Shigella Aotus Challenge Model

Development of an *Aotus nancymaae* model for *Shigella* vaccine immunogenicity and efficacy studies

Michael Gregory¹, Robert W. Kaminski², Luis A. Lugo-Roman¹, Hugo Galvez Carrillo³, Drake Hamilton Tilley¹, Christian Baldeviano¹, Mark Simons¹, Nathanael D. Reynolds¹, Ryan T. Ranallo², Akamol E. Suvarnapunya², Malabi M Venkatesan², and Edwin V. Oaks².

¹ U.S. Naval Medical Research Unit No. 6 (NAMRU-6) Callao, Peru
² Walter Reed Army Institute of Research (WRAIR) Silver Spring, Maryland
³ Universidad Nacional Mayor de San Marcos Lima, Peru

Running Header: Shigella Aotus Challenge Model

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# Address correspondence and reprint requests to Dr. Robert W. Kaminski, Bacterial Diseases Branch, Walter Reed Army Institute of Research, 503 Robert Grant Ave. Silver Spring, MD 20910. Phone: 301-319-9803, Fax: 301-319-9801, Email: Robert.W.Kaminski.civ@mail.mil
Shigella Aotus Challenge Model

Abstract

Several animal models exist to evaluate the immunogenicity and protective efficacy of candidate *Shigella* vaccines. The two most widely used non-primate models for vaccine development include a murine pulmonary challenge model and a guinea pig keratoconjunctivitis model. Non-human primate models exhibit clinical features and gross and microscopic colonic lesions that mimic those induced in human shigellosis. Challenge models for ETEC and *Campylobacter* spp. have been successfully developed in *Aotus nancymaae* and the addition of a *Shigella Aotus* challenge model would facilitate the testing of combination vaccines.

A series of experiments were designed to identify the dose of *Shigella flexneri* 2a, 2457T that induces an attack rate of 75% in the *Aotus*. After primary challenge, the dose required to induce an attack rate of 75% was calculated to be $1 \times 10^{11}$ cfu. *Shigella*-specific immune responses were low after primary challenge and subsequently boosted upon re-challenge. However, pre-existing immunity derived from the primary challenge was insufficient to protect against the homologous *Shigella* serotype.

A successive study in *A. nancymaae* evaluated the ability of multiple oral immunizations with live-attenuated *Shigella* vaccine strain SC602 to protect against challenge. After three oral immunizations, animals were challenged with *S. flexneri* 2a, 2457T. A 70% attack rate was demonstrated in control animals, whereas animals immunized with SC602 were protected from challenge (efficacy = 80%; $p = 0.05$). The overall study results indicate the *Shigella Aotus nancymaae* challenge model may be a valuable tool for evaluating vaccine efficacy and investigating immune correlates of protection.
Shigella Aotus Challenge Model

43
Introduction

Shigellosis, or bacillary dysentery, results in greater than 100,000 deaths globally in 2010, mostly in developing countries (1). Although shigellosis is considered a disease of developing countries, over 14,000 laboratory-confirmed cases are reported to occur in the U.S. annually (2). In the United States, *Shigella* infections constitute the third most common cause of gastroenteritis, after *Campylobacter* and *Salmonella* infections. Populations particularly susceptible are children in day-care centers, migrant workers, travelers to developing countries, and homosexual men (3-6). The low infectious dose, the fecal-oral route of transmission, and the emergence of resistance to multiple antibiotics among *Shigella* isolates pose a major public health problem throughout the developing world and necessitate the development of a safe, efficacious vaccine.

There are several animal models to investigate pathogenic mechanisms utilized by *Shigella* spp. and to evaluate the immunogenicity and protective efficacy of candidate vaccines. The two most widely used models for vaccine development include a murine pulmonary challenge model (7), which is useful for preliminary screening of vaccine candidates, and a guinea pig keratoconjunctivitis model (8). The ability of *Shigella* to invade the corneal epithelium of guinea pigs and spread to contiguous cells causing keratoconjunctivitis, provides a model system that mimics the invasive process which occurs in the mucosal epithelium.

Recently, a guinea pig rectocolitis model has been described (9) that induces bloody, mucoidal stools. Adaptations to the published protocol have facilitated use of the rectocolitis model in vaccination/efficacy studies in larger and older guinea pigs (Kaminski and Oaks, unpublished data).
Non-human primate models also exist for shigellosis and have been used to better understand pathogenesis (10) and to evaluate vaccine immunogenicity and efficacy (11). In the Rhesus model, oral challenge doses are administered at levels of $1 \times 10^{10}$ to $1 \times 10^{11}$ cfu and the animals are administered bicarbonate solution to neutralize stomach acidity. The clinical features combined with gross and microscopic colonic lesions induced by wild-type shigellae in monkeys are similar to those induced in human shigellosis (12). The similar disease course and pathology between human and monkey shigellosis provide an excellent model to study shigellosis. Despite the similarities, several differences remain between the pathology associated with human and monkey shigellosis. For example, gastric mucosal lesions have been observed in Rhesus monkeys after experimental or natural infection with shigellae (10) whereas in humans, lesions are limited to the colonic epithelium (13). Oral feeding of Rhesus monkeys with *S. flexneri* 2a induces an inflammatory reaction in the gastric mucosa that is similar to that in the gut. The gastric lesions could be a result of the high level of bacteria ($10^{10}$ cfu) needed for challenge or differences in rhesus compared to human physiology.

In recent years, oral challenge models have been developed in *Aotus nancymae* monkeys for both *Campylobacter jejuni* and enterotoxigenic *E. coli* (ETEC). Both Aotus challenge models result in reproducible attack rates $\geq 70\%$ and are characterized by colonization of the gastrointestinal tract and the induction of diarrhea (14, 15). The addition of a *Shigella Aotus* challenge model would enable the testing of potential combination vaccines against the three most common enteric bacterial pathogens responsible for traveler's diarrhea.

To that end, the research described herein focuses on determining a dose of *S. flexneri* 2a, 2457T that reproducibly achieved an attack rate of $\geq 75\%$. Once the challenge dose was
established, the immunogenicity and protective efficacy of a well-characterized, live-attenuated Shigella flexneri 2a vaccine strain, SC602, was investigated in the Aotus model.
MATERIALS AND METHODS

Animal use and welfare. Captive-bred *Aotus nancymaae* were purchased from Instituto Veterinario de Investigaciones Tropicales y de Altura (IVITA), University of San Marcos, Iquitos, Peru and shipped to NAMRU-6 in Lima for the study. The animals had not previously been used in a *Shigella* study. The study was conducted in an Association for the Assessment and Accreditation of Laboratory Animal Care, International, accredited vivarium with local approval by the NAMRU-6 Institutional Animal Care and Use Committee (IACUC), second level approval from the Bureau of Medicine (BUMED), and was approved by the Peruvian Dirección General Forestal y de Fauna Silvestre (resolution number 0023-2011-AG-DGFFS-DGEFFS). Animals were identified by unique tattoo numbers on their abdomens and were maintained in paired housing when not required to be individually housed for sample collection. Prior to inclusion in the study, animals were screened by stool cultures for existing infection with *Shigella* spp. and for prior *Shigella* exposure by ELISA for anti-*S. flexneri* 2a LPS serum IgG titers. Those animals meeting the inclusion criteria (negative stool cultures and IgG titers ≤ 20) were randomized to the various treatment groups. *Aotus* used in the challenge dose finding study had a mean (± SD) weight of 840 ± 66 g and a mean age of 19 ± 3 months on day 0 of the study. *Aotus* used in the vaccine immunogenicity and efficacy study had a mean weight of 868 ± 86 g and a mean age of 20 ± 5 months on day 0 of the study.

Preparation and administration of Shigella vaccine and challenge inoculums. *S. flexneri* 2a, 2457T is a wild-type *Shigella* strain that is Sereny-positive (16), pathogenic to monkeys (11, 17)
and virulent in humans (18, 19). A vial of cGMP *S. flexneri* 2a, 2457T was reconstituted in saline, serially diluted, and plated for isolation on trypticase soy agar (TSA) with 0.01% Congo red dye. After overnight incubation at 37°C, three small, smooth, Congo red-positive colonies were used to inoculate TSA plates (without Congo red) for confluent growth. Plates were harvested with 3.0 ml of cold PBS and the suspension diluted based on a standardized OD$_{600}$ value. *Shigella flexneri* 2a vaccine strain SC602 has deletions in virG (icsA) and iuc (encoding aerobactin) genes (19). Strain SC602 is Congo red positive, indicating retention of the virulence plasmid, and unable to cause keratoconjunctivitis in the guinea pig eye (Sereny negative) (20).

*S. flexneri* 2a strain SC602 (19) was propagated using identical procedures used for *S. flexneri* 2a, 2457T.

Monkeys were fasted overnight prior to administration of vaccine or challenge inoculums. Gastric acid production was inhibited with ranitidine (1.5 mg/kg) by IM injection 90 min prior to inoculum delivery. Anesthetized animals (ketamine HCL; 50 mg/ml, 4-5 mg/kg, IM) were orogastrically administered 5 ml of the rice-based buffer CeraVacx I (CeraProducts, Jessup, MD) using a single-use, sterilized, 5 Fr/Ch (1.7 mm) 16” (41 cm) feeding tube to neutralize the stomach contents. Immediately prior to inoculum delivery, the fluid content of the stomach was sampled and measured for pH. The challenge dose of *S. flexneri* 2a, 2457T and immunization dose of *S. flexneri* 2a, SC602 were delivered orogastrically in a 5 mL volume using a new feeding tube.

**Challenge Dose Finding Study Design.** Groups (n = 9 animals/grp) of *A. nancymaae* were orogastrically inoculated with increasing doses (5 x 10$^9$, 5 x 10$^{10}$, or 5 x 10$^{11}$cfu) of *Shigella*
Shigella Aotus Challenge Model

*flexneri* 2a, 2457T. A fourth group of 10 animals was administered PBS. Nine weeks following primary challenge (day 63) all animal were re-challenged with $1 \times 10^{11}$ cfu of *S. flexneri* 2a, 2457T. Animals were observed for 10 days following each inoculation for illness symptoms (described below), then treated with enrofloxacin (5 mg/kg, IM) daily for five days.

**Vaccine Immunogenicity and Efficacy Study Design.** Groups of eight *A. nancymaae* were orogastrically immunized on study days 0, 14, and 42 with $1 \times 10^{10}$ or $1 \times 10^{11}$ cfu of the live-attenuated vaccine strain *Shigella flexneri* 2a, SC602. Another group was immunized with a sub-clinical dose ($1 \times 10^9$ cfu) of *S. flexneri* 2a, 2457T. A control group ($n = 10$ Aotus) was inoculated with PBS on the same schedule. On study day 70, all animals were orogastrically challenged with $1 \times 10^{11}$ cfu of *S. flexneri* 2a, 2457T as described above.

**Observations post vaccination and challenge.** Animals were observed for signs and symptoms of diarrhea twice daily prior to each vaccination or challenge, for five days post vaccination and for ten days post challenge. Observations included activity level, stool consistency and the presence of blood observed in the feces. Activity level was scored on a scale of 0 to 3 as follows: 0, active and responsive; 1, reduced activity; 2, immobile; 3, recumbent. Fecal occult blood was determined by hemoccult test (Hemoccult II SENSA®, Beckman Coulter, Fullerton, CA) according to the manufacturer’s instructions. Stools were graded daily as follows: grade 1 (formed, firm stool pellets), grade 2 (formed but soft stool pellets or droppings), grade 3 (loose, unformed feces), grade 4 (watery, non-clear feces), and grade 5 (watery, clear liquid stools). Stools graded 1 or 2 were considered normal whereas stools graded as 3, 4 or 5 were
considered abnormal. The case definition of a diarrhea episode was defined as the passing of grade 3 or higher stools for at least two consecutive days during the observation period. The duration of diarrhea was defined as the time between the first day of a diarrhea episode and the last day of diarrhea preceding two consecutive diarrhea-free days. Animals meeting the case definition of diarrhea prior to challenge were excluded from data analysis. Clinical symptoms of *Shigella*-induced gastroenteritis were defined as evidence of *Shigella* colonization (PCR or isolation) and either 1.) an episode of diarrhea (as defined above) or 2.) blood in the stool (occult, gross or melena) for two consecutive days or 3.) death.

**Clinical sample collection and processing:** Blood was collected and serum stored at -80°C until assayed by ELISA. Blood was collected from individual animals on study days 0, 7, 14, 21, 49, 70 and 77 in the challenge dose finding study. In the vaccine immunogenicity and efficacy study, blood samples were collected on study days 0, 21, 49, 70, 77 and 84. Stool samples were collected from cage drop pans before immunization or challenge and daily for 10 days after each vaccination or challenge.

**Shigella colonization determination.** Colonization of *Aotus* after vaccination or challenge was determined as previously described for rhesus macaques (21). Briefly, stool was streaked onto Hektoen Enteric Agar plates. Suspected *Shigella* colonies were confirmed by slide agglutination with commercially available *S. flexneri* 2a antiserum (Denka Seiken Co) or by colony immunoblot with the anti-IpaB monoclonal antibody 2F1 (22). Stool samples testing negative for *Shigella* were subjected to PCR analysis targeting the *ipaH* gene (21).
Immunogenicity assessment. Serum antigen-specific antibody responses were assessed by an ELISA as previously described (23) with the following modifications: antigen coating concentrations were 10 µg/ml of *S. flexneri* 2a LPS and 1 µg/ml for *S. flexneri* 2a Invaplex (24), and purified IpaB and IpaC in a total assay volume of 100 µl. Conjugated rabbit anti- *Aotus* IgG and anti- *Aotus* IgA secondary antibodies (Lampire Biological Labs Inc, Pipersville, PA) were used to detected antigen-bound serum antibodies. Seroconversion was defined as ≥ 4-fold increase in titer over baseline.

Statistical Analysis. Intra-group comparisons of clinical and immunologic outcomes were performed using nonparametric tests for continuous outcomes (Wilcoxon Ranked Sum for 2 group comparisons; Kruskal-Wallis for more than 2 group comparisons) and Fisher’s exact tests for nominal outcomes. All statistical tests were interpreted in a two-tailed fashion with acceptance of significance set to the $p<0.05$ level.
RESULTS

Dose finding and re-challenge study: Clinical symptoms, microbiology and challenge results.

Results from a preliminary, pilot study indicated that oral challenge with $5 \times 10^9$ cfu of *S. flexneri* 2a, 2457T induced diarrhea in 1 of 3 animals (33%) and did not cause significant disease in the remaining animals (data not shown). Therefore, three groups of *A. nancymaae* were orally challenged with *S. flexneri* 2a, 2457T at either $5 \times 10^9$, $5 \times 10^{10}$, or $5 \times 10^{11}$ cfu (Table 1) to determine a challenge dose that induced diarrhea in at least 75% of the animals. *Aotus* in the control group were mock-challenged with PBS. One of the ten animals (10%) in the PBS control group was positive for diarrhea for nine days after inoculation with PBS. *Shigella* spp. were not recovered from the animal in fecal cultures and the stools were *ipaH*-negative by PCR at all time points.

In contrast, *S. flexneri* 2a, 2457T induced diarrhea in 25, 56 and 100% of animals orally inoculated with $5 \times 10^9$, $5 \times 10^{10}$, or $5 \times 10^{11}$ cfu, respectively. Disease was characterized as loose, low volume stools with either gross or occult blood present. There was no significant difference in colonization rates or duration between dose groups. Only the group administered $5 \times 10^{11}$ cfu of *S. flexneri* 2a, 2457T had significantly higher number of diarrhea days (Table 2) as compared to PBS controls. On day 2 after challenge, one animal inoculated with $5 \times 10^{11}$ cfu was euthanized due to severe disease. An additional animal that was inoculated with $5 \times 10^{10}$ cfu was euthanized 6 days post challenge due to bloody vomitus and lethargy. Necropsy of the animals revealed hemorrhagic and necrotic small intestines and stomach. Tissue collected from...
Shigella Aotus Challenge Model

\[ 13 \]

the colon, ileum, and stomach was macerated, cultured and tested positive for \textit{S. flexneri}.

Tissues from the peritoneum tested negative for enteropathogens.

The clinical symptoms incidence, which captures disease and death due to the infection,

was used to calculate a dose of \(1 \times 10^{11}\) cfu to result in a 75% attack rate. The dose of \textit{S. flexneri} 2a, 2457T expected to induce the targeted 75% attack rate in naive \textit{Aotus nancymae} was calculated by applying a linear fit to the line generated after plotting the log10 transformed doses (CFU) of \textit{S. flexneri} 2a, 2457T versus the attack rate achieved at each dose and interpolating the expected dose.

The animals were rested for 9 weeks and then all groups were orally challenged with \(1 \times 10^{11}\) cfu of \textit{S. flexneri} 2a, 2457T. Veterans from the primary challenge were used to determine if protection could be achieved after homologous re-challenge whereas the veteran PBS control group was used to confirm that the calculated dose would result in a \(\geq 75\)% attack rate.

Challenge of the veteran PBS groups with \textit{S. flexneri} 2a, 2457T resulted in an 80% attack rate confirming previous results (Table 1). Animals previously infected with \textit{S. flexneri} 2a, 2457T were not protected upon subsequent re-challenge with a homologous strain (Table 1) with the incidence of clinical symptoms ranging for 38% to 71%. There was no significant difference between duration of diarrhea or colonization among the study groups. Three animals were euthanized due to complications from the second challenge, two from the group that previously received PBS during the primary challenge phase of the experiment and one that had received \(5 \times 10^{11}\) cfu. All euthanized animals had occult blood in the stool and bloody vomitus prior to death. Upon necropsy, blood and colitis was noted in the distal colon of two animals (\textit{Shigella} veteran and PBS control veteran) with no pathology in the stomach or small intestines.
intestine. Necropsy of the third animal (PBS control veteran) revealed loose stool without blood in the large intestine and necrosis in the stomach.

Dose finding and re-challenge study: Immunological assessment. Individual animals were bled before challenge on day 0 and on day 7, 14, 21 and 49 after the primary challenge. Blood was also collected one and two weeks (day 70 and 77) after the second challenge. Serum IgG and IgA endpoint titers directed to *S. flexneri* 2a LPS, *S. flexneri* 2a Invaplex, IpaB and IpaC were determined by ELISA (Figure 1). *Shigella*-specific antibodies were not detected in serum of animals treated with PBS at any time point during the primary challenge phase of the study. Furthermore, baseline *Shigella*-specific antibodies were low across all groups prior to challenge. The serum IgG titers directed to LPS, Invaplex, IpaB and IpaC followed a dose-dependent relationship with animals receiving the highest dose of *S. flexneri* 2a, 2457T possessing the highest antigen-specific antibody titers (Figure 1). Seroconversion to Invaplex (which includes LPS, IpaB and IpaC antigens (24)) was challenge dose dependent with 25%, 63% and 88% of *Aotus* challenged with 5 x 10⁹, 5 x 10¹⁰, and 5 x 10¹¹ cfu, respectively having at least a four-fold increase in serum IgG titers. IpaC-specific titers were low to undetectable across all groups after the primary challenge. A slight decline was noted in the LPS and Invaplex-specific IgG titers between samples collected on day 21 and day 49 whereas IpaB and IpaC titers remained constant or slightly increased during the same time period. *Shigella*-specific serum IgG titers increased ~0.5 – 2 logs after re-challenge with a homologous serotype. The kinetics of the serum IgG response directed to the *Shigella* antigens during the second challenge phase of the study in the PBS control group largely mirrored the kinetics of the response previously
demonstrated after challenge with $5 \times 10^{11}$ cfu in the primary challenge phase of the experiment. 

*Shigella*-specific serum IgA responses after primary challenge with $5 \times 10^9$ and $5 \times 10^{10}$ cfu were low, with less than 25% of animals seroconverting to any of the antigens (Figure 1). Moderate levels of *Shigella*-specific IgA were elicited after oral inoculation with $5 \times 10^{11}$ cfu of *S. flexneri* 2a, 2457T, with 38 – 50% of *Aotus* seroconverting after primary challenge. After re-challenge there were a significant increase in Invaplex and IpaB-specific serum IgA titers whereas IgA responses directed to IpaC and LPS were largely unchanged (Figure 1).

Reactogenicity and colonization after oral immunization of *A. nancymae* with SC602 or wild-type *S. flexneri* 2a, 2457T. The reactogenicity, immunogenicity and protective efficacy of a live-attenuated *Shigella flexneri* 2a vaccine strain, SC602 was assessed in the *Aotus nancymae* model. SC602 has been previously shown to be immunogenic and protective against shigellosis in clinical trials (19) and in the *rhesus macaque* model (Venkatesan, unpublished data). In addition, a group of *Aotus* were immunized with *S. flexneri* 2a, 2457T ($1 \times 10^9$ cfu) to test the hypothesis that multiple immunizations with a subclinical dose could induce a protective immune response. As presented above, primary challenge with *S. flexneri* 2a, 2457T at $5 \times 10^9$ cfu followed by re-challenge with $1 \times 10^{11}$ cfu resulted in a strong immune response directed to multiple *Shigella* antigens (Figure 1) conveying partial protection as evidenced by a low diarrhea rate (Table 2) suggesting that low level infections could confer protection against a larger bolus of *Shigella*.
Groups were orally immunized on day 0, 14 and 42 with SC602 (10^{10} or 10^{11} cfu/dose) or S. flexneri 2a, 2457T (10^9 cfu/dose). Clinical symptoms and bacterial colonization were monitored for 10 days after each immunization (Table 3). As expected, animals mock-immunized with PBS were not colonized with shigellae. All animals immunized with SC602 were colonized after each oral immunization for 1 to 8 days. The number of diarrhea cases was also low after each immunization with SC602. In contrast, oral immunization with S. flexneri 2a, 2457T induced diarrhea in 75% (6/8) of animals after each immunization, which was significantly higher than the number of diarrhea cases in PBS controls (p = 0.007; Fishers exact) and colonization rates were also substantial (75-100%).

**Protective efficacy after oral immunization of Aotus with live-attenuated SC602 or wild-type S. flexneri 2a, 2457T.** Animals orally immunized on day 0, 14 and 42 were subsequently challenged with an oral dose of S. flexneri 2a, 2457T (1 x 10^{11} cfu) on day 70 and monitored for 10 days (Table 4). Two animals immunized with SC602 were excluded from analysis due to diarrhea onset prior to challenge. The diarrhea attack rate in the placebo group was 70% (7/10 animals) and 14% (1/7 animals) in groups immunized with 1 x 10^{10} or 1 x 10^{11} cfu of SC602 (80% protective efficacy; p = 0.05). Comparison of the PBS control group to both groups immunized with SC602 (1 x 10^{10} and 10^{11} cfu) resulted in 80% protective efficacy (p = 0.01; Fishers exact). Immunization with S. flexneri 2a, 2457T did not result in significant protection (46%; p = 0.34), a delay in the mean day of onset nor a decrease in the illness duration (Table 5). There was a significant reduction in the duration of diarrhea after challenge in groups orally immunized with 1 x 10^{10} SC602 (p = 0.05; Mann-Whitney) and 1 x 10^{11} cfu SC602 (p = 0.025) as compared to the
PBS control group. There was no difference in colonization rate or duration after oral challenge with \textit{S. flexneri} 2a, 2457T in any of the immunized groups and the placebo controls.

**Immune responses after oral immunization of \textit{Aotus} with live-attenuated SC602 or wild-type \textit{S. flexneri} 2a, 2457T and subsequent oral challenge.** Blood collected on study days 0, 21, 49, 70, 77 and 84 was assayed by ELISA for IgG and IgA endpoint titers directed to \textit{S. flexneri} 2a LPS, Invaplex, IpaB and IpaC (Figure 2). Animals mock-immunized with PBS did not mount an antigen-specific serum IgG or IgA response during the immunization phase of the study. Furthermore, \textit{Shigella} antigen-specific serum IgG and IgA titers on study day 0 were low in all experimental groups. In contrast, robust levels of serum IgG directed to \textit{S. flexneri} 2a LPS, Invaplex and IpaB were induced after three oral immunizations with SC602 or \textit{S. flexneri} 2a, 2457T and significantly higher magnitude ($p < 0.01$) than the responses in the PBS control group. Seroconversion after immunization with SC602 was dose-dependent and most evident in antigen-specific serum IgA. For example, 43% (3/7) of \textit{Aotus} receiving SC602 (1 x $10^{10}$ cfu) seroconverted to LPS as compared to 100% (7/7) of animals receiving SC602 (1 x $10^{11}$ cfu). Similarly, none of the animals immunized with SC602 (1 x $10^{10}$ cfu) had IpaB-specific serum IgA whereas 57% (4/7) of \textit{Aotus} immunized with SC602 (1 x $10^{11}$ cfu) seroconverted to IpaB.

Interestingly, only animals immunized with \textit{S. flexneri} 2a, 2457T had detectable anti-IpaC serum IgG (38% or 3/8) and IgA (13% or 1/8) responses after immunization, albeit at low levels. However, there was no significant difference in seroconversion rates between animals immunized with SC602 (1 x $10^{10}$ or 1 x $10^{11}$ cfu) and animals immunized with \textit{S. flexneri} 2a, 2457T across all antigens assayed. An increase in serum IgG and IgA directed to LPS, Invaplex
Shigella Aotus Challenge Model

and IpaB was demonstrated after challenge indicating vaccination effectively primed the ensuing immune response.
DISCUSSION

The three most common bacterial pathogens responsible for traveler’s diarrhea include ETEC, Campylobacter and Shigella (6). In addition, significant morbidity and mortality is associated with these enteric pathogens in impoverished areas with endemic disease (25).

Substantial efforts over the past decade have resulted in the generation of several vaccine candidates to prevent the diarrhea caused by these enteric bacterial pathogens. Ideally, a combination vaccine capable of protecting against ETEC, Shigella and Campylobacter will be developed and deployed. A single animal model to evaluate immunogenicity and efficacy of a combination enteric vaccine may greatly facilitate development and evaluation.

The A. nancymae model has been used to evaluate the immunogenicity and efficacy of several ETEC (15) and Campylobacter vaccines (14, 26). Attack rates in naïve Aotus orally inoculated with 5-7 x 10^{11} cfu of C. jejuni are typically ≥ 70%. Similar attack rates are achieved after oral inoculation of naïve Aotus with 1-5 x 10^{11} cfu of ETEC. Disease in the ETEC and Campylobacter challenge models is typically characterized by diarrhea and bacterial colonization evidenced by positive stool culture. Although there are similarities between the three challenge models in terms of infectious dose and inducing diarrhea, there are also several key differences between the ETEC and Campylobacter Aotus models as compared to the Shigella model. In the Shigella model, melena or black tarry stool with gross blood is a typical outcome whereas gross blood is rarely seen in the ETEC and Campylobacter models.

Another characteristic of the Shigella Aotus challenge model that differs from the ETEC and Campylobacter Aotus challenge models is death in a subset of animals. Necropsies of Aotus challenged with Shigella revealed hemorrhagic and necrotic small intestines and stomach in a
subset of animals, similar to reports using rhesus macaques (10). Death after oral challenge of non-human primates with shigellae has been documented in several studies (27, 28). Oral challenge of *M. fascicularis* monkeys with 1 x 10^{10} or 1 x 10^{11} cfu of *S. flexneri* 2a, 2457T resulted either in death three and four days post inoculation or a moribund state requiring humane euthanasia (27) (28). After oral challenge of 40 rhesus monkeys with *S. flexneri* 2a, 2457T (3.2 x 10^{10} cfu) five animals died reportedly due to necrotizing enteritis characteristic of acute shigellosis (28). In the *Aotus* model, several animals were humanely euthanized or died within a week of oral challenge with ≥ 5 x 10^{10} cfu of *S. flexneri* 2a, 2457T. In the subsequent study in which *Aotus* were challenged with 1 x 10^{11} cfu of *S. flexneri* 2a, 2457T, none of the animals died or required humane euthanasia. It is difficult to speculate on the disparate results between the two *Aotus* studies due in part to the small number of animals in each study. Future work using more animals should help to address the inconsistency.

The majority of results achieved in the current study are consistent with reports describing oral infection of *Rhesus* monkeys with virulent shigellae. The similarities between the two animal models include the dose (~1 x 10^{11} cfu) required for reproducible infection (27-29), disease time course and severity (27, 29, 30) and protection against infection after immunization of *Rhesus* with live-attenuated SC602 vaccine strain (unpublished results). One difference in the results achieved in the *Aotus* as compared to the *Rhesus* model is protection afforded after homologous re-challenge. A seminal study conducted by Formal and colleagues clearly demonstrated that prior infection with *S. flexneri* 2a protected rhesus monkeys against subsequent challenge with homologous *S. flexneri* 2a but not against heterologous *S. sonnei*, despite serum IgG responses directed to the highly conserved Ipa proteins (31). In the rhesus...
Shigella Aotus Challenge Model

Model, serotype-specific LPS responses were suggested as the protective antigen. In a similar fashion, Aotus monkeys were challenged with increasing doses of *S. flexneri* 2a, rested for 9 weeks, and then re-challenged with *S. flexneri* 2a. In contrast to Rhesus monkeys, no protection was afforded after a homologous back challenge of the Aotus. The discordant results between the two studies may reflect differences in genetic background and susceptibility to infection, but may also be a product of significant differences in the experimental procedures. In the Aotus studies, all monkeys were screened for serum IgG responses to *S. flexneri* 2a LPS to ensure there was no pre-existing immunity whereas Formal et al focused on stool cultures to ensure no carrier state or active infection was identified. The inoculum dose used by Formal et al was reported as $2 \times 10^{10}$ cfu delivered orally in brain heart infusion (BHI) to animals weighing 2.3-3.2 kg. The Aotus from the current study weighed ~850 grams and were inoculated with $\sim 1 \times 10^{11}$ cfu delivered in a rice-based buffer. The challenge inoculum/weight ratio in the Rhesus study ($2 \times 10^{10}$ cfu/2-3 kg) may have resulted in a less robust challenge (54% attack rate). Finally, the BHI nutrient media used in the challenge bolus given in the Formal et al study may have also impacted gene expression and perhaps invasiveness of the shigellae.

In the challenge/re-challenge study, serum IgG responses directed to *S. flexneri* 2a LPS were low after the primary infection with $5 \times 10^9$ or $5 \times 10^{10}$ cfu and of moderate magnitude after challenge with $5 \times 10^{11}$ cfu. However, upon re-challenge all dose groups demonstrated a significant boost in the anti-LPS serum IgG titers. These results suggested that perhaps a single infection of Aotus with *S. flexneri* 2a did not sufficiently prime the immune system to provide
protective efficacy but perhaps repeated infections may be required to produce the necessary protective immune response.

To test this hypothesis, Aotus were immunized with a sub-clinical dose of S. flexneri 2a (5 x 10^9 cfu) on day 0, 14 and 42. Anti-LPS, IpaB and IpaC serum IgG and IgA titers were similar between groups administered S. flexneri 2a twice (challenge/re-challenge study) and groups administered S. flexneri 2a three times. In agreement with previous results, Aotus administered S. flexneri 2a, 2457T were not significantly protected against back challenge with a homologous Shigella serotype. However in the same study, Aotus immunized with SC602 (10^{10} or 10^{11} cfu) were protected against challenge with S. flexneri 2a, 2457T. There was no significant differences in serum IgG or IgA responses specific for Invaplex, LPS and IpaB between groups immunized with SC602 (10^{10} or 10^{11} cfu) and groups immunized with S. flexneri 2a, 2457T. Mucosal immune responses were not assessed in the current study and may be responsible for the differences in protective efficacy obtained between 2457T and SC602. After inoculation with 2457T, 75% of Aotus had episodes of diarrhea whereas only 11-22% of Aotus immunized with SC602 experienced similar loose stools. Episodes of diarrhea did not result in a significant reduction in colonization but may have impacted the generation of robust anti-LPS serum antibodies or affected the induction of gut-homing mucosal IgA in the large intestine if an adequate microenvironment was not maintained.

Several other non-human primate models other than Aotus nancymaeae have been utilized to investigate Shigella pathogenesis and the immunogenicity and efficacy of Shigella vaccines, including both rhesus (Macaca mulatta) and cynomologus (Macaca fascicularis) monkeys. Shigella flexneri (1 x 10^{11} cfu) fed to rhesus monkey resulted in lesions in the colonic
Shigella Aotus Challenge Model

epithelium (32). Rhesus monkeys fed $10^8$ to $10^{10}$ cfu of *S. flexneri* 2a had clinical signs of acute shigellosis within 48 hrs of challenge (33) including lethargy, prostration and diarrhea with liquid or semisolid mucohemorrhagic stools (12). In addition to rhesus, cynomolgus monkeys have been infected with *Shigella* spp. (34). Interestingly, intragastric ($10^{11}$ cfu) but not intraduodenal ($10^9$ cfu) of *S. dysenteriae* 1 was able to induce shigellosis despite colonization and serum antibody responses after intraduodenal administration.

*Shigella flexneri* 2a vaccine strain SC602 carries deletions in *virG* (*icsA*) and *iuc* (encoding aerobactin) genes (20). SC602 is Congo red positive, indicating retention of the virulence plasmid and unable to cause keratoconjunctivitis in the guinea pig eye (Sereny negative) (20).

In the rhesus model, monkeys were orally immunized on day 0, 10 and 20 with $8 \times 10^{10}$ cfu of SC602. The immunized rhesus ($\geq 44\%$) secreted liquid stools with mucous within 72 hrs after each vaccination with SC602 (unpublished results). After the first vaccination, all monkeys shed *S. flexneri* 2a for three days. All immunized animals also shed *S. flexneri* 2a after the second and third vaccinations but the carriage rate was diminished with each successive immunization. On study day 48, control ($n = 8$) and SC602 immunized animals ($n = 16$) were orogastrically challenged with $1 \times 10^{11}$ cfu of *S. flexneri* 2a, 2457T. Vaccination of rhesus with SC602 was associated with 75% protection against overt dysentery ($p = 0.002$). Similar to the rhesus model, 80% protection was achieved in the current study after oral immunization of *Aotus nancymaae* with SC602 on day 0, 14 and 42.

Oral immunization of humans with $\geq 10^6$ cfu of SC602 causes shigellosis in the majority of volunteers (19). In contrast, immunization with $10^4$ cfu results in transient fever or mild diarrhea in a small percentage of volunteers. Moreover, volunteers immunized with SC602 ($10^4$
Shigella Aotus Challenge Model

cfu) and subsequently challenged with wild-type S. flexneri 2a, 2457T were completely protected against fever and severe shigellosis (4) while six of seven controls experienced shigellosis. Expanded safety evaluation of SC602 (10⁴ cfu) resulted in fever and diarrhea in 15% of volunteers, as well as headaches (35%) and abdominal cramps (24%) warranting further attenuation for clinical development (35). Similar levels of loose stools were induced after administration of SC602 to rhesus macaques and Aotus nancymaeae, albeit at higher dose levels, suggesting the monkey models may mimic, in part, the immunogenicity and reactogenicity in humans.

The described Aotus nancymaeae model provides another means to study pathogenesis and Shigella vaccine immunogenicity and efficacy. Moreover, the model opens the possibility for future testing of combination vaccines to combat infection with the three most prevalent enteric bacterial pathogens encountered by travelers, military and most importantly, children living in endemic areas. Significant research needs to focus on building upon immunological evaluation in the Aotus model to include assessing antigen-specific fecal IgA, memory B cells, and IgA-secreting plasma cells in search of immune correlates of protection. Future efforts will also focus on expanding the challenge model to include additional Shigella serotypes to facilitate the efficacy testing of different Shigella vaccine formulations and constructs and exploring the potential of a broad-based immune responses capable of cross-protecting against multiple, relevant Shigella serotypes.
Acknowledgements: The authors are grateful to K. Ross Turbyfill for providing purified proteins, antibodies and *Shigella* LPS, Kristen Clarkson and Gladys Nunez for excellent technical assistance. Research was be conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for Care and Use of Laboratory Animals (NIH, 1986).

Additionally, animal studies were approved by the Naval Medical Research Center Detachment (NMRC) Institutional Animal Care and Use Committee and the Department of the Navy Bureau of Medicine and Surgery. This study was approved via Resolucion Directoral No.378 by the Directorate of Wild Forest and Fauna Management, Peruvian Ministry of Agriculture.

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Shigella Aotus Challenge Model

REFERENCES


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<th>Citation</th>
</tr>
</thead>
</table>


FIGURE LEGENDS

Figure 1. Kinetics of the Shigella-specific serum IgG and IgA responses in *A. nancymaae* after oral challenge with escalating doses *S. flexneri* 2a, 2457T and re-challenge with 1 x 10^{11} cfu of the homologous strain. Groups of *A. nancymaae* were orally challenged on study day 0 with 5 x 10^9 (green), 5 x 10^{10} (red) or 5 x 10^{11} (blue) cfu of *S. flexneri* 2a, 2457T. Another group was mock-challenged with PBS (black). On study day 63, all groups were orally challenged with 1 x 10^{11} cfu of *S. flexneri* 2a, 2457T. Group mean titers and 1 standard deviation are plotted. The dashed lines represent days of challenge.

Figure 2. Kinetics of the Shigella-specific serum IgG and IgA responses in *A. nancymaae* after oral immunization with live-attenuated vaccine SC602 or wild-type *S. flexneri* 2a, 2457T and oral challenge with *S. flexneri* 2a, 2457T. Groups of *A. nancymaae* were orally immunized on study day 0, 14 and 42 with 1 x 10^{10} SC602 (green), 1 x 10^{11} SC602 (red) or 1 x 10^9 (blue) cfu of *S. flexneri* 2a, 2457T (blue). The control group was mock-immunized with PBS (black). On study day 70, all groups were orally challenged with 1 x 10^{11} cfu of *S. flexneri* 2a, 2457T. Group mean titers and 1 standard deviation are plotted. The dotted lines represent days of immunization and the dashed line represents the day of challenge.
<table>
<thead>
<tr>
<th>Grp</th>
<th>Treatment (cfu dose)</th>
<th>n</th>
<th>Diarrhea $^a$</th>
<th>Clinical Symptoms $^b$</th>
<th>P-Value $^c$</th>
<th>Treatment</th>
<th>n</th>
<th>Diarrhea $^a$</th>
<th>Clinical Symptoms $^b$</th>
<th>P-Value $^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>S. flexneri 2a, 2457T (5 x 10$^9$)</td>
<td>8*</td>
<td>25%</td>
<td>50%</td>
<td>0.558</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>S. flexneri 2a, 2457T (5 x 10$^{10}$)</td>
<td>9</td>
<td>56%</td>
<td>56%</td>
<td>0.057</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>S. flexneri 2a, 2457T (5 x 10$^{11}$)</td>
<td>9</td>
<td>100%</td>
<td>100%</td>
<td>0.0001</td>
<td></td>
<td>8*</td>
<td>43%</td>
<td>71%</td>
<td>1.000</td>
</tr>
<tr>
<td>4</td>
<td>PBS</td>
<td>10</td>
<td>10%</td>
<td>10%</td>
<td>---</td>
<td></td>
<td>8</td>
<td>38%</td>
<td>50%</td>
<td>0.321</td>
</tr>
</tbody>
</table>

$^a$ Diarrhea defined as at least one loose-watery stool on at least two consecutive days during observation period (10 days).

$^b$ Clinical symptoms of Shigella-induced gastroenteritis were defined as evidence of Shigella colonization (PCR or isolation) and either 1.) an episode of diarrhea or 2.) blood in the stool (occult, gross or melena) for two consecutive days or 3.) death.

$^c$ Fisher exact test compared to control group inoculated with PBS.

* One animal excluded from data analysis due to diarrhea for two days prior to challenge.

One animal euthanized after the primary challenge.

Table 1. Incidence of diarrhea and clinical symptoms after oral challenge of Aotus nancymae with escalating doses of S. flexneri 2a, 2457T and homologous re-challenge.
Shigella Aotus Challenge Model

### Table 2. Diarrhea and colonization after oral challenge of *A. nancymae* with *S. flexneri* 2a, 2457T

<table>
<thead>
<tr>
<th>Treatment (cfu)</th>
<th>N</th>
<th>Number of cases</th>
<th>Diarrhea</th>
<th>Colonization a</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. flexneri</em> 2a, 2457T (5 x 10⁹)</td>
<td>8</td>
<td>2</td>
<td>4 (3 - 5)</td>
<td>100</td>
</tr>
<tr>
<td><em>S. flexneri</em> 2a, 2457T (5 x 10¹⁰)</td>
<td>9</td>
<td>5</td>
<td>2 (1 - 4)</td>
<td>100</td>
</tr>
<tr>
<td><em>S. flexneri</em> 2a, 2457T (5 x 10¹¹)</td>
<td>9</td>
<td>8</td>
<td>3 (1 - 3)</td>
<td>100</td>
</tr>
<tr>
<td>PBS</td>
<td>10</td>
<td>1</td>
<td>0.9 (0 - 9)</td>
<td>0</td>
</tr>
</tbody>
</table>

**Diarrhea** defined as at least one loose-watery stool on at least two consecutive days during observation period (10 days) post-challenge.

**Colonization** assessed by plating on HE agar with confirmatory slide agglutination or colony blot. Negative samples confirmed with *ipaH*-specific PCR reaction on frozen stool specimens.

* One animal excluded from data analysis due to diarrhea for two days prior to challenge.

** One animal removed from study on day 6 post-challenge; full duration data not collected.

*** One animal removed from study at 2 days post-challenge; excluded from diarrhea incidence data analysis.

p = 0.002 (Mann-Whitney; two-tailed, a = 0.05) as compared to PBS control group.
Table 3. Clinical symptoms after oral immunization with either live-attenuated SC602 or wild-type *S. flexneri* 2a, 2457T.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>SC602 N = 8 (1 x 10^10 cfu)</th>
<th>SC602 N = 8 (1 x 10^11 cfu)</th>
<th><em>S. flexneri</em> 2a, 2457T N = 8 (1 x 10^9 cfu)</th>
<th>PBS N = 10 (---)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Post Vaccination 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cases of Diarrhea</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Mean Day of Onset (range)</td>
<td>7 (na)</td>
<td>1 (na)</td>
<td>3.8 (1 – 8)</td>
<td>7 (1 – 9)</td>
</tr>
<tr>
<td>Mean Days Illness (range)</td>
<td>2 (na)</td>
<td>10 (na)</td>
<td>3.5 (2 – 9)</td>
<td>3.5 (2 – 5)</td>
</tr>
<tr>
<td>% Colonization</td>
<td>100</td>
<td>100</td>
<td>75</td>
<td>0</td>
</tr>
<tr>
<td>Median duration (range)</td>
<td>1 (1 – 3)</td>
<td>4 (1 – 8)</td>
<td>7.5 (2 – 10)</td>
<td>---</td>
</tr>
<tr>
<td><strong>Post Vaccination 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cases of Diarrhea</td>
<td>2</td>
<td>1</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Mean Day of Onset (range)</td>
<td>2.5 (2 – 3)</td>
<td>1 (na)</td>
<td>2 (1 – 5)</td>
<td>0</td>
</tr>
<tr>
<td>Mean Days Illness (range)</td>
<td>3 (2 – 4)</td>
<td>5 (na)</td>
<td>6 (2 – 10)</td>
<td>0</td>
</tr>
<tr>
<td>% Colonization</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Median duration (range)</td>
<td>2 (1 – 3)</td>
<td>2 (2 – 4)</td>
<td>5 (1 – 8)</td>
<td>---</td>
</tr>
<tr>
<td><strong>Post Vaccination 3</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cases of Diarrhea</td>
<td>0</td>
<td>2</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Mean Day of Onset (range)</td>
<td>0 (na)</td>
<td>1 (na)</td>
<td>2.8 (1 – 6)</td>
<td>0</td>
</tr>
<tr>
<td>Mean Days Illness (range)</td>
<td>0 (na)</td>
<td>8 (6 – 10)</td>
<td>4 (2 – 8)</td>
<td>0</td>
</tr>
<tr>
<td>% Colonization</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Median duration (range)</td>
<td>2 (1 – 2)</td>
<td>2 (1 – 3)</td>
<td>6 (1 – 8)</td>
<td>---</td>
</tr>
</tbody>
</table>

*Diarrhea defined as at least one loose-watery stool on at least two consecutive days during observation period (10 days)*

*Colonization assessed by plating of HE agar with confirmatory slide agglutination or colony blot. Negative samples confirmed with *ipaH*-specific PCR reaction on frozen stool specimens.

na = not applicable
Table 4. Diarrhea incidence, clinical symptoms and protective efficacy in *Aotus nancymae* orally immunized with live-attenuated SC602 vaccine or *S. flexneri* 2a, 2457T and then challenged with *S. flexneri* 2a, 2457T.

<table>
<thead>
<tr>
<th>Treatment (cfu dose)</th>
<th>N</th>
<th>Diarrhea Incidence a</th>
<th>Clinical Symptoms b</th>
<th>Protective efficacy c</th>
<th>P-value d</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC602 (1 x 10^10)</td>
<td>7</td>
<td>14%</td>
<td>14%</td>
<td>80%</td>
<td>0.05</td>
</tr>
<tr>
<td>SC602 (1 x 10^11)</td>
<td>7*</td>
<td>14%</td>
<td>14%</td>
<td>80%</td>
<td>0.05</td>
</tr>
<tr>
<td><em>S. flexneri</em> 2a, 2457T (1 x 10^9)</td>
<td>8</td>
<td>38%</td>
<td>38%</td>
<td>46%</td>
<td>0.34</td>
</tr>
<tr>
<td>PBS</td>
<td>10</td>
<td>70%</td>
<td>70%</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>

* Diarrhea defined as at least one loose-watery stool on at least two consecutive days during observation period (10 days)

* Clinical symptom defined as diarrhea, bloody stools for two days, or death

* Protective efficacy = (\% clinical symptoms control - \% clinical symptoms vaccine) / \% clinical symptoms control

* Fisher exact test vs PBS control group

* one animal excluded from data analysis due to diarrhea for two days prior to challenge
Table 5. Diarrhea duration, clinical symptoms and colonization after oral immunization of *A. nancymae* with live-attenuated Shigella vaccine SC602 or *S. flexneri* 2a, 2457T and oral challenge with *S. flexneri* 2a, 2457T

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>Number of cases</th>
<th>Mean days to onset (range)</th>
<th>Mean days illness (range)</th>
<th>% Incidence</th>
<th>Median days to onset</th>
<th>Median days duration (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC602 (1 x 10^10 cfu)</td>
<td>7</td>
<td>1</td>
<td>3</td>
<td>4</td>
<td>100</td>
<td>1</td>
<td>7.9 (3 – 10)</td>
</tr>
<tr>
<td>SC602 (1 x 10^11 cfu)</td>
<td>7</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>100</td>
<td>1</td>
<td>8.4 (4 – 10)</td>
</tr>
<tr>
<td><em>S. flexneri</em> 2a, 2457T (1 x 10^9 cfu)</td>
<td>8</td>
<td>3</td>
<td>5 (3-6)</td>
<td>3 (2-5)</td>
<td>100</td>
<td>1</td>
<td>6.6 (2 – 10)</td>
</tr>
<tr>
<td>PBS</td>
<td>10</td>
<td>7</td>
<td>3.3 (1 - 7)</td>
<td>4.6 (2 - 8)</td>
<td>100</td>
<td>1</td>
<td>8.0 (4 – 10)</td>
</tr>
</tbody>
</table>

*Groups were orally immunized on day 0, 14 and 42 with either live-attenuated Shigella vaccine strain SC602 or with wild-type *S. flexneri* 2a, 2457T at a subclinical dose. Animals were then orally challenged with 1 x 10^11 cfu of *S. flexneri* 2a, 2457T on day 70.

*Diarrhea defined as at least one loose-watery stool on at least two consecutive days during observation period (10 days).

*Colonization assessed by plating of HE agar with confirmatory slide agglutination or colony blot. Negative samples confirmed with IpaH-specific PCR reaction on frozen stool specimens.

*One animal excluded from data analysis due to diarrhea prior to challenge.*
Figure 1.
Gregory/Kaminski et al. Serum IgG Serum IgA
LPS-specific
x-specific
oint Titers
Invaplex
Log10 Endp
IpaB-specific
c
IpaC-specific
Challenge Challenge Challenge Challenge
Figure 1. Kinetics of the *Shigella*-specific serum IgG and IgA responses in *A. nancymaae* after oral challenge with escalating doses *S. flexneri* 2a, 2457T and re-challenge with $1 \times 10^{11}$ cfu of the homologous strain. Groups of *A. nancymaae* were orally challenged on study day 0 with $5 \times 10^9$ (green), $5 \times 10^{10}$ (red) or $5 \times 10^{11}$ (blue) cfu of *S. flexneri* 2a, 2457T. Another group was mock-challenged with PBS (black). On study day 63, all groups were orally challenged with $1 \times 10^{11}$ cfu of *S. flexneri* 2a, 2457T. Group mean titers and 1 standard deviation are plotted. Days of challenge are indicated on the x axis.
Figure 2. Kinetics of the *Shigella*-specific serum IgG and IgA responses in *A. nancymaae* after oral immunization with live-attenuated vaccine SC602 or wild-type *S. flexneri* 2a, 2457T and oral challenge with *S. flexneri* 2a, 2457T. Groups of *A. nancymaae* were orally immunized on study day 0, 14 and 42 with $1 \times 10^{10}$ SC602 (green), $1 \times 10^{11}$ SC602 (red) or $1 \times 10^{9}$ (blue) cfu of *S. flexneri* 2a, 2457T (blue). The control group was mock-immunized with PBS (black). On study day 70, all groups were orally challenged with $1 \times 10^{11}$ cfu of *S. flexneri* 2a, 2457T. Group mean titers and 1 standard deviation are plotted. Days of vaccinations (V) and challenge (C) are indicated on the x axis.