Oropharyngeal mucosal transmission of Zika virus in rhesus macaques

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Abstract

Zika virus (ZIKV) is present in urine, saliva, tears, and breast milk, but the transmission risk associated with these body fluids is currently unknown. We evaluated the risk of ZIKV transmission through mucosal contact in rhesus macaques. Application of high-dose ZIKV directly to the tonsils of 3 rhesus macaques resulted in detectable plasma viremia in all animals by 2 days post-exposure; virus replication kinetics were similar to those observed in animals infected subcutaneously. Three additional macaques inoculated subcutaneously with ZIKV served as saliva donors to assess the transmission risk from contact with oral secretions from an infected individual. Seven naive animals repeatedly exposed to donor saliva via the conjunctivae, tonsils, or nostrils did not become infected. Our results suggest that there is a risk of ZIKV transmission via the mucosal route, but that the risk posed by oral secretions from individuals with a typical course of ZIKV infection is low.

Introduction

Zika virus (ZIKV) is a mosquito-borne flavivirus that is associated with Guillain-Barré syndrome in adults and a range of birth defects, most notably microcephaly, in congenitally infected infants[1–5]. ZIKV has become a subject of global concern as it has rapidly expanded its geographic range in the past 2 years. One of the many surprising aspects of the current ZIKV pandemic is the confirmation of one previous report of sexually transmitted ZIKV infection[6–9]. Because mosquito-borne flavivirus infection has not previously been associated with human-to-human transmission, understanding transmission risks is critical for designing effective prevention and control strategies.

43   In the Americas, ZIKV is transmitted among humans by *Aedes* species mosquitoes, primarily

44   *Aedes aegypti*[10–13]. The explosive epidemic in tropical areas where important vector mosquito

45   species are common has made it challenging to differentiate between vector-borne and sexual

46   ZIKV transmission, so the risk of human-to-human transmission has been difficult to assess. In

47   the continental United States, which has primarily travel-associated cases, detection of non-

48   vector transmission is much more straightforward, and 41 cases of sexually transmitted ZIKV

49   infection have been reported as of January 27, 2017 (https://www.cdc.gov/zika/geo/united-

50   states.html). These cases have included male-to-female, male-to-male, and female-to-male

51   transmission[6,14–16].

52   ZIKV RNA has been detected in blood, semen, and vaginal secretions, consistent with

53   observations of sexual transmission. Viral RNA and/or infectious virus has also been reported in

54   urine, saliva, tears, and breast milk, suggesting that these body fluids may also pose a

55   transmission risk[17–19]. Indeed, virus was transmitted to a caregiver of an individual with high

56   ZIKV viremia who eventually succumbed to infection[20]. Here we used rhesus macaques to

57   evaluate the risk of ZIKV transmission through mucosal membrane contact with saliva

58   (specifically, palatine tonsils, nasal mucosae, and conjunctivae). We applied high-dose ZIKV

59   stock directly to the tonsils of one group of animals to assess what we term the "theoretical" risk

60   of mucosal transmission using a dose that is >20-fold higher than that typically found in saliva.

61   In another experiment, we applied saliva from ZIKV-infected macaques to determine whether

62   ZIKV might also be transmitted by more casual contact.

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Results

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65 ***ZIKV application to tonsils results in systemic infection*.** To evaluate the risk of ZIKV

66 transmission via the oropharyngeal mucosa, we applied 8 x 10^5 PFU Zika virus/H.sapiens-

67 tc/FRA/2013/FrenchPolynesia-3328 (ZIKV-FP;[21]) directly to the palatine tonsils of three Indian-

68 origin rhesus macaques using a pipet (Fig. 1). One macaque infected by this route (664817) had

69 detectable plasma viremia by 1 day post-infection (dpi) and all 3 had detectable plasma viremia

70 by 2 days post-infection (dpi) (Fig. 2a). Peak plasma viremia was observed at 6 dpi in all 3

71 animals and ranged from 2.4 x 10^5 to 1.1 x 10^7 vRNA copies/mL. ZIKV plasma viremia was

72 undetectable in all animals by 14 dpi. Plasma viremia of animals infected after tonsillar

73 inoculation (Fig. 2, orange traces) was compared to plasma viremia of 3 animals infected

74 subcutaneously (Fig. 2, blue traces). Overall, plasma viremia in macaques infected via the oral

75 mucosa was similar in magnitude and duration to that of macaques inoculated subcutaneously

76 with the same stock of ZIKV-FP. Two notable exceptions were that viremia only became

77 detectable two days after infection in 2 of the 3 macaques infected via the tonsils, and the peak

78 plasma viral load in these animals occurred 1-3 days later than in animals infected

79 subcutaneously (Fig. 2a).

80 Zika virus RNA was also detectable in other bodily fluids following tonsil exposure. ZIKV RNA

81 was detected in the urine of animal 664817 at 8 dpi (1.6 x 10^3 vRNA copies/mL) and 590870 at

82 8-10 dpi (range = 7.4 x 10^3 - 1.8 x 10^4 vRNA copies/mL), but not in 511690 (Fig. 2b). We

83 collected saliva when possible, but because the volume of saliva that could be collected was

84 often insufficient to accurately assess viral load, we also collected oral secretions using

85 absorbent swabs that were eluted in a standardized volume of viral transport media (VTM).

86 ZIKV RNA detection in oral swab samples was variable from most animals and undetectable

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87   from animal 511690 (Fig. 2c). We were only able to collect saliva directly on two occasions from

88   animal 664817, and both samples were negative for ZIKV RNA (data not shown). ZIKV RNA

89   was detected in saliva from animal 590870 on days 6-9 post infection and from animal 511690

90   on days 8 and 10 post infection (Fig. 3). Overall, when saliva samples were available, ZIKV

91   RNA was detected more consistently and at more time points than in oral swabs (Fig. 3). By 14

92   dpi, ZIKV RNA was undetectable in the tested body fluids of all animals infected by tonsil

93   inoculation.

94   Sera from macaques that were infected by application of ZIKV to the tonsils neutralized ZIKV-

95   FP across a range of serum dilutions. Indeed, neutralization curves prepared using sera from all

96   3 animals revealed a similar profile as compared to sera from animals infected subcutaneously

97   (see Supplementary Figure 1). All animals developed neutralizing antibodies (nAb) with a 90%

98   plaque reduction neutralization test ($PRNT_{90}$) titer of 1:160 (664817 and 511690) or 1:80

99   (590870) by 28 dpi. Together, these results show that application of high doses of ZIKV to the

100   oral mucosae can result in systemic infection and induce humoral immune responses in a

101   manner similar to subcutaneous infection.

102   ***Inoculation with oral secretions from ZIKV-infected donors does not result in systemic***

103   ***infection.*** The highest concentration of ZIKV RNA we observed in the oral secretions of infected

104   macaques in previous studies was 2.9 x 10^4 vRNA copies/mL, while the dose of ZIKV stock

105   applied to the tonsils of the animals described above contained 8 x 10^5 PFU (approximately 8 x

106   10^8 vRNA copies/mL). To examine whether saliva from ZIKV-infected macaques was infectious

107   and represented an actual transmission risk to uninfected macaques through oropharyngeal

108   mucosa exposure, we first subcutaneously inoculated 3 rhesus macaques with 1 x 10^4 PFU of

109   ZIKV-FP, using the same dose, stock, and strain we have reported previously[21,22]. These

110   animals served as saliva donors to 7 naive recipients—saliva or oral swabs were collected from

111   donors daily from 3-10 dpi (cohorts 1 and 3) or 3-12 dpi (cohort 2) and applied to the tonsils,

112   nostrils, or conjunctivae of 1 or more recipients (See Fig. 1 for details). This 3-10 or 3-12 dpi

113   timeframe encompassed the period of time in which ZIKV RNA was detected in non-blood body

114   fluids of animals infected subcutaneously in our previous studies. All saliva donors had

115   detectable ZIKV plasma viremia by 1 dpi, peaking 3-5 dpi, (range = 7.35 x 10^5 - 5.42 x 10^6

116   vRNA copies/mL) and resolving by 14 dpi (Fig. 2a). ZIKV RNA was detected in the passively

117   collected urine of all 3 donors. Peak urine viral loads were detected 7-10 dpi (range = 2.68 x 10^4

118   - 1.95 x 10^5 vRNA copies/mL) (Fig. 2b).

119   We monitored ZIKV RNA in saliva over time through collection of oral swabs and, whenever

120   possible, saliva, from donor animals, to measure vRNA in the inocula used to challenge the

121   recipients (Fig. 2c and Fig. 3). We detected ZIKV RNA in the oral swab eluate of two saliva

122   donors (448436 and 861138); levels peaked at 2.77 x 10^3 vRNA copies/mL at 7 dpi and 1.18 x

123   10^4 vRNA copies/mL at 10 dpi respectively. The last saliva donor (756591) did not have

124   detectable ZIKV RNA in oral swab samples. We were unable to consistently collect saliva from

125   448436, but ZIKV RNA in the saliva of the 861138 was detectable 4-14 dpi and peaked at 1.12

126   x 10^4 vRNA copies/mL (Fig. 3). 756591 had detectable ZIKV RNA in saliva that peaked at 2.32 x

127   10^3 vRNA copies/mL at 5 dpi. Considered together, all donor animals had at least 2 time points

128   with detectable vRNA in either the saliva or oral swab samples during the time when saliva was

129   used to challenge the recipient animals. At 28 dpi, serum collected from all 3 donor animals

130   yielded $PRNT_{90}$ values of 1:160, similar to the recipients of tonsillar challenges (see

131   Supplementary Figure 1 for neutralization curves). Importantly, ZIKV RNA was undetectable in

132   all 7 recipient animals in all body fluids tested throughout the study. Furthermore, ZIKV-specific

133   neutralizing antibodies were undetectable in recipient animals ≥ 21 days after the final challenge

134   with saliva from infected donors.

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135 ***Infectivity of saliva from subcutaneously infected donors to IFNAR -/- mice.*** Because no

136 recipients of saliva from infected donors had detectable systemic ZIKV infections, we tested the

137 infectivity of donor saliva in *IFNAR-/-* mice. These mice are highly susceptible to ZIKV

138 infection[23,24], even at challenge doses as low as 1×10^2 fluorescent focus units[23] (approximately

139 1×10^2 PFU (personal communication Michael Diamond)). We inoculated 5- to 6-week-old

140 *IFNAR-/-* mice with ZIKV RNA-positive macaque saliva from 861138 collected at 10 dpi (1.12 x

141 10^4 vRNA copies/mL; 50 µl inoculum; $\sim 1 \times 10^{-0.25}$ PFU (estimated from 1:1000 ratio of

142 PFU:vRNA copies/mL of ZIKV stock [21,22])) or oral swab eluate from 448436 collected at 7 dpi

143 (2.77×10^3 vRNA copies/mL; 50 µl inoculum; $\sim 1 \times 10^{-0.86}$ PFU (estimated)), and monitored them

144 for 25 days for weight loss and mortality. All control and experimental mice survived with no

145 evidence of morbidity (Supplementary Fig. 2). Furthermore, ZIKV RNA was undetectable in

146 mouse serum at 3 dpi (the approximate peak of viremia in mice infected previously[23,24]) and the

147 animals did not seroconvert by 25 dpi, as assessed by PRNT (data not shown). These data

148 suggest that saliva from ZIKV-infected macaques did not contain enough replication-competent

149 virus to initiate infection in these sensitive hosts. The same saliva and oral swab samples were

150 concurrently inoculated onto Vero cells to attempt to quantify the infectious dose the mice might

151 have received; however, both the saliva and oral swab sample used for the mouse challenge

152 experiments gave no plaques on these cells. Our plaque assay and mouse inoculation doses

153 contained on the order of 100-500 vRNA copies, while our previous studies suggest that the

154 ratio of vRNA copies to plaque-forming units is approximately 1000:1, further supporting the

155 conclusion that very little infectious virus was present in these samples[21,22]. Together these

156 results suggest that macaques with ZIKV virus loads in the usual range after subcutaneous

157 infection shed very small quantities of infectious ZIKV in saliva.

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Discussion

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160   Humans and animals infected with ZIKV are known to shed virus (or vRNA) in multiple body

161   fluids, including blood, urine, saliva, and genital secretions[21,25–27]. Sexual transmission of ZIKV

162   between humans has been documented in several cases, but the risk of transmission from more

163   casual contact has been difficult to evaluate. Human-to-human transmission via non-sexual

164   contact has been reported in a single case to date, though this case involved a source patient

165   with extremely high viremia; in this case contact with tears or sweat from the source patient was

166   hypothesized to be the mode of transmission[20]. Here we used a nonhuman primate model to

167   investigate whether exposure to ZIKV via mucous membranes, particularly the oropharyngeal

168   mucosa, represents an infection risk.

169   Application of a high dose (8×10^5 PFU) of Asian-lineage ZIKV to the palatine tonsils resulted in

170   systemic infection in 3 of 3 rhesus macaques, suggesting that productive infection can indeed

171   be initiated at the oropharyngeal mucosa. The kinetics and magnitude of ZIKV replication in

172   plasma in these three animals were similar to those observed in animals infected

173   subcutaneously with the same stock of ZIKV-FP (Fig. 2a), and all 3 animals developed strong

174   nAb titers against homologous ZIKV-FP by 28 dpi. Detection of ZIKV RNA in oral swabs, saliva,

175   and urine was variable in the animals that received a direct tonsil challenge, findings that were

176   also similar to those from animals infected subcutaneously in this and a previous study[21].

177   Limitations associated with sample collection could have influenced our ability to detect vRNA in

178   fluids other than blood. For example, time from urination to sample collection varied because

179   urine was collected passively from pans in the animals' housing. Similarly, there is wide

180   variation in the degree to which individual animals salivate while under sedation. As a result, it

181   was not always possible to collect saliva from each animal at each time point. Oral swabs

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182   provided a more consistent means for collecting oral secretions, but as the relatively small

183   amount of secretions absorbed by the swabs must be eluted in medium, detection of ZIKV RNA

184   or infectivity may not be as sensitive in swab sample as in undiluted saliva. Accordingly, ZIKV

185   RNA was more consistently detected in the saliva versus oral swabs when both sample types

186   were available (Fig. 3). Although ZIKV RNA was detectable in the saliva of ZIKV donor animals

187   861138 and 756591 and in oral swab samples from 861138 and 448436, transfer of saliva from

188   donors to the mucosae of naive recipients did not result in ZIKV transmission. In addition, donor

189   saliva produced no detectable plaques on Vero cells. Moreover, *IFNAR-/-* mice inoculated with

190   saliva or oral swab samples from infected donor macaques showed no overt signs of disease

191   and did not seroconvert. Our previous studies suggest that the proportion of vRNA copies to

192   infectious particles from both virus stock and sera from infected animals is approximately

193   1000:1[21,22]. The highest viral load that we detected in a saliva sample in this study was only 1.1

194   x 10^4 vRNA copies/mL; given that we were never able to collect a full 1mL of saliva, it seems

195   likely that saliva transferred to recipients (tonsil maximum of 100-200μL, conjunctivae maximum

196   of 50μL, and nasal passage maximum of 100μL) in our study likely contained less than three

197   infectious ZIKV virions.

198   In addition to sample collection limitations, saliva may represent a natural barrier to virus

199   transmission in the oral cavity. A number of different viruses, such as HIV and influenza A, are

200   detected in the saliva of infected individuals, but mucosal infection in many cases is considered

201   low risk[28–30]. This may be due in part to the antimicrobial constituents present in saliva, which

202   include mucins, lysozyme, peroxidase, defensins, and salivary agglutinin[30]. However, even with

203   these natural barriers to infection, challenge of animals with a higher dose of ZIKV directly to the

204   tonsils resulted in productive infection. A bolus challenge of SIV directly to the oral cavity has

205   also been shown to infect rhesus macaques[31]. The authors of that study suggested that their

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206 oral bolus transmission model might approximate HIV transmission through oral-genital

207 contact[31]. The finding that an oral bolus of virus may overcome natural mucosal barriers to

208 infection is especially interesting because ZIKV has been detected in semen for many months

209 following acute infection and at viral loads significantly higher than those detected in blood

210 plasma (8.6 $\log_{10}$ copies/mL)[32,33]. ZIKV RNA and infectious virus have also been detected in

211 breast milk, another potential route of oral mucosal exposure[34]. The potential for transmission of

212 HIV through breast milk is well documented in humans and is associated with the milk viral load

213 [35,36]. Although there have been no documented cases of transmission between a nursing mother

214 and her infant, a ZIKV viral load of greater than 8×10^6 vRNA copies/mL has been reported in

215 the breast milk in a woman with acute ZIKV infection[18].

216 Taken together, our results suggest that there is a risk of ZIKV transmission via the oral

217 mucosal route, as shown by systemic infection in 3 of 3 macaques after application of high-dose

218 infectious virus to the tonsils. However, the actual risk of transmission via mucosal exposure to

219 saliva may be low—saliva from donor animals with typical plasma viremia harbored little or no

220 infectious virus and could not mediate transmission of ZIKV in our study. However, it must be

221 noted that secretions, including saliva from individuals with unusually high viral load, semen,

222 and breast milk, could pose a transmission risk.

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Materials and methods

225 ***Study Design***. This study was designed as a proof of concept study to examine whether ZIKV

226 transmission may occur in the absence of vector-borne or sexual transmission, via saliva, in the

227 rhesus macaque model. Nothing is currently known about the potential saliva transmission of

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228   ZIKV in vivo so we selected 3 animals for direct application of ZIKV stock to the palatine tonsils,

229   3 animals for subcutaneous inoculation with a well characterized dose of ZIKV[21] to serve as

230   saliva donors, and 7 animals (a minimum of 2 per donor) to serve as recipients of saliva from

231   infected donors. Available animals were allocated to experimental groups based on qualitative

232   assessment of salivation while under ketamine sedation as communicated by staff at the

233   Wisconsin National Primate Research Center. Investigators were not blinded to experimental

234   groups.

235   *Ethical approval.* This study was approved by the University of Wisconsin-Madison Institutional

236   Animal Care and Use Committee (Animal Care and Use Protocol Number G005401 and

237   V5519).

238   *Nonhuman primates.* Six male and seven female Indian-origin rhesus macaques (*Macaca*

239   *mulatta*) utilized in this study were cared for by the staff at the Wisconsin National Primate

240   Research Center (WNPRC) in accordance with the regulations, guidelines, and

241   recommendations outlined in the Animal Welfare Act, the Guide for the Care and Use of

242   Laboratory Animals, and the Weatherall report. In addition, all macaques utilized in the study

243   were free of Macacine herpesvirus 1, simian retrovirus type D, simian T-lymphotropic virus type

244   1, simian immunodeficiency virus, and had no history of exposure to any dengue virus serotype.

245   Animals ranged in age from 3 years to 17 years old (mean = 7.8 years). For all procedures,

246   animals were anesthetized with an intramuscular dose of ketamine (10mL/kg). Blood samples

247   were obtained using a vacutainer or needle and syringe from the femoral or saphenous vein.

248   *Virus.* Macaques in this study were inoculated with Asian-lineage Zika virus/H.sapiens-

249   tc/FRA/2013/FrenchPolynesia-01_v1c1 (ZIKV-FP) obtained from Xavier de Lamballerie

250   (European Virus Archive, Marseille, France). This virus was originally isolated from a 51-year-

251   old female in France after travel to French Polynesia in 2013 and passaged a single time on

252   Vero cells (African green monkey kidney cells; CCL-81). The virus stock used in this study was

253   prepared by inoculation onto a confluent monolayer of C6/36 mosquito cells (*Aedes albopictus*

254   larval cells; CRL-1660). Cell lines were obtained from American Type Culture Collection

255   (ATCC), were not further authenticated, and were not specifically tested for mycoplasma. A

256   single, clarified harvest of virus, with a titer of 5.9 x 10^6 PFU/mL (3.9 x 10^9 vRNA copies/mL)

257   ZIKV-FP was used as stock for all subcutaneous inoculations.

258   ***Tonsil challenges.*** The ZIKV-FP stock was thawed, diluted in PBS to 1 x 10^6 PFU/mL, and

259   maintained on ice until inoculation. Each animal was anesthetized and 8 x 10^5 PFU ZIKV-FP

260   was applied directly to the palatine tonsils (maximum of 400μL per tonsil) via pipet after

261   visualization with a laryngoscope. Animals were closely monitored by veterinary and animal care

262   staff for adverse reactions and signs of disease. Animals were examined, and blood, urine, oral

263   swabs, and saliva were collected from each animal daily from 1 through up to 12 dpi and then

264   weekly thereafter through 28 dpi.

265   ***Subcutaneous inoculations.*** The ZIKV-FP stock was thawed, diluted in PBS to 1 x 10^4

266   PFU/mL, and loaded into a 3mL syringe maintained on ice until inoculation. For subcutaneous

267   inoculations, each of three Indian-origin rhesus macaques was anesthetized and inoculated

268   subcutaneously over the cranial dorsum with 1mL virus stock containing 1 x 10^4 PFU. All

269   animals were closely monitored by veterinary and animal care staff for adverse reactions and

270   signs of disease. Animals were examined, and blood, urine, oral swabs, and saliva were

271   collected from each animal daily from 1 through up to 12 dpi and then weekly thereafter through

272   28 dpi.

273   ***Mucosal membrane challenges***. Oral swabs or saliva were collected from subcutaneously

274   inoculated, ZIKV-infected donor macaques for mucosal membrane challenge of uninfected

275   recipient animals beginning at day three post-infection and continuing through day 10 (cohorts 1

276   and 3) or 12 (cohort 2) post-infection of the donor animals. For recipients challenged with saliva

277   from the first donor, oral swabs were held under the tongue of the anesthetized donor animal for

278   up to 2 minutes to collect saliva. Swabs were then placed in 750μL of viral transport media

279   (tissue culture medium 199 supplemented with 0.5% FBS and 1% antibiotic/antimycotic),

280   swished vigorously for at least 15 seconds, and then swabs were discarded. Viral transport

281   media containing saliva was then applied via pipet to the palatine tonsils (100μL per tonsil)

282   (after visualization via laryngoscope), conjunctivae (50μL per eye), or nasal passages (100μL

283   per nostril) of the corresponding recipient animal. For recipients challenged with saliva from the

284   second and third donors, saliva was collected via pipet from under the tongue of an

285   anesthetized donor animal and applied directly via pipet to the palatine tonsils (200μL per tonsil)

286   of each recipient animal. Mucosal challenges were repeated daily for up to 10 days. Animals

287   were closely monitored by veterinary and animal care staff for adverse reactions and signs of

288   disease. As described previously, animals were examined, and blood, urine, saliva, and oral

289   swabs were collected from each animal daily during challenge and then weekly thereafter

290   through 28 days after final challenge.

291   ***Plaque reduction neutralization test (PRNT).*** Macaque serum samples were screened for

292   ZIKV neutralizing antibody utilizing a plaque reduction neutralization test (PRNT) on Vero cells

293   (ATCC #CCL-81). Endpoint titrations of reactive sera, using a 90% cut off ($PRNT_{90}$), were

294   performed as described in[37] against ZIKV-FP. Neutralization curves were generated using

295   GraphPad Prism software. The resulting data were analyzed by non-linear regression to

296   estimate the dilution of serum required to inhibit 50% of infection.

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297   ***Live virus isolation***. Mice deficient in the alpha/beta interferon receptor on the C57BL/6

298   background (*IFNAR-/-*) were bred in the pathogen-free animal facilities of the University of

299   Wisconsin-Madison School of Veterinary Medicine. Four groups (n=12) of 5- to 6-week-old,

300   mixed sex mice were used to test infectivity of ZIKV RNA in macaque saliva. Mice were

301   inoculated in the left, hind foot pad with 50μL of saliva or saliva swab eluate in viral transport

302   media (VTM) from samples that tested positive for ZIKV RNA by qRT-PCR. Mock-infected

303   experimental control mice received saliva or saliva swab eluate in VTM collected from ZIKV-

304   negative control macaques. Following inoculation, mice were monitored twice daily for the

305   duration of the study. Sub-mandibular blood draws were performed and serum was collected to

306   verify viremia via qRT-PCR and nAb titers via PRNT.

307   ***Viral RNA isolation***. Plasma was isolated from EDTA-anticoagulated whole blood collected the

308   same day by Ficoll density centrifugation at 1860 rcf for 30 minutes. Plasma was removed to a

309   clean 15mL conical tube and centrifuged at 670 rcf for an additional 8 minutes to remove

310   residual cells. Urine was opportunistically collected from a pan beneath each animal's cage and

311   centrifuged at 500 rcf for 5 minutes to remove cells and debris. Saliva was collected via pipet

312   from under the tongue or using sterile oral swabs run under the tongue while animals were

313   anesthetized. Saliva collected via pipet was used to directly inoculate recipient animals in

314   cohorts 2 and 3. Additional saliva, when available, was removed from the collection pipet,

315   centrifuged at 500 rcf for 5 minutes, and added to 650μL viral transport media at a ratio of no

316   more than one part saliva to three parts viral transport media. Swabs were placed in viral

317   transport media for 60-90 minutes, then vortexed vigorously and centrifuged at 500 rcf for 5

318   minutes to elute saliva. Prior to extraction, oral swab eluates were pelleted by centrifugation at

319   14000 rpm and 4°C for an hour. After centrifugation, supernatant was removed, leaving virus in

320   200μL media. Viral RNA was extracted from 300μL plasma or urine using the Viral Total Nucleic

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321 Acid Kit (Promega, Madison, WI) on a Maxwell 16 MDx instrument (Promega, Madison, WI).

322 Viral RNA was extracted from 200µL oral swab-derived samples using the QIAamp MinElute

323 Virus Spin Kit (Qiagen, Germantown, MD) with all optional washes. RNA was then quantified

324 using quantitative RT-PCR. Viral load data from plasma and urine are expressed as vRNA

325 copies/mL. Viral load data from oral swabs are expressed as vRNA copies/mL eluate. Viral load

326 data from saliva in viral transport media are expressed as vRNA copies/mL total volume.

327 ***Quantitative reverse transcription PCR (qRT-PCR).*** For ZIKV-FP, vRNA from plasma, urine,

328 saliva, and oral swabs was quantified by qRT-PCR using primers with a slight modification to

329 those described by Lanciotti et al. to accommodate African lineage ZIKV sequences[38]. The

330 modified primer sequences are: forward 5'-CGYTGCCCAACACAAGG-3', reverse 5'-

331 CACYAAYGTTCTTTTGCABACAT-3', and probe 5'-6fam-

332 AGCCTACCTTGAYAAGCARTCAGACACYCAA-BHQ1-3'. The RT-PCR was performed using

333 the SuperScript III Platinum One-Step Quantitative RT-PCR system (Invitrogen, Carlsbad, CA)

334 on a LightCycler 480 instrument (Roche Diagnostics, Indianapolis, IN). The primers and probe

335 were used at final concentrations of 600 nm and 100 nm respectively, along with 150 ng random

336 primers (Promega, Madison, WI). Cycling conditions were as follows: 37°C for 15 min, 50°C for

337 30 min and 95°C for 2 min, followed by 50 cycles of 95°C for 15 sec and 60°C for 1 min. Viral

338 RNA concentration was determined by interpolation onto an internal standard curve composed

339 of seven 10-fold serial dilutions of a synthetic ZIKV RNA fragment based on ZIKV-FP.

340 ***Data availability.*** Primary data that support the findings of this study are available at the Zika

341 Open-Research Portal (https://zika.labkey.com). The authors declare that all other data

342 supporting the findings of this study are available within the article and its supplementary

343 information files, or from the corresponding author upon request.

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448   T.C.F., C.M.N., D.M.D., M.T.A., and D.H.O. designed the experiments. C.M.N., D.M.D., M.T.A.,

449   and T.C.F analyzed data and drafted the manuscript. M.T.A. provided and prepared viral stocks,

450   performed plaque assays, and performed the mouse experiments. A.M.W., G.L.B. and T.C.F.

451   developed and performed viral load assays. M.S.M., 3M.E.B., L.M.S., C.R.B, C.M.N., E.L.M and

452   D.M.D. coordinated and processed macaque samples for distribution. M.E.G. maintained the

453   Zika Open Portal site where data was stored and shared. J.P., N.S-D., E.P., S.C., and W.N.,

454   coordinated and performed macaque infections and sampling.

455

456   Competing financial interests

457   The authors declare no competing financial interests.

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Figure Legends

Figure 1. Study design. Three cohorts of animals were inoculated with Zika virus either by application of stock virus to the tonsils (orange filled symbols) or subcutaneously (blue filled symbols). Saliva from animals infected subcutaneously was used to challenge naive recipient animals (open symbols) either to the palatine tonsils, conjunctivae or nasal passages. Blood plasma, urine and oral swabs (and/or saliva) were tested for Zika virus RNA by qRT-PCR in all animals.

Figure 2. Longitudinal detection of Zika vRNA in plasma, urine and oral swabs in subcutaneously infected animals (blue) or animals inoculated directly to the tonsils (orange). a. Legend for graphs represented in parts b-d. Orange lines represent animals infected via the tonsil and blue lines represent animals infected via subcutaneous injection. b. Zika vRNA copies per mL of peripheral blood plasma. c. Zika vRNA copies per mL of passively collected urine from pans. d. Zika vRNA copies per oral swab. The gray box indicates the time frame in which saliva or an oral swab sample from saliva donor animals was used to challenge recipient animals. The y-axis starts at the limit of quantification of the qRT-PCR assay (100 vRNA copies/mL).

Figure 3. Longitudinal Zika virus load detected in saliva and oral swabs. Two of three animals infected either via the tonsil (under orange monkey) or subcutaneously (under blue monkey) had sufficient saliva collected for viral load testing. Only time points in these four animals at which both saliva (gray) and oral swabs (black) were tested simultaneously are shown. All points not distinguishable above the limit of quantification (100 vRNA copies/mL) are present on the x-axis.

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493   **Supplementary Figure 1. Neutralization by ZIKV immune sera from tonsil inoculated and**

494   **subcutaneously inoculated macaques.** Immune sera from macaques inoculated

495   subcutaneously (blue) or via the tonsils (orange) were tested for their capacity to neutralize

496   ZIKV-FP. ZIKV was mixed with serial 2-fold dilutions of serum for 1 hour at 37°C prior to being

497   added to Vero cells. Infection was measured by plaque reduction neutralization test (PRNT) and

498   is expressed relative to the infectivity of ZIKV-FP in the absence of serum. The concentration of

499   sera indicated on the x-axis is expressed as Log_{10} (dilution factor of serum). The dilution of sera

500   at half-maximal neutralization of infection (EC_{50}) was estimated by non-linear regression

501   analysis and were 2.803 (861138), 3.081 (448436), 2.479 (756591), 2.638 (664817), 2.991

502   (511690), and 2.746 (590870). Neutralization curves for each animal at 28 dpi are shown.

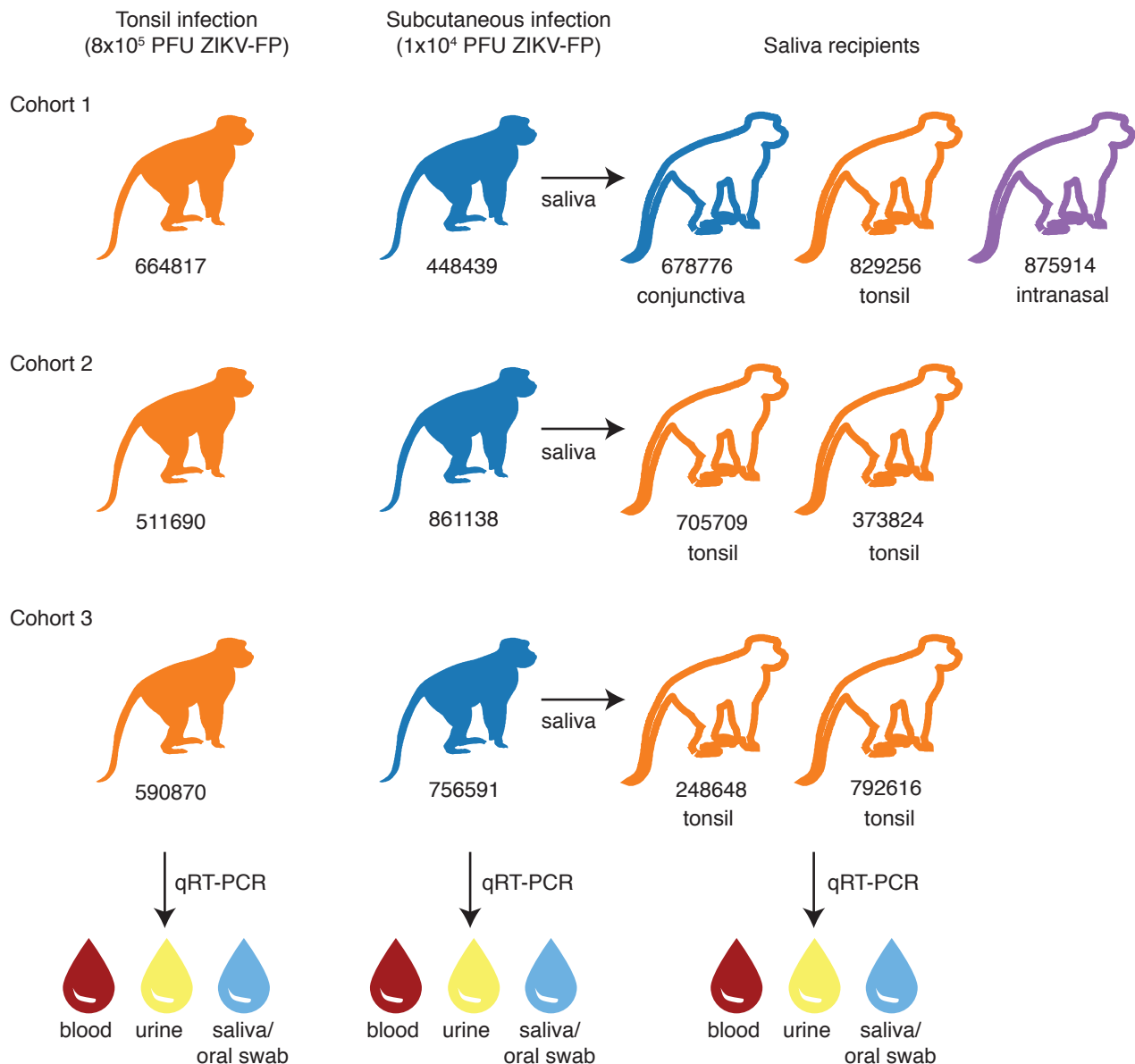503   **Supplementary Figure 2. Injection of ZIKV+ oral swab eluate or ZIKV+ saliva does not**

504   **cause mortality or morbidity in *IFNAR-/- mice.*** *IFNAR-/-* mice (n=3 per group) were

505   inoculated in the left hind footpad with ZIKV+ or ZIKV- oral swab eluate (black) or ZIKV+ or

506   ZIKV- saliva (gray). Mice were monitored until 25 dpi; all survived without signs of morbidity.
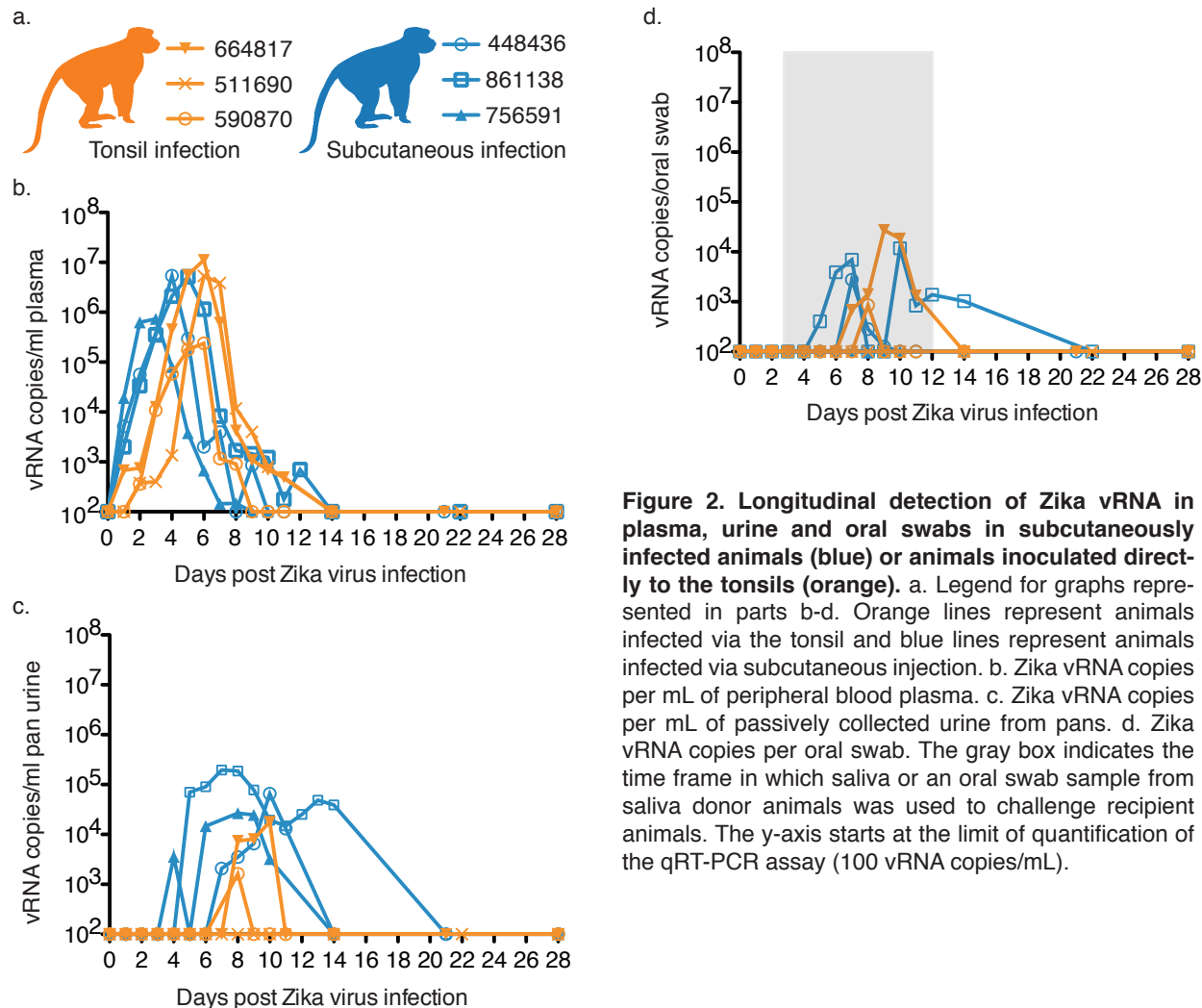
507   Changes in weight were calculated daily for ZIKV+ and ZIKV- oral swab eluate and ZIKV+ or

508   ZIKV- saliva inoculated mice. Error bars represent the standard deviation of the mean.

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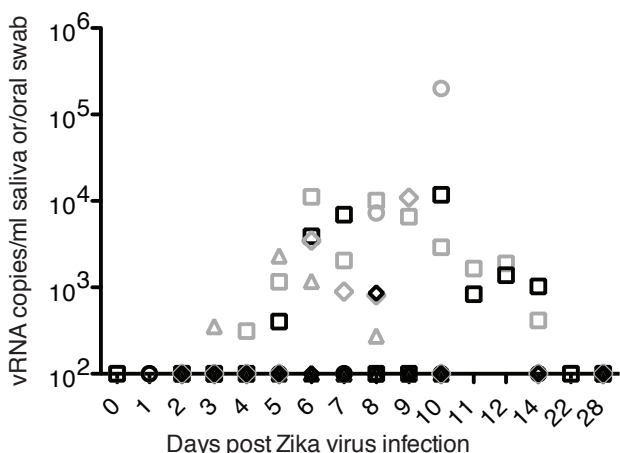
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