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1 **Oral administration of an anti-CfaE secretory IgA antibody protects against**
2 **Enterotoxigenic *Escherichia coli* diarrheal disease in a non-human primate model**

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6

7 Running Title: Oral anti-CfaE SIgA protects against ETEC

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27 **ABSTRACT**

28 Enterotoxigenic *Escherichia coli* (ETEC) is a leading cause of diarrhea-associated illness in
29 developing countries. There is currently no vaccine licensed to prevent ETEC and the
30 development of an efficacious prophylaxis would provide an intervention with significant
31 impact. Recent studies suggested that effective protection could be achieved by inducing
32 immunity to block colonization of ETEC. Here, we evaluated the efficacy of secretory (s) IgA2
33 and dimeric (d) IgA2 of an anti-colonization factor antigen antibody, 68-61, in the *Aotus*
34 *nancymaae* non-human primate (NHP) ETEC challenge model via oral and parental delivery.
35 Thirty-nine animals were distributed across 3 groups of 13, and challenged with 5.0×10^{11} cfu of
36 H10407 on Day 0. Group 1 received a dIgA2 68-61 subcutaneously on day 0. Group 2 received
37 a SIgA2 68-61 orally on days -1, 0, and +1, and Group 3 received an irrelevant SIgA2 antibody
38 orally on days -1, 0, and +1. All animals were observed for symptoms of diarrhea, and stools
39 were collected for ETEC colony counts. SIgA2 treatment significantly lowered the attack rate,
40 resulting in a protective efficacy of 71.4% ($p=0.025$) in Group 2 as compared to Group 3. Anti-
41 CfaE dIgA2 treatment group reduced the diarrheal attack rate, although the reduction did not
42 reach significance (57.1%; $P=0.072$) as compared to the irrelevant SIgA2 Group 3. Our results
43 demonstrated the feasibility of oral administration of SIgA as a potential immunoprophylaxis
44 against enteric infections. To our knowledge, this is the first study to demonstrate the efficacy of
45 administrated SIgA in a non-human primate model.

46

47 **Key words:** ETEC diarrhea; SIgA; dIgA, non-human primates

48

49 INTRODUCTION

50 Enterotoxigenic *Escherichia coli* (ETEC) is the most common cause of diarrheal illness in
51 infants in the developing world and in travelers to endemic countries. An estimated 10 million
52 cases per year occur among travelers and military personnel deployed in endemic regions [1].
53 ETEC is a non-invasive pathogen that mediates small intestine adherence through filamentous
54 bacterial surface structures known as colonization factors (CF). Once bound to the small
55 intestine, the bacteria produce toxins causing a net flow of water from enterocytes, leading to
56 watery diarrhea [2, 3]. Previous approaches to prevent ETEC infection have targeted bacterial
57 attachment and colonization. However, poor responses to vaccines and difficulties in the
58 establishment of protective mucosal immunity against diverse types of CFs have hindered the
59 licensing of ETEC vaccines.

60
61 CFA/I is one of the most prevalent CFs expressed by pathogenic ETEC strains. CFA/I is
62 composed of a stalk consisting of a long homopolymeric subunit and a minor adhesin subunit
63 (CfaE) at the tip of the fimbria. Recent studies have demonstrated that the adhesin subunit can
64 induce anti-adhesive immunity against ETEC infection [4, 5]. In human immunoprotection trials,
65 oral administration of anti-CfaE bovine IgG provided protection in over 60% of the test group,
66 suggesting that an adhesin-based vaccine could be effective to elicit endogenous production of
67 protective antibodies [6].

68
69 IgG and secretory IgA (SIgA) are both present in the small intestine as effector molecules of
70 *mucosal* immune system. SIgA is considered to be one of the most important effector molecules
71 because it constitutes the primary immune defense against pathogens at the mucosal surface [7].

72 In secretory IgA, two IgA monomers are covalently linked by a joining chain (J-chain), and
73 stabilized by a polypeptide called the secretory component that make the molecule more resistant
74 to digestion in the small intestine than IgG [8]. Early studies also have suggested that the
75 secretory component may have its own antimicrobial activity to block epithelial adhesion of
76 ETEC [9].

77
78 Our laboratory has recently identified a panel of anti-CfaE human monoclonal antibodies IgGs
79 that could be employed as an immunoprophylaxis to prevent ETEC diarrhea [10]. We performed
80 isotype switch to SIgAs and we are investigating the potential of these SIgAs to serve as
81 immunoprophylaxis against ETEC diarrhea. Here, we evaluated the efficacy of one lead anti-
82 CfaE SIgA, 68-61, in the *A. nancymaae* non-human primate ETEC challenge model (ETEC
83 strain H10407) [11, 12]. In this model, *A. nancymaae* has been shown to be susceptible to
84 diarrhea in response to experimental infection with ETEC expressing CFA/I, mimicking ETEC
85 pathogenesis in human (Jones 2006). 68-61 was administered to *Aotus* either as a dimeric IgA2
86 (dIgA2) via a single subcutaneous dose (SC) on the day of challenge (day 0) or as a secretory
87 IgA2 (SIgA2) via oral delivery on days -1, 0 (challenge day), and +1. Animals were then
88 monitored for diarrhea as previously described [12]. Our results demonstrate that oral
89 administration of SIgA2 can protect animals from diarrhea associated with ETEC infection.

90

91 **RESULTS**

92 **Production and characterization of 68-61 SIgA2 and dIgA2 antibodies for NHP studies**

93 Large scale production for anti-CfaE 68-61 dIgA2 and SIgA2 antibodies were set up to generate
94 sufficient material for NHP studies using an established IgA production platform in our

95 laboratory [10]. To verify the antibody quality, purified antibodies were analyzed by SDS-PAGE
96 and western blots (Fig. 1A). MRHA and Caco-2 cell adhesion assays were also conducted to test
97 the antibody in vitro functionalities. Similar to what was reported previously [10], both purified
98 68-61 dIgA2 and SIgA2 showed functional activity in both hemagglutination assay (minimal
99 inhibitory concentration of 0.04ug/ml and 0.08ug/ml) and Caco-2 adhesion assay (Fig. 1B and C,
100 respectively).

101

102 **Antibody efficacy study in a non-human primate model challenged with ETEC**

103 Dimeric (Group 1) and secretory (Group 2) anti-CfaE IgA2 antibodies were administered to
104 *Aotus nancymae* monkeys to determine their efficacy against ETEC H10407 strain. Animals
105 administered the irrelevant control SIgA2 antibody (Group 3, oral) had a diarrheal attack rate of
106 58% (7/12), within the range of the reported attack rate in this animal model [12]. Anti-CfaE
107 dIgA2 treatment (Group 1; S.C.) lowered the attack rate to 23% (3/13), while SIgA2 treatment
108 (Group 2; oral) significantly lowered the attack rate to 15% (2/13) as compared to Group 3. One
109 animal in Group 3 was excluded from analysis due to the onset of diarrhea prior to challenge
110 (Table 2). There was no significant difference in the colonization rate or the duration of shedding
111 between the treatment groups. Based on the diarrheal attack rates, oral anti-CfaE SIgA2 (Group
112 2) treatment resulted in a protective efficacy of 71.4% (P=0.025) compared to the irrelevant
113 SIgA2 (Group 3). Treatment with a subcutaneous injection of anti-CfaE dIgA2 (Group 1)
114 reduced the diarrheal attack rate, although the reduction did not reach significance (57.1%;
115 P=0.072) as compared to Group 3 (Table 3). Of note, Group 1 animals did not receive any of the
116 oral rehydration drink on days -1 and +1 that was used to orally administer the SIgA antibodies
117 in Groups 2 and 3.

118

119

120 **DISCUSSION**

121 While both IgG and IgA are expressed at the mucosa, IgA is usually more effective on a molar
122 basis and thus are the natural choice for mucosal passive immunization. The avidity of mucosal
123 IgA, due to multimeric structure, enhances antibody binding with antigens and increases
124 antibody mediated conformational or structural changes in the antigen. The diverse, high level
125 of glycosylation of IgA antibodies, in comparison to IgG, further protects the mucosal surface by
126 non-specific interference with microbial adherence. Here, we further explored the feasibility of
127 administration of anti-CfaE IgA for protection against an ETEC challenge in the *A. nancymaae*
128 non-human primate model. Oral delivery of an anti-CfaE SIgA2 resulted in 71.4% protective
129 efficacy against ETEC diarrhea in animals.

130

131 The administration of a single dose of anti-CfaE dIgA antibody subcutaneously resulted in a
132 57.1% reduction of ETEC diarrhea. Though not significant ($P=0.072$), in contrast to the control
133 animals (Group 3), Group 1 animals did not receive any of the oral rehydration drink, which may
134 have imparted a small therapeutic effect to the control animals not recapitulated in Group 1.
135 Eliminating this difference, dose optimization, and/or temporal administration experiments may
136 reveal a significant reduction in diarrhea and further experimentation is clearly warranted.

137

138 Nevertheless, fecal shedding of H10407 was observed in all animals regardless of the treatment
139 (Table 2). These results are consistent with observations in previous vaccine studies in animals
140 and human challenge volunteers [12, 13], suggesting that the anti-ETEC immunity may inhibit

141 bacterial adherence and/or pathogenicity without affecting fecal shedding. Histological analysis
142 in future experiments may verify the prevention of intestine colonization and better define the
143 protective mechanism. Regardless, these results are promising and further definition of dose and
144 temporal kinetics of SIgA oral administration should further increase efficacy.

145

146 IgA antibodies function in mucosal immunity as the first line of defense against pathogens
147 making them attractive as candidate therapeutics [14]. However, efficacy studies of passively
148 administered IgA in animal models has been limited by the capabilities of large-scale production
149 in the laboratory. In this study, we were able to utilize our recently developed IgA production
150 methods [10] to generate high quality dIgA and SIgA to support a well-powered non-human
151 primate animal study. Through our study, we established the feasibility of oral administration of
152 SIgA2 as a potent immunoprophylaxis against enteric infections. We further demonstrate the
153 potential feasibility of subcutaneous administered dIgA2 in preventing ETEC diarrhea. These
154 results are of great interest as they demonstrate for the first time that SIgA2 can be used as a
155 preventative against bacterial diarrhea.

156

157 **MATERIALS AND METHODS**

158 **68-61 SIgA and dIgA antibody production and characterization**

159 68-61 dIgA2 and SIgA2 antibodies were produced and characterized as previously described
160 [10]. To generate dIgA2, the heavy and light chain vectors were co-transfected with a J chain
161 expressing vector with equal molar ratio in CHO cells. For SIgA2, a secretory component
162 expressing vector was added to the dIgA2 transfection reaction (equal molar ratio for all
163 vectors). Supernatant was run through a column of Capture Select IgA (ThermoFisher) or
164 CptoL resin (GE Life Sciences) for dIgA and SIgA respectively, followed by size exclusion
165 chromatography (HiLoad 26/600 Superdex 200 pg size exclusion column; GE Life Sciences) to
166 separate out the desired dimeric or secretory antibodies. Desired fractions were pooled,
167 concentrated and quality tested by SDS-PAGE, western blots and through mannose-resistant
168 hemagglutination (MRHA) and Caco-2 cell adhesion assays.

169

170 **Mannose resistant hemagglutination assay of human group A erythrocytes**

171 ETEC cultures were taken from frozen cell banks and diluted in sterile 0.15 M saline solution
172 until reaching an OD_{600nm} of 1 for the assay. Human erythrocytes type A+ stored in K3EDTA
173 were washed three times with 0.15 M saline solution and resuspended in the same solution to a
174 final concentration of 1.5% (vol/vol). In a U-bottom 96-well plate (Nunc Thermo Scientific) 100
175 µl of IgA antibodies were added in duplicate to the top row and diluted 1:2 down the plate in
176 0.15 M saline solution. 50 µl of appropriately diluted ETEC was added to each well together
177 with 50 µl of 0.1 M D-mannose solution (sigma). The plate was incubated for 10 minutes at
178 room temperature. After incubation, 50 µl of blood solution was added to the plate and mixed
179 well (200 µl final volume). Plates were allowed to sit stagnant at 4°C for two hours.

180 Hemagglutination was then observed without the aid of magnification. The absence of a pellet of
181 red blood cells at the bottom of the well is indicative of positive hemagglutination. Blood was
182 ordered fresh every week (BioreclamationIVT).

183

184 **Caco-2 adhesion assay**

185 Caco-2 cells seeded at 1×10^5 cells/mL were grown in 24-well tissue culture plates containing
186 Dulbecco's modified Eagle's medium (DMEM), at 37°C in 5% CO₂ static. Frozen bacterial banks
187 were streaked on CFA agar plates and grown overnight at 37°C. The next day, bacteria were
188 resuspended in PBS and diluted until reaching an OD_{600nm} of 0.1. Antibody dilutions were set up
189 in a deep well plate. Antibody dilutions and bacteria were combined in a 1:10 ratio and allowed
190 to shake at 300 rpm for one hour at room temperature. After incubation, 0.2 mL of
191 antibody/bacteria mixture was added to each well containing Caco-2 cells. The cells were then
192 incubated statically for 3 hours at 37°C. Cells were then washed four times with 1 mL PBS to
193 remove non-adherent ETEC cells. Afterwards, Caco-2 cells were dislodged with 0.2 mL 0.25%
194 trypsin. Cells were collected via gentle centrifugation and resuspended in 1mL PBS. Dilutions
195 were plated on CFA agar plates and colonies counted the next day. IC₅₀ was defined as
196 concentration of antibody needed to inhibit 50% of ETEC adhesion to the Caco-2 cells,
197 compared to an irrelevant isotype antibody.

198

199 **Ethics statement**

200 The non-human primate (NHP) research was conducted in an AAALAC-accredited Laboratory
201 Animal Facility, in compliance with the Animal Welfare Act and in accordance with principles
202 set forth in the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory

203 Animals Resources, National Research Council, National Academy Press, 1996, and other U.S.
204 Federal Government statutes. Local approval was by the U.S. Naval Medical Research Unit No
205 6 (NAMRU-6) Institutional Animal Care and Use Committee (IACUC), second-level approval
206 from the U.S. Navy Bureau of Medicine and Surgery, and the study was approved via
207 Resolucion Directoral No. 0452-2017 SERFOR-DGGSPFFS by the Forestry and Wild Fauna
208 Service, Peruvian Ministry of Agriculture

209

210 The *A. nancymae* used in this study were purchased from the Instituto Veterinario de
211 Investigaciones Tropicales y de Altura (IVITA), University of San Marcos, Lima, Peru, and
212 shipped to NAMRU-6 in Lima, Peru. Animals were maintained in pairs when not required to be
213 individually housed for sample collection, fed a standard monkey diet supplemented with fruit
214 and provided water *ad libitum*. Following the study, antibiotic treated and ETEC-free animals
215 were returned to the IVITA colony.

216

217 **Administration of antibody and ETEC challenge inoculums**

218 The ETEC challenge model has been previously described [10, 12]. Briefly, *A. nancymae*
219 monkeys were screened by enzyme-linked immunosorbent assay (ELISA). Animals deemed
220 seropositive were excluded from the study. The remaining thirty-nine animals were distributed
221 across three groups of 13 according to age, sex, and weight. Following a 21 day acclimation
222 period, the animals were fasted overnight and on study day 0 all animals were anesthetized with
223 ketamine hydrochloride (10mg/kg weight, Ketalar, Parke-Davis) and an orogastric feeding tube
224 was placed (5Fr/Ch, 1.7 mm X 41 cm). All animals also received ranitidine (1.5 mg/kg) by
225 intramuscular injection 90 minutes prior to challenge to inhibit gastric acid production, and 5 ml

226 CeraVacxII (CeraProducts, Jessup, MD) was given 30 minutes prior to challenge to neutralize
227 stomach pH. All animals were then challenged with 5×10^{11} cfu ETEC CFA/I+H10407 (5 ml
228 volume).

229 All groups received an antibody treatment (9 mg/kg) prior to challenge on Day 0. Group 1
230 received an anti-CfaE dIgA2 antibody by subcutaneous (S.C.) injection. Group 2 received an
231 anti-CfaE SIgA2 antibody via the orogastric line. Group 3 received a control SIgA2 antibody
232 against an HIV target (no cross-reactivity with H10407 in vitro, data not shown) via the
233 orogastric line. Group 2 and Group 3 also received antibody treatment one day prior to challenge
234 (day -1) and one day post challenge (day +1). These additional treatments (9 mg/kg) were
235 prepared by diluting the antibody (anti-CfaE SIgA2 for Group 2 and control SIgA2 for Group 3)
236 into 5 mL total volume of Prang oral rehydration drink (Bio-Serv; orange flavor), and the diluted
237 antibody was then administered orally by syringe via voluntary consumption. All animals were
238 observed for 10 days and then treated with Enrofloxacin until ETEC H10407 was not detected in
239 stool samples. The Study design of the challenge model is illustrated in Figure 1. The
240 demographic variables of animals in each individual group is listed in Table 1.

241

242 **Observation after passive immunoprophylaxis and challenge**

243 Animals were observed twice daily for signs and symptoms of diarrhea starting on study day -3
244 and continuing for 10 days after challenge. Stools were graded as follows: grade 1 (formed, firm
245 stool pellets), grade 2 (formed but soft stool pellets or droppings), grade 3 (loose, unformed
246 feces), grade 4 (watery, non-clear feces), and grade 5 (watery, clear liquid stools). Stools graded
247 1 or 2 were considered normal, whereas stools graded 3, 4, or 5 were considered abnormal. The
248 case definition of a diarrhea episode was defined as the passing of grade 3 or higher stools for at

249 least two consecutive days during the observation period. The duration of diarrhea was defined
250 as the time between the first day of a diarrhea episode and the last day of diarrhea preceding two
251 consecutive diarrhea-free days. Animals meeting the case definition of diarrhea prior to the
252 challenge were excluded from data analysis.

253

254 Fecal cultures for H10407 ETEC were performed daily for 10 days after challenge by streaking
255 fresh stool and plating serial dilutions of stool directly onto MacConkey agar. Presumptive
256 H10407 isolates (lactose-positive) were confirmed by colony blot using rabbit antisera against
257 CFA/I. Stool was considered negative for H10407 if no lactose positive *E. coli* colonies were
258 isolated, or if 10 presumptive colonies were negative by immunoblot. A period of fecal shedding
259 was defined as isolation of H10407 (CFA/I positive colonies) from stool collected after
260 challenge, beginning (onset) as early as the first day after challenge, and ending (duration) on the
261 last day that H10407 is detectable in stool, up to day 10 post challenge.

262

263 **Statistical analyses**

264 Intergroup comparison of clinical outcomes were performed using nonparametric tests for
265 continuous outcomes (Kruskal-Wallis test for comparing the values for more than two groups)
266 and Fisher's exact test for nominal outcomes. All statistical tests were interpreted in a two-tailed
267 fashion with acceptance of significance set at the $P < 0.05$ level.

268

269

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282 17 U.S.C. § 101 defines a U.S. Government work as a work prepared by a military service
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- 329

330 **Figure Legend:**

331 Figure 1: Study design of the challenge experiment. FO = fecal observation and scoring, all
332 animals; F = fecal collection, all animals; C = challenge; O = oral antibody administration; Q=
333 SubQ antibody administration.

334

335 Figure 2: Characterization of 68-61 dimeric and secretory IgA. A) SDS-PAGE and Western blot
336 of 68-61 dIgA (line 2) and 68-61 SIgA (line 3). Antibody specific to secretory component and
337 SIgA was used for western blot. B) Activity of 68-61 dIgA2 and SIgA2 in a mannose resistant
338 hemagglutination assay of human group A erythrocytes. The minimal inhibitory concentration to
339 prevent hemagglutination is 0.04 and 0.08 $\mu\text{g/ml}$ for dIgA2 and SIgA2 respectively. The assay
340 was repeated three times using different blood donors. C) Functionality of 68-61 dIgA2 and
341 SIgA2 tested in a representative Caco-2 adhesion assay

Table 1. Demographic variable and study design

Group	Treatment	N° of Animals	N° males/ females	Mean age, Months (SD)	Mean weight, grams (SD)	Study Design				
						Route	Dose	Time Points of Administration	Challenge Strain	Challenge Day
1	dIgA2 anti-CfaE	13	6/7	16.1 (1.38)	853.5 (111.5)	Sub-Q	9 mg/Kg	SD 0	H10407	SD 0
2	sIgA2 anti-CfaE	13	7/6	15.9 (1.32)	819 (42.6)	Oral-OG	9 mg/Kg	SD -1, 0, 1	H10407	SD 0
3	Control IgA2	12 ^a	7/6	16.1 (1.28)	812.5 (72.3)	Oral-OG	9 mg/Kg	SD -1, 0, 1	H10407	SD 0

^aOne animal excluded from data analysis due to diarrhea for 3 days prior to challenge.

Table 2. Diarrhea and colonization after oral challenge of *A. nancymaae* with ETEC strain H10407

Treatment	N° of Animals	Diarrhea ^a			Fecal Shedding ^b			
		N° of Cases	Incidence (%)	Mean N° of days to onset (range)	Mean N° of days to illness (range)	Incidence (%)	Mean N° of days to onset (range)	Mean N° of days of duration (range)
dIgA2 anti-CfaE	13	6/7	16.1 (1.38)	853.5 (111.5)	Sub-Q	9 mg/Kg	SD 0	H10407
sIgA2 anti-CfaE	13	7/6	15.9 (1.32)	819 (42.6)	Oral-OG	9 mg/Kg	SD -1, 0, 1	H10407
Control IgA2	12 ^c	7/6	16.1 (1.28)	812.5 (72.3)	Oral-OG	9 mg/Kg	SD -1, 0, 1	H10407

^aDiarrhea defined as at least one loose-watery stool on at least two consecutive days during the observation period (10 days) postchallenge

^bFecal shedding assessed by plating on MacConkey agar with confirmatory colony blotting.

^cOne animal excluded from data analysis due to diarrhea for 3 days prior to challenge

Table 3. Protective Efficacy in *A. nancymaae*

Group	Vaccine	N° with diarrhea/N (%)	Protective Efficacy (%)	<i>P</i> value ^a
1	Dimeric anti-CfaE IgA2	3/13 (23)	57.1	0.072
2	Secretory anti-CfaE IgA2	2/13 (15)	71.4	0.025
3	Control IgA2	7/12 (58)	-	-

^aFisher Exact Test, two-tailed, comparing frequency of diarrhea test groups to control group.

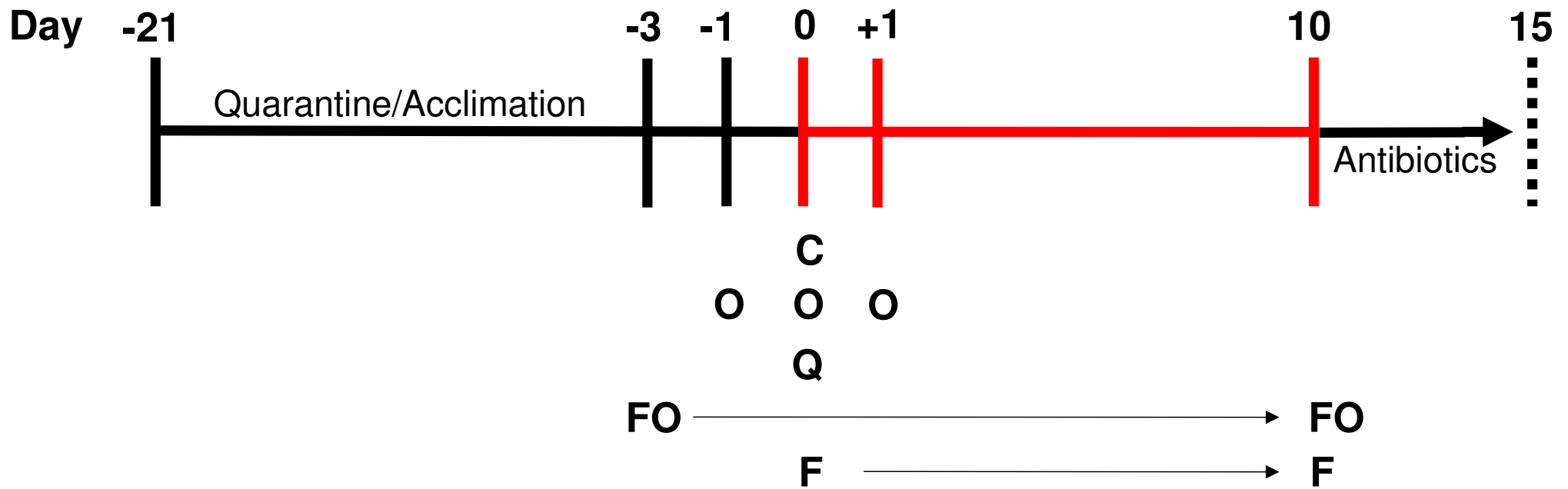
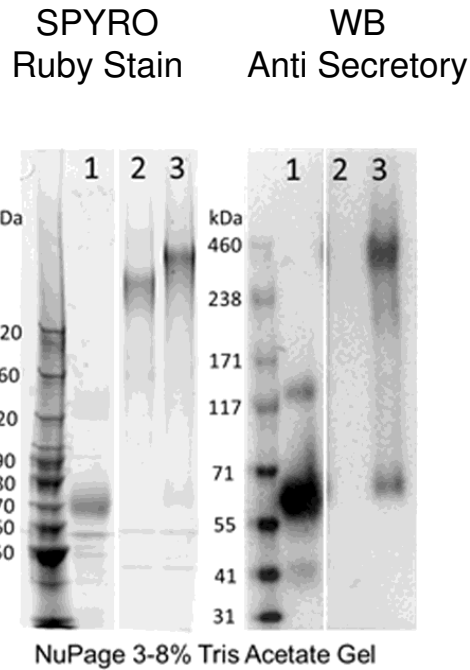


Figure 1: Study design of the challenge experiment. FO = fecal observation and scoring, all animals; F = fecal collection, all animals; C = challenge; O = oral antibody administration; Q= SubQ antibody administration.

Panel A

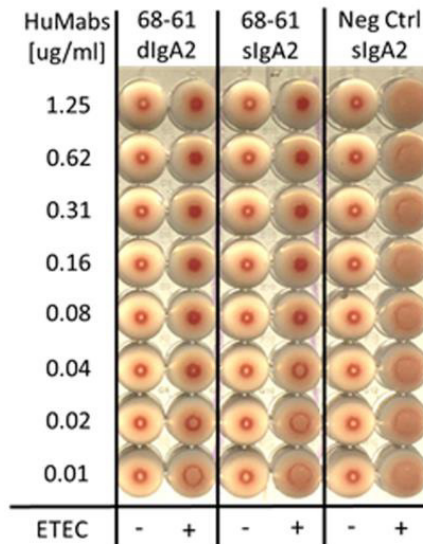


Line:

1. Secretory Component Control
2. 68-61 dIgA2
3. 68-61 sIgA2

Panel B

Hemagglutination assay



Panel C

Caco-2 adhesion assay

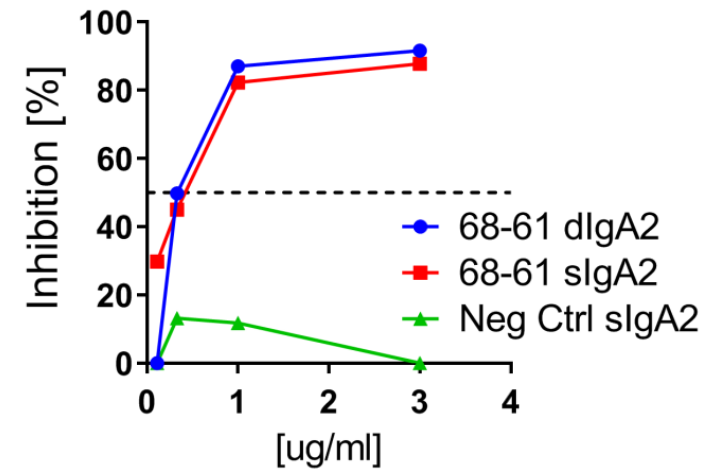


Figure 2: Characterization of 68-61 dimeric and secretory IgA. A) SDS-PAGE and Western blot of 68-61 dIgA (line 2) and 68-61 SlgA (line 3). Antibody specific to secretory component and SlgA was used for western blot. B) Activity of 68-61 dIgA2 and SlgA2 in a mannose resistant hemagglutination assay of human group A erythrocytes. The minimal inhibitory concentration to prevent hemagglutination is 0.04 and 0.08 $\mu\text{g/ml}$ for dIgA2 and SlgA2 respectively. The assay was repeated three times using different blood donors. C) Functionality of 68-61 dIgA2 and SlgA2 tested in a representative Caco-2 adhesion assay