversity of virus
229 in natural influenza infections in vaccinated and unvaccinated individuals, suggesting that
230 the immune system does not put significant pressure on the influenza virus to evolve over
231 the course of an individual infection [12, 13]. Our results agree with this and imply there is
232 little positive selection on the LAIV H3N2 HA in the nasopharynx within the first week and
233 that reversion of egg-adaptation mutations such as K160 is unlikely.

234

235 Although egg adaptation is likely to be an important factor, increasing data suggest several
236 factors contribute to the low VE to H3N2 observed in some years [14]. Developing
237 alternatives to egg-based methods of vaccine production is clearly important as current
238 vaccines may result in protective H3N2 responses only in sub-populations of individuals.

239

240 **Funding**

241 This work was supported by a Wellcome Trust Intermediate Clinical Fellowship award to TdS
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244 Wellcome fellowship (216353/Z/19/Z).

245

246 **Acknowledgements**

247 We gratefully acknowledge the study participants and parents who took part in the study. We
248 also acknowledge the dedicated field team involved in carrying out the clinical trial. We thank
249 Christine Carr, Monali Patel and Hirushi Rajapakse for their technical support at the
250 Respiratory Virus Unit (PHE NIS, Colindale).

251

252

253 **Figure legends**

254 **Figure 1. a)** Geometric Mean Fold Rise (GMFR) to egg-cultured and cell-cultured HK14 **b)**
255 Fold-change from day 0 to day 21 post-LAIV in HA1-specific IgA to proteins representing
256 egg-cultured and cell-cultured HK14 **c)** GMFR to egg and cell-cultured HK14 for each
257 individual. Dotted line represents 4-fold increase in HAI titre between day 0 and day 21
258 which defines seroconversion.

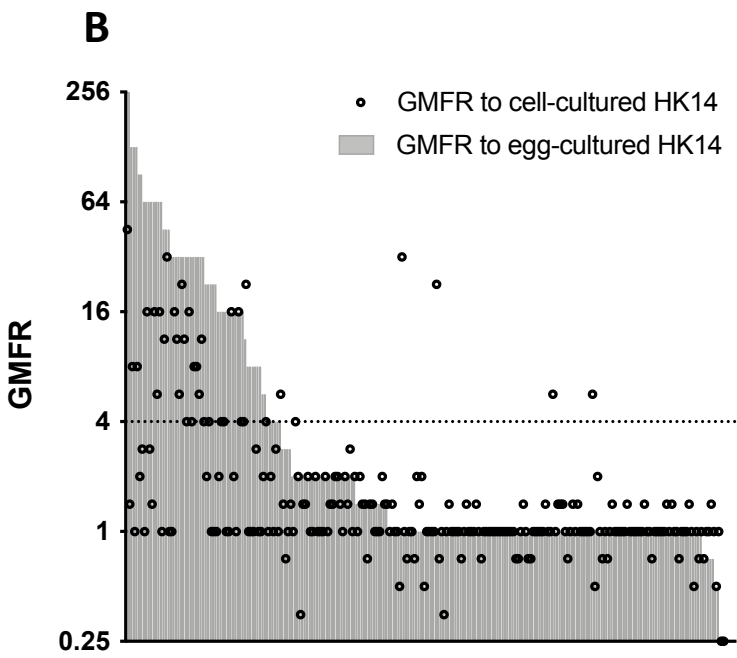
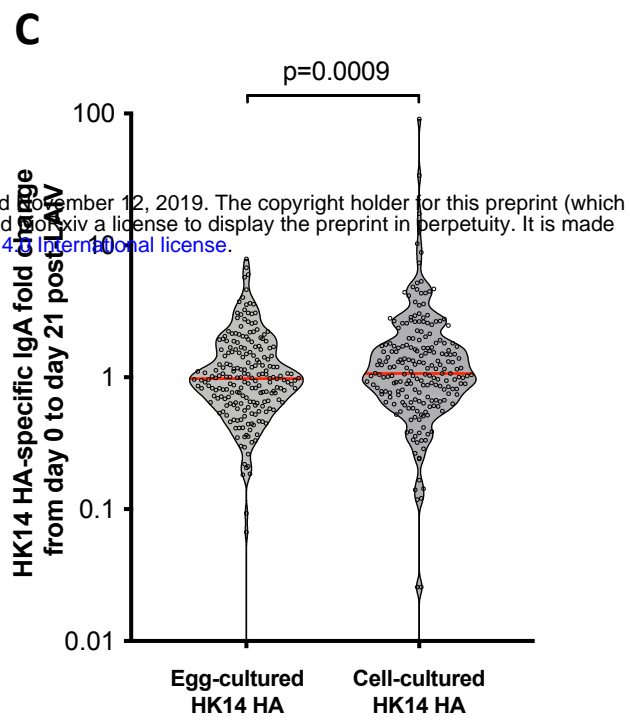
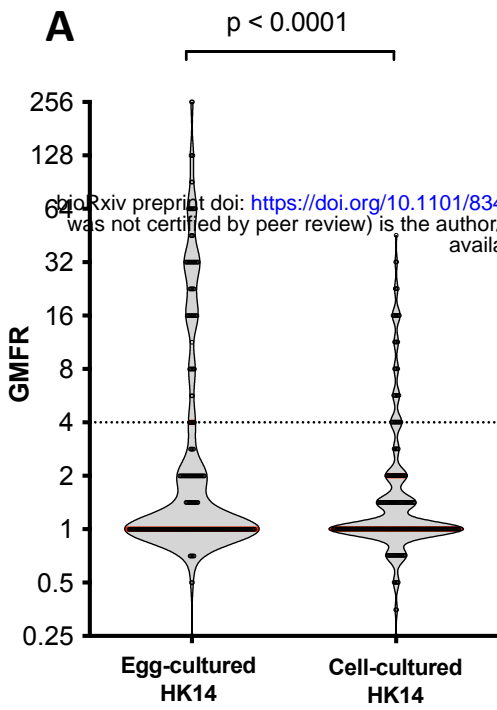
259 **Figure 2. a)** GMFR to egg and cell-cultured HK14 comparing children seropositive and
260 seronegative to cell-cultured (i.e. wild-type) HK14 prior to receiving LAIV. Dotted line
261 represents 4-fold increase in HAI titre between day 0 and day 21 which defines
262 seroconversion. **b)** Fold-change from day 0 to day 21 post-LAIV in HA1-specific IgA to
263 proteins representing egg-cultured and cell-cultured HK14, comparing seropositive and
264 seronegative children. **c)** Shed virus from 20 samples from either day 2 or day 7 and the
265 vaccine from 2017 and 2018 were sequenced using Primer ID. The percentage of mutations
266 are shown at each sequenced nucleotide position in the HA where 1 refers to the first base
267 of the signal peptide. The sample ID and day of sample collection are shown.

268

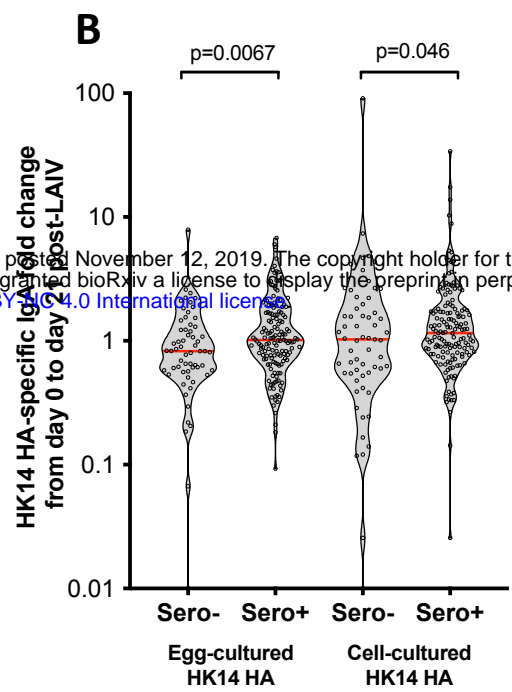
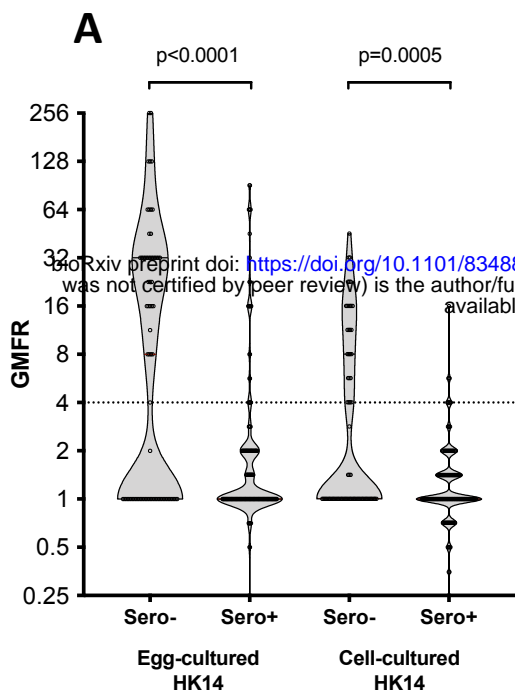
269 **References**

- 270 1. Zost SJ, Parkhouse K, Gumina ME, et al. Contemporary H3N2 influenza viruses have a
271 glycosylation site that alters binding of antibodies elicited by egg-adapted vaccine strains.
272 Proceedings of the National Academy of Sciences **2017**; 114:12578-83.
- 273 2. Chambers BS, Parkhouse K, Ross TM, Alby K, Hensley SE. Identification of hemagglutinin
274 residues responsible for H3N2 antigenic drift during the 2014–2015 influenza season. Cell
275 reports **2015**; 12:1-6.
- 276 3. Lindsey BB, Jagne YJ, Armitage EP, et al. Effect of a Russian-backbone live-attenuated
277 influenza vaccine with an updated pandemic H1N1 strain on shedding and immunogenicity
278 among children in The Gambia: an open-label, observational, phase 4 study. Lancet
279 Respiratory Medicine **2019**; 7:665-76.
- 280 4. Ellis J, Zambon M. Molecular analysis of an outbreak of influenza in the United Kingdom.
281 European journal of epidemiology **1997**; 13:369-72.
- 282 5. Koopmans M, De Bruin E, Godeke G-J, et al. Profiling of humoral immune responses to
283 influenza viruses by using protein microarray. Clinical Microbiology and Infection **2012**;
284 18:797-807.
- 285 6. de Silva TI, Gould V, Mohammed NI, et al. Comparison of mucosal lining fluid sampling
286 methods and influenza-specific IgA detection assays for use in human studies of influenza
287 immunity. Journal of immunological methods **2017**; 449:1-6.
- 288 7. Jabara CB, Jones CD, Roach J, Anderson JA, Swanstrom R. Accurate sampling and deep
289 sequencing of the HIV-1 protease gene using a Primer ID. Proc Natl Acad Sci U S A **2011**;
290 108:20166-71.
- 291 8. Goldhill DH, Langat P, Xie H, et al. Determining the mutation bias of favipiravir in influenza
292 virus using next-generation sequencing. Journal of virology **2019**; 93:e01217-18.

- 293 9. Morokutti A, Muster T, Ferko B. Intranasal vaccination with a replication-deficient
294 influenza virus induces heterosubtypic neutralising mucosal IgA antibodies in humans.
295 *Vaccine* **2014**; 32:1897-900.
- 296 10. Muramatsu M, Yoshida R, Yokoyama A, et al. Comparison of antiviral activity between
297 IgA and IgG specific to influenza virus hemagglutinin: increased potential of IgA for
298 heterosubtypic immunity. *PLoS One* **2014**; 9:e85582.
- 299 11. Leonard AS, McClain MT, Smith GJ, et al. Deep sequencing of influenza A virus from a
300 human challenge study reveals a selective bottleneck and only limited intrahost genetic
301 diversification. *Journal of virology* **2016**; 90:11247-58.
- 302 12. Debbink K, McCrone JT, Petrie JG, et al. Vaccination has minimal impact on the intrahost
303 diversity of H3N2 influenza viruses. *PLoS pathogens* **2017**; 13:e1006194.
- 304 13. McCrone JT, Woods RJ, Martin ET, Malosh RE, Monto AS, Luring AS. Stochastic
305 processes constrain the within and between host evolution of influenza virus. *Elife* **2018**;
306 7:e35962.
- 307 14. Cobey S, Gouma S, Parkhouse K, et al. Poor immunogenicity, not vaccine strain egg
308 adaptation, may explain the low H3N2 influenza vaccine effectiveness in 2012–2013. *Clinical*
309 *Infectious Diseases* **2018**; 67:327-33.
- 310



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