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Deletions in *guaBA* and *htrA* but not *clpX* or *rfaL* constitute a live-attenuated vaccine strain of *Salmonella* Newport to protect against serogroup C₂-C₃ *Salmonella* in mice

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ABSTRACT

Non-typhoidal *Salmonella* (NTS) are a leading cause of foodborne infections worldwide, and serogroups B, C_1, C_2-C_3 and D are the most common serogroups associated with human disease. While live vaccine candidates that protect against *S.* Typhimurium (serogroup B) and *S.* Enteritidis (serogroup D) have been described by us and others, far less effort has been directed towards vaccines that target either serogroup C_1 or C_2-C_3 *Salmonella*. Here we describe a *Salmonella* Newport-based live-attenuated vaccine (serogroup C_2-C_3). Deletion of the genes *clpX* or *rfaL*, previously used in live vaccines to attenuate *S.* Typhimurium and/or *S.* Enteritidis, failed to attenuate *S.* Newport. However, we found that deletion of either *guaBA* or *htrA* raised the 50% lethal dose of *S.* Newport in an intraperitoneal infection model in BALB/c mice. Our live-attenuated vaccine candidate CVD 1966 (*S.* Newport Δ*guaBA* Δ*htrA*) elicited strong antibody responses against COPS, flagellin and outer membrane proteins when administered intraperitoneally or orally. Following lethal challenge with the parental virulent strain of *S.* Newport, we observed vaccine efficacies of 53% for immunization via the intraperitoneal route and 47% for immunization via the oral route. Following intraperiteonal immunization, the vaccine also significantly reduced the bacterial burden of challenge organisms in the liver and spleen. Interestingly, reducing the LPS chain length by deleting *rfaL* did not induce a stronger immune response towards surface antigens, and failed to elicit any protection against lethal homologous challenge. In conclusion, we have developed a live-attenuated *Salmonella* serogroup C_2-C_3 vaccine that we are further evaluating.

Introduction

Non-typhoidal *Salmonella* (NTS) is one of the leading causes of foodborne infections, and is estimated to have caused 78.7 million cases of illness worldwide in 2010.[1,2] While *S.* Typhimurium (serogroup B; O:4) and *S.* Enteritidis (serogroup D; O:9) are the most common NTS serovars worldwide, serogroups C_1 (O:6,7) and C_2-C_3 (O:8) serovars are also highly relevant to human and animal health.[3] In the U.S., collectively, serogroups C_1 and C_2-C_3 are the most common serogroups associated with human infection. In 2012, 34.7% of NTS were serogroups C_1 and C_2-C_3, 28.1% were serogroup D and 27.6% were serogroup B.[3] They are also increasing in prevalence in Europe, Africa and Asia.[3] While most NTS cause self-limiting gastroenteritis in healthy adults, some serovars are associated with higher rates of invasive disease characterized by disseminated, focal infections. In particular, *S.* Choleraesuis (C_1) is highly invasive (up to 56% of human-associated isolates are isolated from blood), and is prevalent in certain parts of Asia such as Taiwan and Thailand.[4–9] According to the U.S. Centers for Disease Control and Prevention (CDC), the most common serogroup C_1 or C_2-C_3 serovars isolated in the U.S. in 2015 were *S.* Newport (C_2-C_3), *S.* Muenchen (C_2-C_3) and *S.* Infantis (C_1).[10]

While live-attenuated vaccine candidates that protect against *S.* Typhimurium and *S.* Enteritidis have been described in the literature, comparatively few vaccines that target serogroup C_1 or C_2-C_3 *Salmonella* have been described, and none are approved for use in humans.[11–13] Four vaccines have been licensed to protect swine against *S.* Choleraesuis, and one licensed vaccine targets serogroup C_2-C_3 (*S.* Newport) in cattle.[14–16,3] Two multivalent vaccines targeting serogroups B, C_1, D and E are in development for use in poultry.[17,18]

Our overall goal is to develop a multivalent vaccine consisting of live-attenuated serogroup B, D, C_1 and C_2-C_3 strains that can protect against the majority of infections due to NTS. The aim of the current study was to develop a live-attenuated *S.* Newport vaccine using mutations that were previously successful for *S.* Typhimurium, *S.* Enteritidis, *S.* Paratyphi A and *S.* Typhi vaccines. We previously showed that deleting genes involved in guanine biosynthesis (*guaBA*) or coding for the master regulatory protease ClpPX (*clpPX*) had a strong attenuating effect in *S.* Typhimurium, *S.* Enteritidis and *S.* Paratyphi A in mouse models.[11,13,19] Another mutation found to be strongly attenuating in *S.* Typhimurium and *S.* Typhi is the deletion of *htrA* (also known as *degP*), coding for the periplasmic protease/chaperone HtrA.[20–22] We assessed, here, whether

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ⓘ Supplemental data for this article can be accessed on the publisher's website.

S. Newport with deletions in *guaBA*, *clpX* and *htrA* would be well tolerated and immunogenic in mice. Additionally, we evaluated whether deletion of *rfaL,* coding for the O-antigen ligase responsible for polymerization of O-units onto the lipid A core, could improve immunogenicity as mutations that target LPS biosynthesis have been shown to enhance the immunogenicity of surface proteins.[23–26]

Results

S. Newport mouse model

We administered WT *S.* Newport strain Chile 361 (10^8 CFU) perorally (p.o.) or intraperitoneally (i.p.) to 6-8-week-old BALB/c mice, and recorded mortality daily for up to 21 days. All three mice survived oral infection but none of the three mice survived i.p. challenge with *S.* Newport. Next we determined the i.p. 50% lethal dose (LD_{50}) in BALB/c mice, and found it to be 5×10^6 CFU. We selected the intraperitoneal route of infection of BALB/c mice as our challenge model.

Deletion of guaBA, clpX and htrA from S. Newport to create a live-attenuated vaccine

We deleted *guaBA*, *clpX* and *htrA* from *S.* Newport, leaving no antibiotic resistance marker in the chromosome. We verified the phenotypes of the mutants *in vitro* and confirmed that deletion of *guaBA* caused the mutant strain to become auxotrophic for guanine (Fig. 1A) and deletion of *clpX* resulted in hyperflagellation with increased motility (Fig. 1B). We subsequently evaluated the virulence of these mutants in our BALB/c mouse model (Table 1). The LD_{50} of the *guaBA* mutant was > 2 log_{10} more than the wild-type *S.* Newport parental strain (> 8×10^8 CFU and 5×10^6 CFU, respectively). However, deletion of *clpX* had no effect on virulence (LD_{50} of 7×10^6 CFU). Deletion of *htrA* increased the LD_{50} of the mutant strain (1.5×10^7 CFU), although not to the same extent as the *guaBA* mutation. We constructed the double mutant *S.* Newport Δ*guaBA* Δ*htrA* (named CVD 1966) to create a live vaccine candidate and verified that this strain is attenuated compared to the WT parental strain (Table 1).

Immunogenicity and protective efficacy of CVD 1966 via the intraperitoneal route

We immunized mice i.p. three times with 10^8 CFU of CVD 1966, three weeks apart. No adverse effects were observed following each immunization. Serum was collected four weeks after the last immunization and IgG titers against core-O polysaccharide (COPS), flagellin (FliC) as well as a crude mixture of outer membrane proteins (OMPs) were measured by ELISA (Fig. 2A; Figure S1). All animals that received CVD 1966 seroconverted (15/15), with geometric mean titers (GMT) of 9.7×10^4 ELISA units (EU)/ml (COPS), 5.5×10^4 EU/ml (FliC) and 5.5×10^5 EU/ml (OMPs), compared to a GMT lower than 25 EU/ml for each of these antigen preparations in animals that received PBS.

We next assessed whether vaccine-induced antibodies were able to opsonize and promote uptake of *S.* Newport by mouse macrophages. We found that serum from mice immunized i.p.
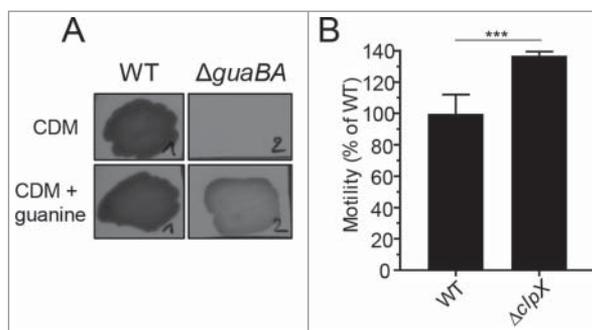

Figure 1. Phenotype of Δ*guaBA* and Δ*clpX* mutants in *S.* Newport. Panel A, guanine auxotrophy of the Δ*guaBA* mutant was confirmed by patching bacteria onto Chemically-Defined Media (CDM) or CDM supplemented with 0.02% (w/v) guanine. Panel B, hyperflagellation of the Δ*clpX* mutant was assessed by measuring the motility on soft agar plates; *** $p < 0.001$ (Student's t-test).

with CVD 1966 was able to significantly ($p < 0.001$, Student's t-test) promote uptake of *S.* Newport by J774 macrophages *in vitro*, compared to the serum of mice that received PBS (Fig. 2B).

Four weeks after the last immunization, mice were challenged i.p. with 8 x LD_{50} of WT *S.* Newport. All PBS-immunized mice succumbed to the infection between days 3 and 7, whereas 53% of mice that received CVD 1966 survived, producing a vaccine efficacy of 53% ($p = 0.002$, Fisher's exact test; Fig. 2C). Moreover, we found that CVD 1966-immunized mice that were not protected against mortality died at a significantly later time-point ($p < 0.001$, Log-rank test) compared to PBS-immunized mice (median survival of 8 days, versus 5 days).

Finally, we assessed bacterial clearance following WT *S.* Newport infection of mice immunized with CVD 1966 compared to PBS. Two days after challenge with a sub-lethal dose of *S.* Newport WT (10^6 CFU i.p.), animals were euthanized and bacterial burden was determined in their liver and spleen (Fig. 2D). Control mice had bacterial counts up to 10^7 CFU per gram in both organs, while CVD 1966-immunized mice had less than 10^3 CFU per gram of organ. Moreover, 50% of CVD 1966-vaccinated animals (5 mice out of 10) had no detectable bacteria in their liver, and 40% of vaccinated animals (4 out of 10) had no detectable bacteria in their spleen.

We did not observe a correlation between anti-FliC, anti-OMPs or anti-COPS IgG titers and protection ($p = 0.48$, 0.20 and 0.80, respectively) or uptake by J774 cells ($p = 0.76$, 0.46 and 0.81, respectively). There was, however, a correlation between opsonophagocytic uptake and delayed time to death (Spearman coefficient r = 0.78; p = 0.04).

Immunization and vaccine efficacy via the oral route

Since live-attenuated bacterial vaccines can be orally delivered, we investigated whether protection would be maintained after

Table 1. 50% lethal dose of *S.* Newport strains.

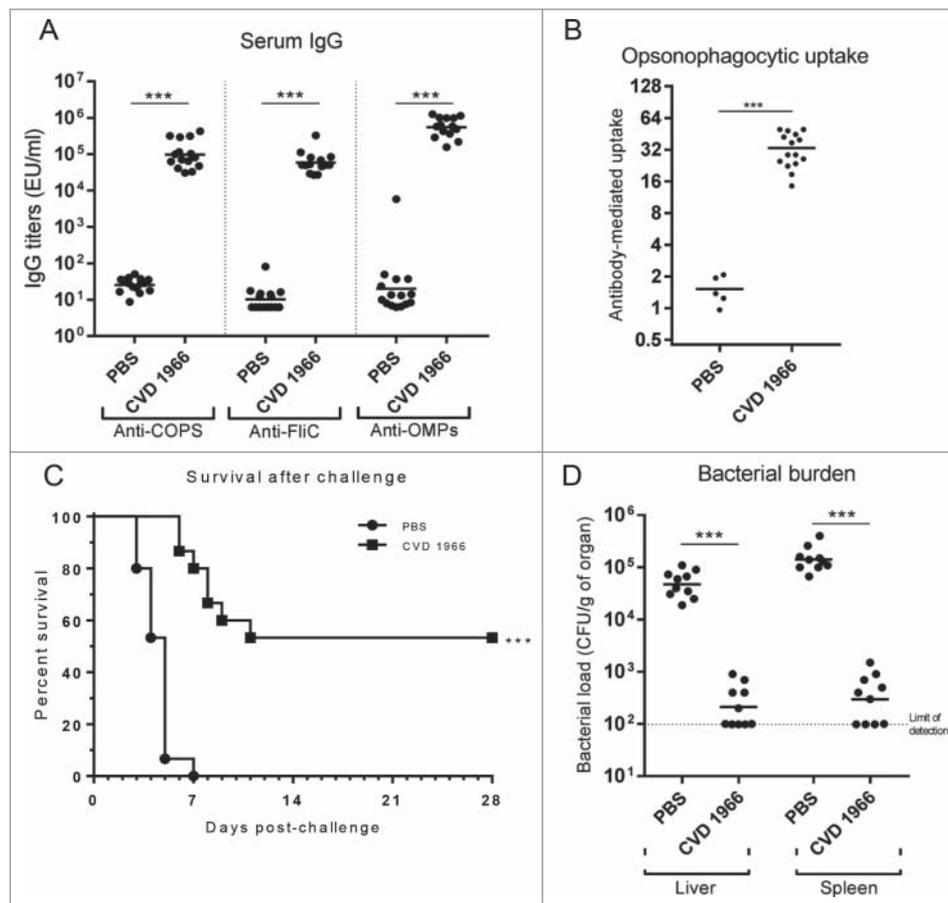| Strain	Genotype	Intraperitoneal LD_{50}
S. Newport (Chile361)	Wild-type	5×10^6 CFU
SNE-guaBA	Δ*guaBA*	> 5×10^8 CFU
SNE-clpX	Δ*clpX*	7×10^6 CFU
SNE-htrA	Δ*htrA*	1.5×10^7 CFU
CVD 1966	Δ*guaBA* Δ*htrA*	6.1×10^8 CFU
SNE-rfaL	Δ*rfaL*	3×10^7 CFU

Figure 2. Serum IgG titers, opsonophagocytic uptake, survival and organ burden following intraperitoneal immunization with CVD 1966. Mice were immunized 3 times intraperitoneally with PBS or 10^8 CFU of CVD 1966 and bled 4 weeks after the last immunization. Panel A, serum IgG titers against COPS, FliC and outer membrane proteins (OMPs) were measured by ELISA and reported as EU/ml; bars represent the geometric mean titers; *** $p < 0.001$ (Student's t-test). Panel B, antibody-mediated uptake of *S.* Newport WT by J774 macrophages was assessed using serum of immunized mice, and normalized against the no-serum control; bars represent the mean; *** $p < 0.001$ (Student's t-test). Panel C, survival of mice after lethal challenge with 4×10^7 CFU of *S.* Newport WT; *** $p < 0.001$ (Log-rank test). Panel D, immunized mice were challenged with a sub-lethal dose of *S.* Newport WT (10^6 CFU), euthanized 48 hours later and bacterial burden in the liver and spleen was assessed and reported as CFU per gram of organ; bars represent the geometric mean *** $p < 0.001$ (Mann-Whitney rank-sum test).

oral immunization with CVD 1966 (*S.* Newport Δ*guaBA* Δ*htrA*). Moreover, since removal of the O-antigen has been shown to enhance responses towards surface antigens and to increase protective efficacy in live-attenuated bacterial vaccines, we assessed the addition of a mutation in *rfaL* (coding for the O antigen ligase) to produce CVD 1977 (*S.* Newport Δ*guaBA* Δ*htrA* Δ*rfaL*). We found that deletion of *rfaL* was moderately attenuating in *S.* Newport strain Chile 361 (Table 1), and confirmed the alteration of the LPS profile in CVD 1977, compared to the WT strain or parent CVD 1966 (Figure S2). We observed that 4 of 30 mice showed a balance defect following peroral immunization with CVD 1966 but fully recovered.

Serum was collected and analyzed by ELISA four weeks after the last immunization (Fig. 3A). Both vaccine candidates CVD 1966 and CVD 1977 elicited comparable anti-flagellin and anti-OMPs serum IgG titers. As expected, CVD 1977 elicited very low IgG production against COPS compared to CVD 1966 (56 EU/ml versus 2.9×10^4 EU/ml, respectively). Overall, fewer mice seroconverted for anti-flagellin IgG when they were immunized orally compared to i.p. immunization with CVD 1966, (67% [20/30] versus 100% seroconversion, $p = 0.02$, Fisher's exact test). The seroconversion rate for anti-OMP antibody was statistically not different between the two routes, however, a trend was seen

towards greater seroconversion with i.p. immunization (100% for i.p. versus 78% for oral immunization [21/27], $p = 0.06$). All mice seroconverted for anti-COPS serum IgG when immunized with CVD 1966, regardless of the immunization route.

When we examined antibody-mediated uptake of *S.* Newport by J774 macrophages, we found that sera from mice immunized orally with CVD 1966 elicited significantly more uptake than mice than received PBS ($p < 0.001$), whereas no uptake-promoting functionality was noted for sera from mice immunized with CVD 1977 (Fig. 3B).

We assessed the protective efficacy of CVD 1966 or CVD 1977 by immunizing mice three times orally with 10^9 CFU of either vaccine. Mice were challenged with *S.* Newport WT (4×10^7 CFU i.p., i.e., $8 \times LD_{50}$; Fig. 3C), four weeks after the final immunization. Following infection, all of the PBS-immunized mice died, between days 3 and 6, as did mice that received CVD 1977 (vaccine efficacy of 0%). There was no difference in the median time to death between the two groups (5 days in both cases). Forty-seven percent of mice immunized orally with CVD 1966 survived challenge (vaccine efficacy of 47%, $p = 0.002$, Fisher's exact test). There was no significant delay in time to death for the CVD 1966-immunized mice that eventually succumbed to infection, compared to PBS-immunized mice (6 days).
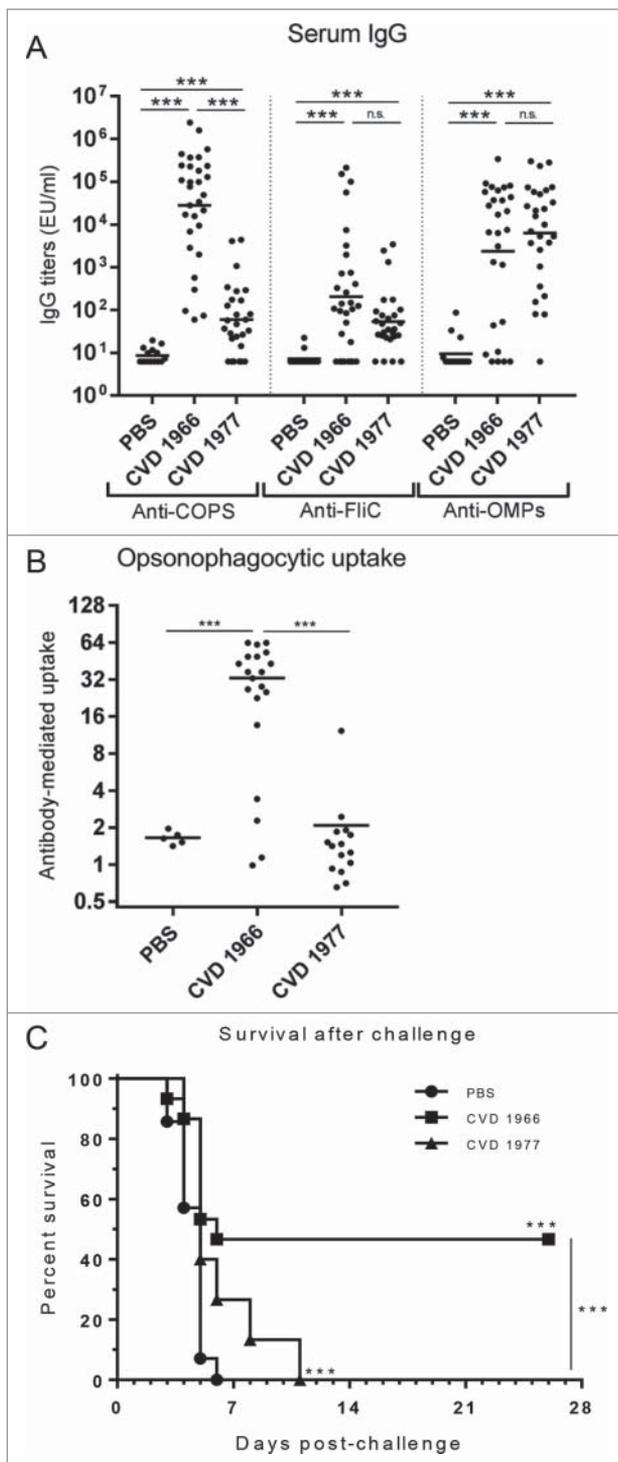
Figure 3. IgG titers, opsonophagocytic uptake and survival, following oral immunization with CVD 1966 or CVD 1977. Mice were immunized 3 times orally with PBS, 10^9 CFU of CVD 1966 or 10^9 CFU of CVD 1977 and bled 4 weeks after the last immunization. Panel A, serum IgG titers against COPS, FliC and outer membrane proteins (OMPs) were measured by ELISA and reported as EU/ml; bars represent geometric mean titers; *** $p < 0.001$, n.s. not significant (One-way ANOVA with Holm-Sidak multiple comparison test). Panel B, antibody-mediated uptake of *S.* Newport WT by J774 macrophages was assessed and normalized against the no-serum control; bars represent the mean; *** $p < 0.001$ (One-way ANOVA with Holm-Sidak multiple comparison test). Panel C, survival of mice after lethal challenge with 4×10^7 CFU of *S.* Newport WT; *** $p < 0.001$ (Log-rank test).

We did not observe a correlation between serum antibody levels against COPS, FliC or OMPs and protection ($p = 0.84$, 0.77 and 0.29, respectively). IgG titers against surface antigens (COPS and OMPs) were found to correlate with uptake of opsonized bacteria by J774 macrophages (Spearman coefficient r = 0.63 and 0.62, respectively; $p < 0.001$).

Shedding of orally-administered CVD 1966 and CVD 1977

A requirement of any live oral vaccine is that it is not excreted in stool for an extended duration to prevent its dissemination. We assessed the duration of the shedding of the kanamycin-resistant precursors to CVD 1966 and CVD 1977, *S.* Newport $\Delta guaBA$ $htrA::kan^R$ and *S.* Newport $\Delta guaBA$ ΔhrA $rfaL::kan^R$, respectively. We found that shedding of both strains was strongly reduced by day 3 post-immunization (less than 400 CFU per gram of stool), and below the detection limit after day 7 (Fig. 4). Additionally, no bacteria could be detected inside the liver and spleen of immunized mice euthanized at days 2 and 7 post-immunization.

Discussion

We have constructed a live-attenuated *Salmonella* Newport vaccine, CVD 1966 that was immunogenic and protected mice against lethal challenge. By combining the two independently attenuating mutations $\Delta guaBA$ and $\Delta htrA$, we were able to create a strain that had an i.p. LD_{50} greater than 100-fold higher than the parental wild-type strain. Unlike other NTS serovars such as *S.* Typhimurium and *S.* Enteritidis, *S.* Newport was not virulent in BALB/c mice when administered orally. Interestingly, none of the serogroup C_1 or C_2-C_3 serovars we tested initially (*S.* Muenchen [C_2-C_3], *S.* Infantis [C_1], *S.* Virchow [C_1] and *S.* Brazzaville [C_1]) were lethal when administered orally, at up to 10^9 CFU (data not shown). We therefore had to use intraperitoneal challenge to test our vaccine.

Mice immunized with three doses of CVD 1966 produced high serum IgG titers against *S.* Newport COPS and surface antigens such as flagellin and outer membrane proteins, regardless of the route of administration. It is worth noting, however, that titers against protein antigens were significantly lower when CVD 1966 was administered orally rather than intraperitoneally, while IgG responses against COPS were comparable for both routes. Additionally, there was higher variation among mice in response to oral versus i.p. immunization. Moreover, 22% (6/27) of orally-immunized mice did not mount an immune response to OMPs, and 30% (9/30) did not respond to flagellin (9/30), whereas complete seroconversion (15/15) was achieved for both antigens with intraperitoneal immunization. Although serum IgG responses against FliC and OMPs were higher in animals vaccinated i.p. versus perorally, vaccine efficacy was similar for both routes of immunization (53% for i.p. immunization and 47% for peroral immunization). Our results support those of Kinnear and Strugnell who found that despite a live-attenuated *S.* Typhimurium vaccine eliciting comparable protection following vaccination by two different routes (oral and intravenous), the mechanisms of protection differed.[27]
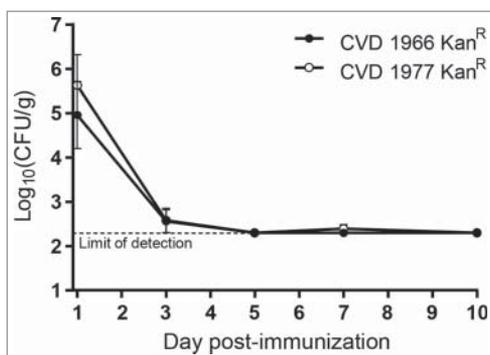
Figure 4. Shedding of Kan^R derivatives of CVD 1966 and CVD 1977. Mice were immunized orally with 10^9 CFU of CVD 1966 Kan^R or CVD 1977 Kan^R and stools collected every two days to measure shedding in feces. Results are reported as CFU per gram of stool.

The variable immunogenicity of the vaccine via the oral route may be a consequence of the requirement for transit through the stomach prior to reaching the small intestine, the site of inductive immune responses. The oral LD$_{50}$ of S. Newport is higher than 10^8 CFU, suggesting that bacteria may not persist long enough in the gastrointestinal tract to consistently induce a robust immune response. Indeed, we observed a limited duration of shedding of CVD 1966 (<5 days), which is greatly reduced relative to our prior findings for a highly immunogenic and protective live oral S. Typhimurium candidate vaccine that was shed in feces of mice for at least 14 days.[11] We also found that our vaccine could not be detected in the spleen and liver two or seven days after oral immunization. We observed that a small number of mice showed a balance defect following peroral immunization, which has previously been reported for S. Typhimurium, but fully recovered.[28] This response was not observed in mice vaccinated intraperitoneally.

While we did not observe a correlation between serum antibody levels against COPS, FliC or OMPs and protection in our mouse model, IgG titers against surface antigens (COPS and OMPs) were found to correlate with uptake of opsonized bacteria by J774 macrophages when mice were immunized orally. Such correlation was not observed when mice were immunized i.p., possibly due to the lower number of sera analyzed (15 sera versus 25 sera). It is worth noting, however, that in i.p.-immunized mice that eventually succumbed to the infection, there was a correlation between functionality of the antibodies and delayed time to death. This correlation was not found in orally-immunized mice, suggesting that the i.p. and p.o. routes of immunization may induce different types of immune responses. More importantly, our observations are in agreement with recent findings that antibody-mediated serum bactericidal activity, while not correlating with protection against infection, was associated with delayed disease onset and reduced disease severity in a human S. Typhi challenge model.[29,30]

In an effort to increase the immunogenicity and protective efficacy of our live-attenuated vaccine when delivered orally, we assessed the effect of removing the O-antigen chain from the LPS by deleting *rfaL*. Previous studies showed that removal of the O-antigen in S. Typhimurium can enhance the antibody response towards surface antigens and increase protective efficacy.[31,23,25] Surprisingly, ablation of OPS expression only moderately attenuated S. Newport, contrary to the avirulent

phenotype observed for other serovars such as S. Typhimurium in mice.[26,32] As anticipated, we found no anti-OPS antibody after oral immunization with CVD 1977, however, we also did not observe a concomitant increase in antibody responses towards OMPs or flagellin. Moreover, protection against lethal challenge was abrogated when *rfaL* was deleted from the vaccine, suggesting that OPS is an important protective antigen. Interestingly, antibodies elicited by CVD 1977 did not promote any opsonophagocytic uptake of WT S. Newport by mouse macrophages *in vitro*, suggesting the importance of anti-LPS antibodies for uptake in this assay. These observations are compatible with previous findings that anti-COPS antibodies induced by conjugate vaccines are protective in mice against S. Typhimurium or Enteritidis.[33–36] Additionally, it was reported recently that a live-attenuated S. Typhimurium vaccine expressing the S. Newport O-antigen cluster (*wzx-wbaR-wbaL-wbaQ-wzy-wbaW-wbaZ*) conferred full protection against challenge with S. Newport, compared to less than 42% vaccine efficacy with the unmodified live-attenuated strain, strengthening the importance of anti-LPS antibodies in providing protection.[37]

In conclusion, we constructed a live-attenuated vaccine candidate using mutations known to be attenuating in other serovars, and showed that it is immunogenic and protective in mice, either orally or i.p. Moreover, we showed for the first time that anti-OPS antibodies play a critical role for protection against S. Newport, as deletion of *rfaL* abrogated *in vitro* uptake and *in vivo* protection. We are currently evaluating the effect of an additional mutation to improve the tolerability of the vaccine. We will ensure that the optimized vaccine retains its immunogenicity while improving its safety profile. Future work will also examine the protective efficacy conferred by CVD 1966 in a gastroenteritis model, such as pigs, rabbits or rhesus macaques.[38–40] Ultimately, this vaccine will be combined with other candidate vaccines against *Salmonella* serogroups B, D and C$_1$ to provide broad protection against the most common serogroups of non-typhoidal *Salmonella*.

Materials and methods

Ethics statement

All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Maryland School of Medicine.

Bacterial strains, plasmids and culture conditions

Bacterial strains used in this study are listed in Table 2. Plasmids pKD13, pKD46 and pCP20 were used for mutagenesis of specific chromosomal loci.[41] All *Salmonella* strains were maintained on animal-product-free Hy-Soy medium (10 g/l soytone [Teknova # S9052], 5 g/l Hy-yest [Kerry BioScience # 5Z10313] and 5 g/l sodium chloride [AmericanBio # AB01915]). When needed, agar (AmericanBio # AB01185) was added at 15 g/l. Supplementation of medium with antibiotics (carbenicillin [Corning # 46-100-RG] or kanamycin [Sigma # K-4000], 50 μg/ml final) or guanine (Sigma-Aldrich # G11950, 0.02% [w/v] final) was added when necessary. Guanine auxotrophy was assessed using fully Chemically-Defined Media (CDM) as previously described.[11]

Table 2. Bacterial strains used in this study.

Strain	Source/characteristics	Reference
Chile 361	Clinical isolate of *Salmonella* Newport	44–46
SNE-guaBA	*S.* Newport Chile 361 Δ*guaBA*	This work
SNE-clpX	*S.* Newport Chile 361 Δ*clpX*	This work
SNE-htrA	*S.* Newport Chile 361 Δ*htrA*	This work
SNE-rfaL	*S.* Newport Chile 361 Δ*rfaL*	This work
SNE-OMPs	*S.* Newport Chile 361 Δ*fliC* Δ*fljBA rfaL::kan*^{R}	This work
CVD 1964	*S.* Newport Chile 361 Δ*guaBA* Δ*clpX* Δ*fliD* Δ*fljBA*	This work
CVD 1966	*S.* Newport Chile 361 Δ*guaBA* Δ*htrA*	This work
CVD 1966 Kan^R	*S.* Newport Chile 361 Δ*guaBA* Δ*htrA::kan*^{R}	This work
CVD 1977	*S.* Newport Chile 361 Δ*guaBA* Δ*htrA* Δ*rfaL*	This work
CVD 1977 Kan^R	*S.* Newport Chile 361 Δ*guaBA* Δ*htrA* Δ*rfaL::kan*^{R}	This work

Mutagenesis of S. Newport

We used lambda red-mediated homologous recombination to delete specific loci in the bacterial chromosome as described previously.[11,41] Primers used to construct the mutants in this study are described in Table S1.

Motility assay

Motility assays were performed as described previously.[11] Briefly, bacteria were stab inoculated on motility agar plates (10 g/l soytone, 5 g/l sodium chloride, 4 g/l agar) and the plates were incubated overnight (18 to 20 hours) at 37°C. The diameter of the zone of motility was recorded, and motility expressed as percent of the motility of the wild-type strain.

LPS gel electrophoresis

One-and-a-half milliliters of overnight culture grown at 37°C under agitation (250 rpm) were centrifuged and pellets were frozen at -80°C. Following a quick thaw at room temperature, pellets were resuspended in 100 μl of lysis buffer (400 mM Tris HCL pH 7, 10% [v/v] glycerol, 4% [v/v] beta-mercaptoethanol, 2% [w/v] SDS and 0.02% [w/v] bromophenol blue), and boiled for 10 minutes. Fifty micrograms of proteinase K (New England Biolabs # P8107) were then added and samples were incubated for one hour at 60°C. One hundred microliters of liquefied phenol (Sigma # P9346) were added to each sample, followed by vortexing and incubation for 15 minutes at 60°C. After centrifugation for 10 minutes at 16000 *x g*, 20 μl of the upper phase was mixed with 20 μl of lysis buffer, and half of this mixture was resolved on a 4–20% mini-Protean TGX gel (Bio-Rad # 456–1093). The gel was then stained using the Pro-Q Emerald 300 LPS gel stain kit (ThermoFisher # P20495) according to the manufacturer's instructions and LPS patterns were visualized at a wavelength of 300 nm with a Bio-Rad ChemiDoc MP imager.

S. Newport infection of mice

Bacteria were streaked from a frozen glycerol stock onto Hy-Soy agar (supplemented when necessary with 0.02% guanine) and grown overnight at 37°C (18-20 hours). Bacteria were then collected with a sterile loop and resuspended in sterile PBS. Viable counts were performed on Hy-Soy agar. To determine the most appropriate route of infection, 6-8-week-old BALB/c mice (3/group) (Charles River Laboratories) were infected either perorally (p.o.) by gavage or intraperitoneally (i.p.) with 10^8 CFU of *S.* Newport wild-type (WT) and monitored for 21 days. To determine the 50% lethal dose (LD$_{50}$), tenfold or five-fold dilutions (typically, 6 doses were tested for each strain) of the bacterial strains were then administered i. p. to groups of 5 mice. Following infection, mice were monitored daily for 28 days. Any mouse that lost 20% or more of its initial body weight or that showed signs of severe morbidity (e.g., shallow breathing, lack of motility) was euthanized and scored as having succumbed to infection. The 50% lethal dose (LD$_{50}$) of each strain was calculated using linear regression analysis.

Immunization and challenge of mice

Groups of 15 (for survival) or 10 (for organ burden) 6-8-week-old BALB/c mice were immunized i.p. with 10^8 CFU of CVD 1966, or by oral gavage with 10^9 CFU of CVD 1966 or CVD 1977, three times with three week intervals between each immunization. Blood samples were collected one day before each immunization, and two days before challenge. Four weeks after the last immunization, mice were challenged i.p. with 4×10^7 CFU of WT *S.* Newport to evaluate survival. Mice were monitored daily for 28 days for any sign of distress, as described for the LD$_{50}$ experiment. To quantitate bacterial burden, mice were challenged four weeks after the last immunization with a sub-lethal dose of WT *S.* Newport (10^6 CFU i.p.), euthanized two days later and their liver and spleen collected and weighed. Following homogenization of organs in PBS with a hard-tissue homogenizer (Omni Inc.), suspensions were diluted and plated onto Hy-Soy agar for viable counts. After incubation overnight at 37°C, bacterial burden in these organs were reported as CFU per gram of tissue.

Shedding of vaccine strains in feces

Three groups of five 6-8-week-old BALB/c mice were immunized by oral gavage with 10^9 CFU of *S.* Newport Δ*guaBA htrA::kan*, *S.* Newport Δ*guaBA* Δ*htrA rfaL::kan* or PBS. Bacteria used for infection were prepared as described above. At days 1, 3, 5, 7 and 10, fresh fecal pellets were collected from each individual mouse and suspended in PBS at 50 mg/ml. After vortexing, disaggregation of the fecal pellets and short centrifugation (5 seconds at 500 *x g*), 100 μl were spread onto Hy-Soy plates supplemented with kanamycin and guanine. Colony counts were determined after overnight incubation at 37°C, and shedding reported as CFU per gram of stool.

Purification of Salmonella Newport flagellin, COPS and OMPs

Flagellin and core O-polysaccharide (COPS) were purified from a specially engineered strain of *S.* Newport Chile 361 carrying deletions of genes *guaBA*, *clpX*, *fliD* and *fljBA*, named CVD 1964 and constructed by lambda red mutagenesis as described above, using primers listed in Table S1. These deletions are responsible for virulence attenuation, flagellin overexpression, absence of flagellar capping protein FliD and absence of Phase

2 flagellin FljB, respectively. Monomeric phase 1 flagellin FliC and COPS were then purified from fermentation cultures as described previously.[36,42]

Outer membrane proteins (OMPs) were purified using a protocol adapted from the saline extraction method described by Logan and Trust.[43] Briefly, OMPs were purified from an *S.* Newport strain genetically engineered to lack Phase 1 ($\Delta fliC$) and 2 ($\Delta fljB$) flagellin and to lack O-antigen ($\Delta rfaL$). *S.* Newport $\Delta fliC \Delta fljBA \ rfaL::kan^R$ was grown overnight at 37°C on Hy-Soy plates supplemented with 50 μg/ml kanamycin and resuspended in sterile PBS. Bacteria were then washed twice with PBS, resuspended in 2.5% (w/v) NaCl and incubated for one hour at 56°C. After centrifugation for 15 minutes at 14,000 $x \ g$, cell-free supernatants were adjusted to 0.01% (v/v) Tween 20 and dialyzed against 0.01% (v/v) Tween 20 in water for 6 hours at 4°C with one change of buffer. The crude OMPs mixture was then analyzed by 4–20% SDS-PAGE and Coomassie staining to assess purity (Figure S1) and concentration was estimated by spectrophotometry.

Measurement of serum antibodies

Blood was collected from the retro-orbital plexus, processed using Z-gel tubes (Sarstedt # 41.1500.005), and serum stored at -80°C until use. Levels of serum IgG antibodies against flagellin (FliC), core-O-polysaccharide (COPS) and OMPs were determined by enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well, medium-binding ELISA plates (Greiner # 655001) were coated overnight at 4°C with flagellin (5 μg/ml in 0.05 M Na_2CO_3, pH 9.6), COPS (5 μg/ml in PBS pH 7.4) or OMPs (15 μg/ml in PBS pH 7.4). Plates were washed twice with PBS pH 7.4 containing 0.05% (v/v) Tween 20 (PBST), blocked for 1 hour at 37°C with 10% (w/v) nonfat dried milk in PBST (PBST-M), and washed twice with PBST. Serial dilutions in PBST-M of the sera to be tested were then added (in duplicate), plates were incubated for 1 hour at 37°C and washed four times with PBST. Peroxidase-labeled goat anti-mouse antibody (KPL # 474–1802) diluted 1:2000 in PBST-M was then added, and plates were incubated for 1 hour at 37°C. Following six washes with PBST, tetramethylbenzidine substrate solution (KPL # 5120-0047) was added and plates were incubated for 15 minutes in the dark at room temperature, before the reaction was stopped by adding an equal volume of 0.5 M phosphoric acid. Titers were calculated by interpolation on a standard curve as the inverse of the serum dilution that produces an absorbance value of 0.2 above the blank and reported as ELISA units per ml. Seroconversion was defined as a four-fold increase in titer after immunization, compared to pre-immunization titers.

Opsonophagocytic uptake by J774 macrophages

This assay was performed as described previously.[11] Briefly, bacteria grown to an OD_{600} of 0.25 were diluted 1:20 in PBS and 90 μl of this suspension were added to 10 μl of heat-inactivated (20 minutes at 56°C) mouse serum. After incubation for 20 minutes at room temperature, 10 μl of the serum-bacteria mixture were added to semi confluent monolayers of J774 cells in a 24-well plate. After 45 minutes incubation at 37°C, 5% CO_2, cells were washed once with PBS, and Dulbecco's Modified Eagle Medium (Corning # 10-013-CV) supplemented with 100 μg/ml gentamicin (Gibco # 15750-060) was added to each well. Following incubation for 1 hour at 37°C, 5% CO_2, cells were washed 3 times with PBS, and lysed with 500 μl per well of 0.5% (wt/vol) Triton X-100 (Sigma # T8787). Neat and 3-fold dilutions of the cell lysate were then plated on agar plates for viable bacterial counts.

Statistical methods

Data were analyzed and plotted using GraphPad Prism software. For two-tailed Student's *t*-test and Fisher's exact test with alpha = 0.05, a p-value below 0.05 was considered significant, unless specifically stated. For data that was not normally distributed, two-tailed Mann-Whitney rank-sum test or one-way ANOVA were used instead. When appropriate, Holm-Sidak's multiple comparisons test was used to account for multiple comparisons.

Disclosures

S.M. Tennant and R. Simon are holders of the following patents that describe development of NTS vaccines: US patent 9,050,283, "Broad spectrum vaccine against non-typhoidal *Salmonella*"; US patent 9,011,871, "Broad spectrum vaccine against typhoidal and non-typhoidal *Salmonella* disease"; and European Patent Number 2387417, "Broad spectrum vaccine against non-typhoidal *Salmonella*".

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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