In Vivo Models to Evaluate Toxin Neutralizing Therapies to Protect Against Systemic Shiga Toxicosis

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Shiga toxin (Stx)-producing *Escherichia coli* (STEC) is involved in cases of foodborne illness across the world and can lead to serious complications such as hemolytic uremic syndrome (HUS). This can cause profound damage on the kidneys and gastrointestinal tract, is potentially fatal, and untreatable. The Tzipori and Shoemaker labs have developed a strategy using a camelid antibody heavy-chain-only V_H domain (VHH) to create a VHH tetramer that neutralizes Stx1 and Stx2 in mice and protects them from toxicosis. The VHH tetramer is encoded in replicon mRNA (mRNA 747) and delivered by lipid organic nanoparticles (LION). We previously found that when mRNA 747 is administered to mice 24 hours before lethal Stx2a challenge, it is protective against systemic toxicosis. Here instead of directly administering the toxin, we used *Citrobacter rodentium* (CR), a natural murine pathogen, engineered to express Stx2d to evaluate the efficacy of mRNA 747 against this variant. Intramuscular (IM) injection of mRNA 747 given 6 days after inoculation with CR-Stx protected against systemic intoxication in 4 of 5 male mice and 5 of 10 total mice demonstrating that the VHH tetramer was protective in male mice but not female mice when given at that timepoint. However, IM injection of mRNA 747 given 4 days after inoculation with CR-Stx protected against systemic intoxication in female mice as the probability of survival in the days post-inoculation with CR-Stx was significantly greater in female mice that were treated with mRNA 747 compared with the untreated female mice. In conclusion, the VHH tetramer is effective in protecting mice from fatal systemic intoxication after challenge with CR-producing Stx2d. Overall, the effectiveness of the VHH tetramer at neutralizing Stx2a and Stx2d open possibilities for it to eventually be a treatment option for patients with STEC-induced HUS.

Shiga toxin (Stx) is a bacteriophage-encoded cytotoxin with two major variants, Stx-1 and Stx-2[1]. A subset of *Escherichia coli* producing this toxin known as Shiga toxin producing *E. coli* (STEC) are bacteria that can cause diarrheal disease in humans resulting in intestinal and systemic diseases[2, 3, 4]. Shiga toxin producing *E. coli* have been implicated in outbreaks and scattered cases of food-borne illness and diarrheal illness around the world[2, 3, 4]. A severe and sometimes fatal sequelae of STEC infection is hemolytic uremia syndrome (HUS) which is distinguished by thrombocytopenia, hemolytic anemia, and renal failure[5]. Children and the elderly are more likely to be afflicted with HUS[6]. The STEC that express Stx-2 are associated with a higher risk of developing HUS[1]. There are currently no effective preventives or therapeutics for STEC- induced HUS and use of antibiotics is potentially harmful[7]. An acute model of toxicity where Shiga toxin is directly administered systemically to mice can be used to evaluate preventives and therapeutics. Models of STEC in mice do not produce the same markers of diseases as seen in humans. The direct toxin model results in rapid mortality with a short window for therapeutic administration and limited opportunity to evaluate changes in histopathology. Thus, there is a need for additional small animal models that are more representative of the natural disease to evaluate potential preventatives and therapeutics. A model of toxicity using Stx-expressing *Citrobacter rodentium*, which is a pathogen natural to mice, can be used as an alternative that is more representative of the natural course of infection[1]. Using the *Citrobacter* model, we treat the mice prior to systemic disease using fecal cultures to inform colonization of the gastrointestinal tract. In humans, this is the equivalent of administering a therapy at the onset of hemorrhagic diarrhea which is typically a week before development of HUS[5].

Currently, there is no therapy for STEC infections that lead to the development of HUS. While it has been demonstrated that human monoclonal antibodies are protective in animal models, the treatments are costly, have a short shelf life, and require intravenous administration[8, 9, 10]. An alternative to this are VHH antibodies that are delivered by RNA nanoparticles to stop the development of HUS. The Stx neutralizing therapy we propose is a tetramer that utilizes the V_H domain of heavy chain only antibodies from camelids (VHH)[11]. Camelid antibodies contain only heavy chains, and the lack of requirement for light chain domain pairing makes them bind antigen more readily[11]. In addition, their antibodies are small and tightly folded making them more resistant to temperature and pH outside of the normal range than monoclonal antibodies[10,

[11]. Multiple VHHs can be linked together to enhance toxin neutralization of Stx1, Stx2, and Stx2v[12]. Overall, these benefits offer the possibility for this treatment to be used before onset of systemic disease as well as prophylactically for individuals who were exposed to the contaminated food, water, or environment during an outbreak.

VHHs encoded in replicon mRNA allow for further replication of the mRNA and greater transcription of the gene of interest[13]. Lipid inorganic nanoparticles (LION) can be used to deliver the mRNA. The advantage of LION compared with lipid nanoparticles is that RNA does not need to be specifically formulated for it, and therefore an RNA of choice can be utilized and mixed with it before use[10, 13]. It is also stable for up to a year stored at 4 degrees Celsius allowing for more practicality in usage[10, 13]. Additionally, the benefit of this treatment is that it will be a lower cost and easier to administer (single intramuscular injection rather than intravenous administration)[10]. The VHH tetramer designed by this laboratory and produced by HDT Bio is a heterotetramer that consists of the VHHs JETA9, JFGH6, JFDA5, and JGHG1. JETA9 is specific to Stx1. JFGH6 is JFDA5 is cross-specific for Stx2a and Stx2d. JFDA5 is cross-specific and can neutralize Stx1 and Stx2. JGHG1 is Stx2 specific with some cross-specificity with Stx2d. The structure of the VHH tetramer allows it to neutralize several variants of Stx instead of having to be specific to solely one variant. This is particularly important since diagnosing the specific variant of Stx causing disease is challenging[5].

This project builds off earlier work done to develop the VHH tetramer to neutralize Stx in this lab. In a previous study, we found that when mRNA 747 was administered to the mice 24 hours prior to direct Stx2a administration, there was a significant difference in survival between mice infected with Stx that were treated compared with those that were untreated ($P < 0.0001$). This demonstrates that the VHH tetramer protects against the lethal Stx2a challenge. However, the efficacy of the VHH tetramer against Stx2d challenge is unknown. However, Stx2d will not be functional if toxin is administered systemically as it requires activation in the gastrointestinal tract. Therefore, we utilized a strain of CR engineered to express Stx2d (CR-Stx) in a mouse model to evaluate the efficacy of a Stx neutralizing VHH tetramer expressed by mRNA 747 against Stx2d induced systemic disease in mice. Previously, we have found that this CR-Stx model results in colonic inflammation and edema as well as renal tubular necrosis similar to potential damages HUS can cause in afflicted humans[1, 5]. The goal of this study was to evaluate mRNA 747 expressing anti-Stx VHH tetramer to protect mice against CR-Stx challenge.

MATERIAL AND METHODS

Ethics Statement. In vivo studies were performed were performed in accordance with the applicable guidelines and regulations instituted by the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and were approved by the Institutional Animal Care and Use Committees (IACUCs) of Tufts University (Protocol G2021-160).

***Citrobacter rodentium*.** *Citrobacter rodentium* without (DBS771) and with (DBS770) Stx2d activated (Stx2dact) expression provided by John Leong (Tufts Medical Center), were taken from frozen glycerol stocks, and streaked onto Luria-Bertani (LB) agar dishes respectively containing either chloramphenicol alone (DBS770), or chloramphenicol with kanamycin (interrupting Stx expression in DBS771). Plates were incubated overnight at 37 degrees Celsius, and from each plate a single colony was inoculated into LB broth without antibiotics. Next, LB broth tubes were incubated at 37 degrees Celsius in a shaking incubator at 150 rpm for 5 hours to grow to around an OD of 0.9. Optical density at 600 nm was measured using a spectrophotometer. Ten-fold dilutions were made and plated for quantification of CFU. Plates were incubated overnight at 37 degrees Celsius, and colonies were counted to calculate volume of phosphate-buffered saline (PBS)-sucrose needed to reconstitute the bacteria to the appropriate concentration based on the goal dose (1×10^8 CFU in 20 μL in the first trial and 5×10^7 in 20 μL in the second trial). Each LB broth tube was centrifuged at 2500 rpm for 20 minutes at 4 degrees Celsius to pellet the bacteria. The supernatant was discarded, and the pellet was resuspended with the appropriate amount of PBS-sucrose according to the calculation. Ten-fold dilutions of the inocula were plated and incubated overnight at 37 degrees Celsius to quantify CFU and confirm the dose that was administered compared with the goal.

Mouse model. Two mouse trials were performed for the study.

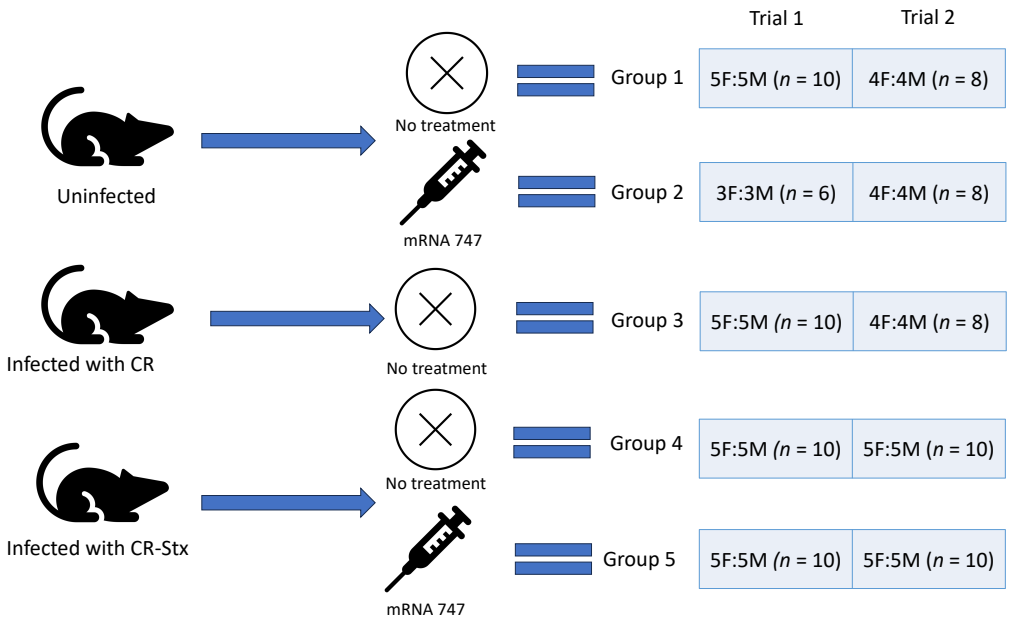

FIG 1 Experimental treatment groups. The study consisted of 5 groups: negative control, mRNA treatment only, CR infection, CR-Stx infection, and CR-Stx infection with mRNA treatment.

Trial 1: We acquired 3-week-old C57BL/6 mice (n= 46, 23 females [F], 23 males [M]; Jackson Laboratories). The study consisted of 5 groups: uninfected with no treatment (group 1; n= 10, 5F:5M), uninfected and treated with mRNA 747 (group 2; n= 6, 3F:3M), infected with CR 771 and untreated (group 3; no Stx; n=10, 5F:5M), infected with CR 770 and untreated (group 4; with Stx; n= 10, 5F:5M), and infected with CR 770 and treated with mRNA 747 (group 5; n= 10, 5F:5M; see FIG 1). The mice were inoculated in a series of two treatments over 2 days with either PBS-sucrose (uninfected), CR 771 (no Stx), or CR 770 (with Stx). Before each treatment, the mice were fasted for 6 hours. On day 6, 40ug of the mRNA treatment was given under anesthesia with four 50 ul intramuscular injections (2 per leg). Mice were monitored daily for clinical signs of disease including reduced activity or responsiveness and dehydration. Body weights were taken daily, and mice were humanely euthanized by overdose of isoflurane followed by cervical dislocation if they lost 15% of their maximum body weight or showed signs of severe illness. All surviving animals were humanely euthanized 15 days after challenge with *C. rodentium.*

Trial 2: We acquired 4-week-old C57BL/6 mice (n= 44, 22 F, 22 M; Jackson Laboratories). The goal dose of 5 x 10^7 CFU to be given to the mice orally in 20 µl of PBS-sucrose. The study

consisted of 5 groups: uninfected with no treatment (group 1; n= 8, 4F:4M), uninfected and treated with mRNA 747 (group 2; n= 8, 4F:4M), infected with CR 771 and untreated (group 3; no Stx; n=10, 4F:4M), infected with CR 770 and untreated (group 4; with Stx; n= 10, 5F:5M), and infected with CR 770 and treated with mRNA 747 (group 5; n= 10, 5F:5M; see FIG 1). The mice were inoculated in a series of two treatments over 2 days with either PBS-sucrose (uninfected), CR 771 (no Stx), or CR 770 (with Stx). Before each treatment, the mice were fasted for 6 hours. On day 4, the mRNA treatment was administered under anesthesia with four intramuscular injections (2 per leg). Mice were monitored daily for clinical signs of disease. Body weights were taken daily, and mice were humanely euthanized by overdose of isoflurane followed by cervical dislocation if they lost 15% of their maximum body weight or showed signs of severe illness. All surviving animals were humanely euthanized 21 days after challenge with *C. rodentium.*

Formulated repRNA preparation. In the first trial, sixteen mice received a 40 μg dose of mRNA 747. The mRNA:LION was formulated in the following manner: 317.7 μL of 1980 ng/mL mRNA 747 stock (HDT Bio) was combined with 1468.3 μL RNAse free water. Separately, 246.4 μL of LION (HDT Bio), 184 μL of 100 mM citrate, 940 μLof 40% sucrose, and 490 μL of RNAse free water were combined, and then mixed with the mRNA 747 stock and RNAse free water. The mRNA: LION was kept at room temperature for 15 minutes before usage. In the second trial, the volumes differed as there were eighteen total mice receiving a 40 μg dose of mRNA 747. The volumes were as follows: 418.2μL of 1980 ng/mL mRNA 747 stock (HDT Bio) combined with 1651.8 μL RNAse free water, 277.3 μL of LION (HDT Bio), 207 μL of 100 mM citrate, 1035 μL of 40% sucrose, and 551 μL of RNAse free water.

Fecal culture. In the first trial, feces was collected from mice infected with CR or CR-Stx on day 5 to determine fecal shedding before administration of mRNA treatment. Fecal collection was done for mice infected with either CR or CR-Stx on days 4, 7, and 11 for the second trial. Fecal samples were also collected from surviving mice from group 5 on day 11 post inoculation and from groups 4 and 5 upon death/termination of study. Mice were held individually in containers, and fecal pellets were collected and placed in pre-weighed 1 mL microcentrifuge tubes with 100 μL of Dulbecco's PBS (DPBS). The tubes were vortexed to ensure adequate mixing and weighed with the sample. Serial dilutions were made and plated onto agar plates containing either chloramphenicol to culture CR 770 or chloramphenicol and

kanamycin to culture CR 771. Plates were incubated overnight at 37 degrees Celsius. Colonies were counted after incubation, and CFU/g feces was calculated.

ELISA for the detection of Stx in mouse feces. The fecal ELISA was performed to quantify the amount of Stx2d present in the feces of CR-Stx infected mice. In the first trial, fecal samples were taken on days 5 and 11 post inoculation, and upon death/ termination of study. In the second trial, fecal samples were collected on days 7 and 11 post inoculation and upon the endpoint for the individual mouse. Mice were individually held in containers, and fecal pellets were collected and placed in pre-weighed 1 mL microcentrifuge tubes with 200 µL of DPBS and 1x Halt Protease Inhibitor (250 µL was used in the second trial). The tubes were vortexed to ensure adequate mixing and weighed with the sample. Samples were stored at -80 degrees Celsius before usage. Greiner Bio-One MICROLON High Binding 96-well ELISA Assay Microplates ELISA plates were coated with 100 µL/well of capture antibody (1 µg/mL LC16:SC12 antibody) and incubated at 4 degrees Celsius for 2-3 hours. Capture antibody was removed and 200 µL/well of blocking solution was added (4% nonfat dry milk powder [NFDM] in PBS and 0.1% Tween-20). Plates were incubated at room temperature for 1-2 hours, and then blocking was washed off using a plate washer (PBS with Tween-20 [PBST], 3 washes). Stx2act protein was used as the standard and applied to the well with nine 2-fold dilutions. Blocking solution was used as blanks. Standards and samples on the plates incubated overnight at 4 degrees Celsius and then were washed off using a plate washer (PBST, 3 washes). One µg/mL JKB-2 in blocking solution was used as the detection antibody and 100 µL was added per well. Plate was then incubated at room temperature for one hour on a rocker. Detection antibody was washed off the plate (PBST, 6 washes) and 100 µL/well of 1:10,000 ∝Etag-HRP in blocking solution was added as a secondary antibody. After a 45-minute incubation at room temperature on the rocker, secondary antibody was washed off (PBST, 6 washes) and 100 µL/well of Thermo TMB 1 Step Ultra was added. Plate was protected from light and incubated at room temperature for 15 minutes before 100 µL/well of Invitrogen ELISA stop solution was added. Plate was read in the plate reader at 450 nm.

Serum Stx2a VHH Tetramer ELISA. In the first trial, two mice from the group receiving mRNA 747 only were euthanized and serum was collected on days 3, 6, and 9 post-administration of mRNA to demonstrate the course of VHH concentrations the days post-

administration. Serum was also collected from the surviving mice that were infected with CR-Stx and received mRNA 747 on day 15 post-inoculation to determine if the mice still had protective concentrations of VHH. In the second trial, mice that received mRNA 747 only were all euthanized on day 8 post-administration of mRNA to collect serum. Serum was also collected at the endpoints from mice that were infected with CR-Stx and received mRNA 747. Mice were euthanized via overdose of isoflurane. Blood was collected from the heart and placed in serum separator tubes. Tubes were centrifuged at 1000 rpm for 5 minutes and serum was collected. Greiner Bio-One MICROLON High Binding 96-well ELISA Assay Microplates plates were coated with 50 µL/well of Stx2a stock and incubated at room temperature for 2.5 hours. Stx2a was removed and plates were washed with the plate washer. Two hundred µL/well of blocking buffer (4% NFDM in PBS and 0.1% Tween-20) was added, and plates were incubated for 3 hours at room temperature. Blocking buffer was then removed and plates were washed using a plate washer (PBST, 3 washes). One nM JKB-2 in blocking buffer was used as the first standard. Nine 2-fold dilutions were made in the dilution plate, and 150 µL of each dilution was transferred to the ELISA plate. Samples were added to the plates in duplicate and plates were sealed and stored overnight at 4 degrees Celsius. Plates were washed in the plate washer (PBST, 3 washes). Rabbit ∝ VHH sera was used as the detection antibody (1:2000 in blocking solution), and 100 µL/well was added. Plates were placed on a plate rocker at room temperature for one hour, and then washed using the plate washer (PBST, 6 washes). One hundred µL/well of secondary antibody (1:5000 in blocking goat ∝ rabbit IgG HRP, human adsorbed) was added. Plates were placed on the plate rocker at room temperature for 45 minutes, and then washed using the plate washer (PBST, 6 washes). Next, 100 µL/well of Thermo TMB 1 Step Ultra was added to each plate. Plates were protected from light and incubated at room temperature for 15 minutes. Finally, 100 µL/well of Invitrogen ELISA stop solution was added and plates were read at 450 nm in the plate reader.

Histopathology. Mice were euthanized via overdose of isoflurane. Necropsies were performed on all mice excluding those in group 2. Kidneys and gastrointestinal tract were collected for histopathology and stored in 10% neutral buffered formalin. Cecum, small intestine, colon, and kidneys were placed on cassettes and slides were made to evaluate tissues.

Statistics. The survival data were analyzed by applying a log-rank (Mantel-Cox) test using GraphPad Prism (version 10.0.0; GraphPad Software, Inc.). Resulting p values of < 0.05

were considered significant. Statistical comparison for percent mean bodyweight change comparing CR-Stx and CR-Stx + mRNA was done using a mixed effects model using GraphPad Prism (version 10.0.0; GraphPad Software, Inc.).

RESULTS

Efficacy of mRNA 747 treatment in *C. rodentium* mouse model. In the first trial, the negative control mice in groups 1 and 2 all survived. Two mice out of ten in group 3 (CR no toxin) were euthanized, one for greater than fifteen percent weight loss and the other for signs of severe illness (1M and 1F). All the female mice that were infected with CR-Stx and not treated were euthanized due to >15% weight loss. Four of the male mice in infected with CR-Stx and untreated were euthanized due to >15% weight loss and one was found dead. For mice infected with CR-Stx and treated, four out of five of the male mice survived. One was euthanized due to greater than fifteen percent weight loss. However, only one out of the five females infected with CR-Stx and treated survived until the end of the study. All other female mice in the group were euthanized due to greater than fifteen percent weight loss. The overall survival (including males and females), and the survival in females was not significantly different between the group infected with CR-Stx and untreated and the group infected with CR-Stx and treated with mRNA 747 ($P > 0.05$). However, the survival in males did significantly differ between these groups ($P = 0.0253$) which demonstrates that for the male mice there was a significant difference in survival between mice infected with CR-Stx that were treated compared with those that were untreated. In addition, statistical analysis of mean percent bodyweight change overtime for days 7 through 10 in male mice showed that treatment (CR-Stx versus CR-Stx + mRNA) had a significant effect on bodyweight variation ($P = 0.0307$) whereas time did not have a significant effect ($P > 0.05$).

In the second trial, we increased the length of the study so that the mice that received the treatment to determine the length of time that the protection from the VHH tetramers would last. The males that were infected with CR-Stx and not treated were excluded from the study because they did not shed the bacteria in their feces and no Stx was found upon ELISA of the fecal samples indicating that they did not become infected. Four out of the five female mice infected with CR-Stx and untreated were euthanized on days 11 and 12, and one was euthanized on day 17. One group 5 female was euthanized on day 9, but the rest were euthanized between day 17 to day 21. The group 5 males were all euthanized between days 17 to 21. None of the control mice

(negative control, mRNA only, or infected with CR without Stx) mice were euthanized due to >15% weight loss. There was a significant difference in the survival between female mice that were infected with CR-Stx (males were excluded) and mice infected with CR-Stx and treated with mRNA 747 ($P = 0.0037$). There was also a significant difference in survival considering only the female mice in these groups ($P = 0.0374$).

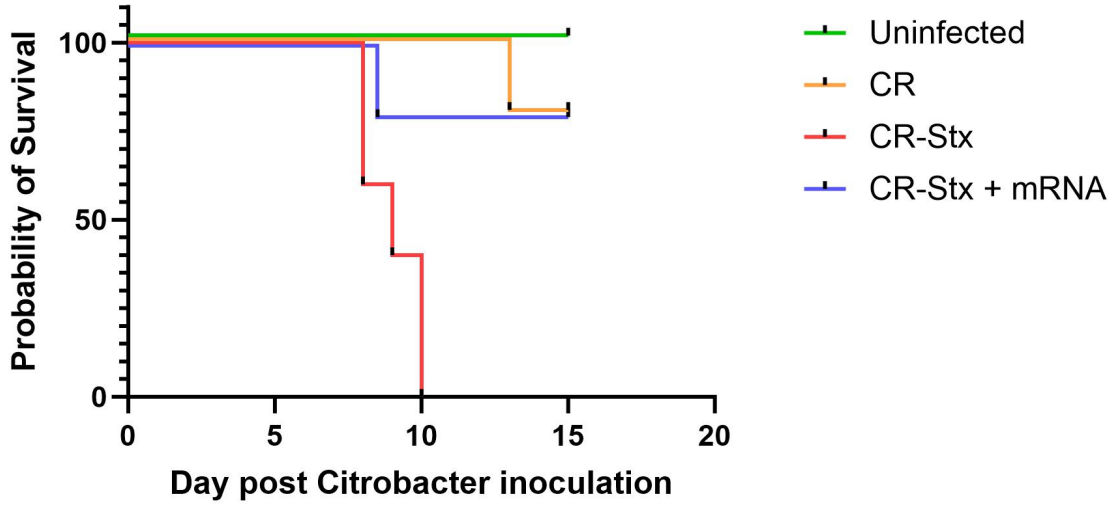

FIG 2 Survival curve for male mice in trial 1. There was a significant difference in survival between male mice infected with CR-Stx that were treated compared with those that were untreated ($P = 0.0253$). Therefore, male mice were protected from CR-Stx challenge when treated with mRNA 747 at 6 days post infection.

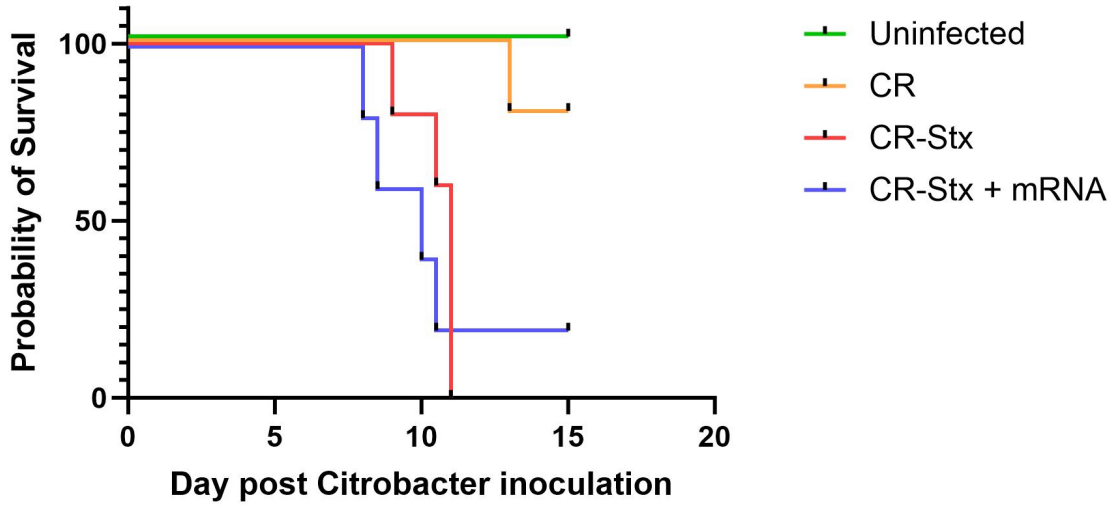

FIG 3 Survival curve for female mice in trial 1. The difference in survival between CR-Stx and CR-Stx + mRNA females was not significant. Therefore, female mice were not protected from CR-Stx challenge when treated with mRNA 747 at 6 days post infection.

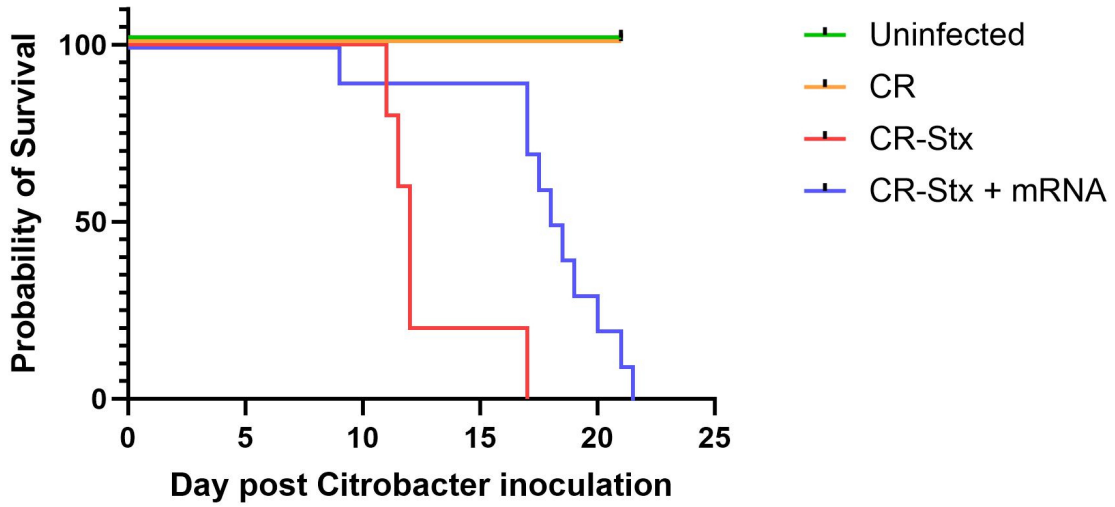

FIG 4 Survival curve for mice in trial 2. There was a significant difference in survival between female mice that were infected with CR-Stx (males were excluded) and mice infected with CR-Stx and treated with mRNA 747 ($P = 0.0037$). Therefore, mRNA 747 protects mice against CR-Stx challenge when administered 4 days post infection.

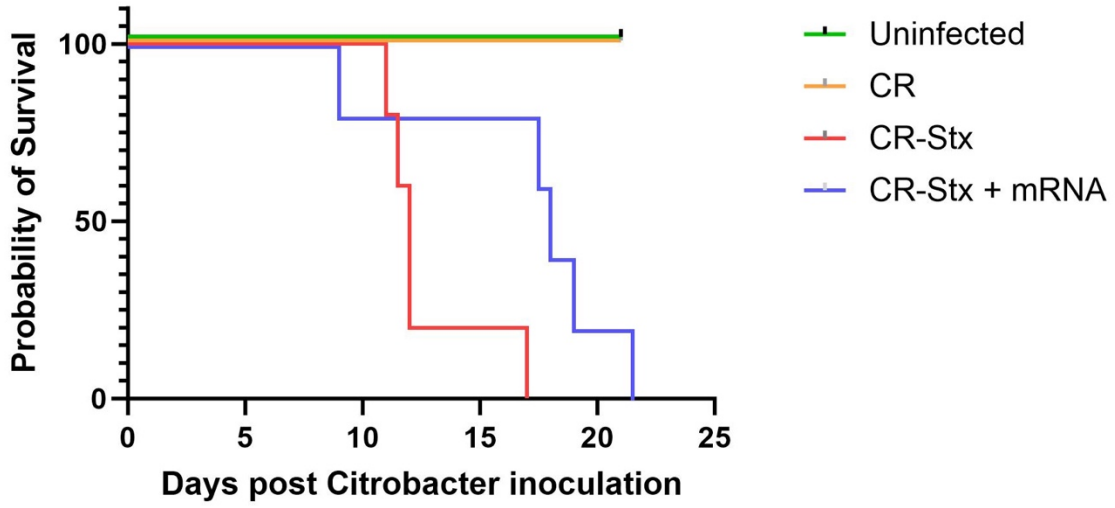

FIG 5 Survival curve for female mice in trial 2. There was a significant difference in survival between female mice that were infected with CR-Stx and female mice infected with CR-Stx and treated with mRNA 747 ($P = 0.0374$). Therefore, mRNA 747 protects female mice against CR-Stx challenge when administered 4 days post infection, but not when administered 6 days post infection.

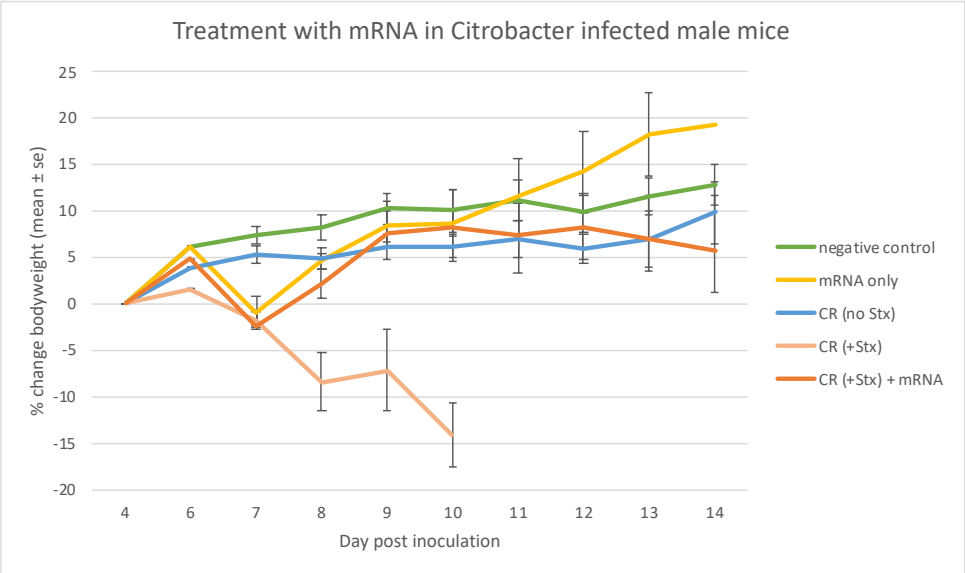

FIG 6 Comparison between mean percent change in bodyweight for male mice in trial 1. When comparing between CR + Stx groups and CR-Stx + mRNA groups on days 7 through 10 post inoculation, time did not contribute significantly to the variation in bodyweight seen ($P > 0.05$). However, treatment group did significantly contribute to the variation ($P = 0.0307$).

Fecal culture. In the first trial, all mice infected with either CR or CR-Stx were confirmed to be shedding Citrobacter in their feces. The mice from the control groups did not shed Citrobacter in their feces. In the second trial, all mice infected with CR or CR-Stx were confirmed to be shedding bacteria in their feces, except for the male mice that were inoculated with CR-Stx. These male mice did not shed Citrobacter in their feces for the duration of the study. The mice from the control groups did not shed any Citrobacter in their feces. In both studies, fecal shedding did not differ between CR and CR-Stx and it increased overtime.

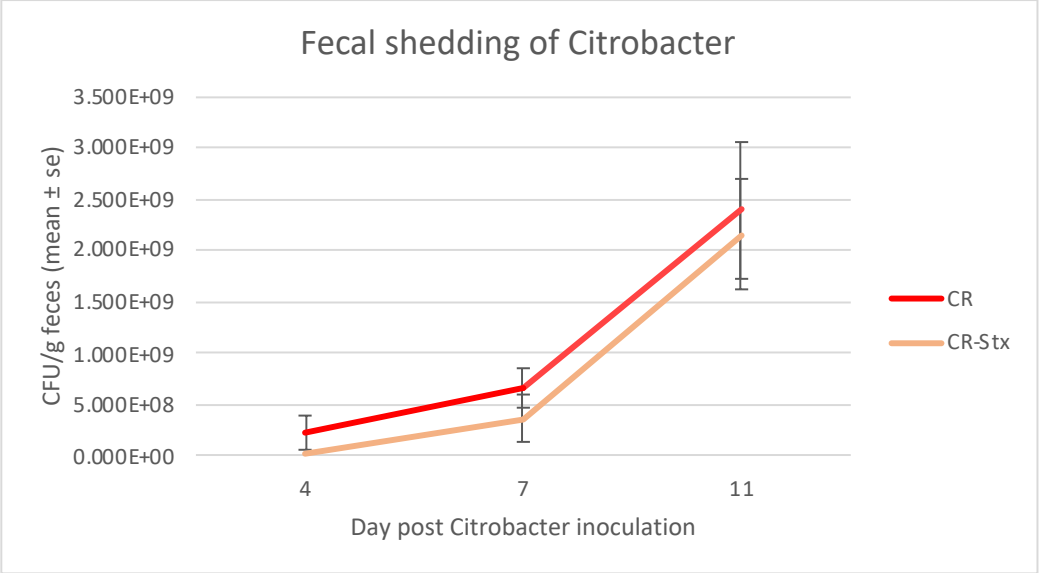

FIG 7 Fecal shedding of Citrobacter in trial 2. Fecal shedding did not differ between mice inoculated with CR and mice inoculated with CR-Stx, and it increased overtime.

ELISA for the detection of Stx2d in mouse feces. In the first trial, it was confirmed that all the mice infected with CR 770 had Stx present in their feces (groups 4 and 5). The mice infected with CR 771 (group 3) did not have Stx present in their feces.

In the second trial, it was confirmed that the mice infected with CR 770 and the male mice infected with CR-Stx did not have Stx present in their feces. The amount of Stx present in the feces increased from day 7 to day 11 to each individual mouse's endpoint.

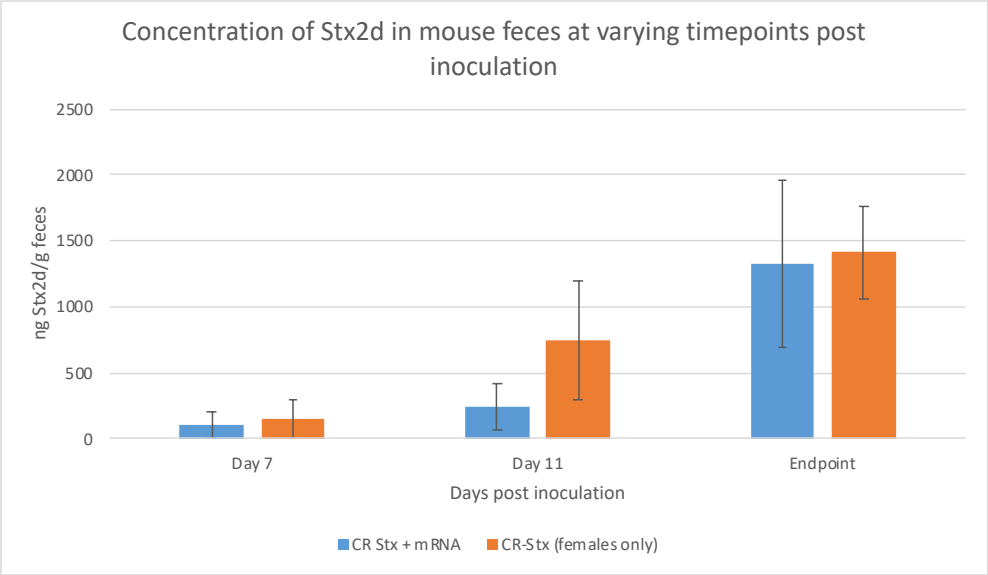

FIG 8 Concentration of Stx2d in mouse feces. In the second trial, concentration of Stx in mouse feces increased in the days past inoculation for mice inoculated with CR-Stx (males excluded) and mice inoculated with CR-Stx and treated with mRNA 747.

Serum Stx2a VHH Tetramer ELISA. Results were inconclusive due to poor assay performance.

Histopathology. Results are pending.

DISCUSSION

In the first study, we demonstrated that when the mRNA was given on day 6 post inoculation with CR-Stx, treated male mice were protected from severe disease compared with the male mice that were inoculated and untreated. These treated male mice were also able to maintain their bodyweight between days 7 and 10 compared with the untreated males that were losing weight and reaching 15% body weight loss due to systemic toxicity. In both studies, the mice that received the mRNA 747 had noticeable weight loss the day after treatment. While their body weight recovered slightly from that initial drop, the female mice in group 5 never recovered their weight fully and did not have a large increase in mean percent body weight change over the study as the negative control group did. The male mice infected with CR-Stx and treated with mRNA 747 did not have as large of an increase in mean percent body weight change over the

study as the negative control group did, but were able to recover their bodyweight compared with the female counterparts that continued to lose weight.

Adjustments were made for the second trial based on the results of the first trial to optimize the murine model. The main differences between the two trials were the age of the mice at the time of inoculation, the dose of CR that the mice were inoculated with, and the day that the mRNA was given post-inoculation. The mice from the second trial were one week older at the time of inoculation (5 weeks of age at inoculation vs. 4 weeks of age at inoculation). The goal dose of CR from the second trial was also lower (5×10^7 CFU vs. 1×10^8 CFU). Additionally, the dose of mRNA was given on day 4 post-inoculation in the second trial compared with day 6 post-inoculation in the first trial. We decided to give the dose of mRNA 747 earlier to allow time for Citrobacter to colonize the gut but for VHH tetramer to be produced prior to systemic dissemination of Stx. In addition, we decreased the amount of *C. rodentium* given to the mice and used mice that were a week older so they would not become ill from Citrobacter alone.

In the second trial, the issue that we encountered was the variability in the course of infection depending on the individual mouse as demonstrated by fecal shedding. Prior studies done in this lab have demonstrated that by day 9 after administering the mRNA 748, another version of the VHH tetramer, the concentrations of VHHs in the serum are undetectable. This means that once the mice reached a certain point, they would no longer protected by the VHH tetramer. However, the *C. rodentium* still continued to produce Stx since the mice do not clear the bacteria. While some of the mice in group 5 were protected by the VHH tetramer during the period in which the untreated mice became ill, this protection was lost with time, and they began to lose weight again and reached the 15% body weight loss terminal endpoint. In the first trial, we euthanized the mice before they reached this point. In the second trial, one female mouse in group 5 was lost early on (day 9 post inoculation, day 5 post mRNA administration), but the rest were able to survive longer than four out of five mice in group 4 (survival time $\geq$ 17 days post infection, 13 days post mRNA administration). This demonstrates that for the mice that received the mRNA 747, there is a point where VHH tetramer no longer neutralizes Stx and the mice are no longer protected so they reach the 15% body weight loss endpoint due to systemic toxicity. We predict that measurement of serum VHH levels will decrease prior to onset of illness. One female mouse infected with CR-Stx and untreated took longer to shed as much CR in its feces as

the rest of the female mice in its group. The variability in the course of infection made it difficult to compare between mice infected CR-Stx that were treated versus those that were untreated.

To prove that the mRNA 747 treatment is effective using the *Citrobacter* model in mice, additional studies could be performed. A second dose of mRNA 747 could be a potential solution as the CR-Stx infected and treated mice would not begin to lose weight again if they had another dose of the mRNA 747 to neutralize the Stx. Antibiotics could also be utilized initially when the mice are inoculated with CR. This might allow for the Stx to colonize the gastrointestinal tract more effectively for a faster and more uniform course of infection to optimize the *Citrobacter* model. In addition, since the positive control for the male mice that were inoculated with CR-Stx and treated with mRNA 747 was lost since the male mice infected with CR-Stx and untreated failed to become infected with CR-Stx, another study would be beneficial to establish the efficacy of the mRNA 747 for both male and female mice. In addition, a pharmacokinetic study to investigate the concentrations of VHH across different timepoints after administration of mRNA 747 would be beneficial to determine at what exactly point the mice lose protection from the VHHs and are vulnerable to toxicosis.

In summary, despite challenges with the variability in the *Citrobacter* model, we have demonstrated that mRNA 747 is protective against lethal challenge with Stx2a and CR-producing Stx2d in mice. Further studies are needed to determine the duration of protection that is provided by the VHHs and to optimize the *Citrobacter* model. This data will inform future studies in the gnotobiotic piglet model to evaluate efficacy of VHH against natural STEC strains which are more pertinent to human patients.

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