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Refined Live Attenuated *Salmonella enterica* Serovar Typhimurium and Enteritidis Vaccines Mediate Homologous and Heterologous Serogroup Protection in Mice

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Invasive nontyphoidal *Salmonella* (NTS) infections constitute a major health problem among infants and toddlers in sub-Saharan Africa; these infections also occur in infants and the elderly in developed countries. We genetically engineered a *Salmonella enterica* serovar Typhimurium strain of multilocus sequence type 313, the predominant genotype circulating in sub-Saharan Africa. We evaluated the capacities of *S.* Typhimurium and *Salmonella enterica* serovar Enteritidis Δ*guaBA* Δ*clpX* live oral vaccines to protect mice against a highly lethal challenge dose of the homologous serovar and determined protection against other group B and D serovars circulating in sub-Saharan Africa. The vaccines *S.* Typhimurium CVD 1931 and *S.* Enteritidis CVD 1944 were immunogenic and protected BALB/c mice against 10,000 50% lethal doses (LD_{50}) of *S.* Typhimurium or *S.* Enteritidis, respectively. *S.* Typhimurium CVD 1931 protected mice against the group B serovar *Salmonella enterica* serovar Stanleyville (91% vaccine efficacy), and *S.* Enteritidis CVD 1944 protected mice against the group D serovar *Salmonella enterica* serovar Dublin (85% vaccine efficacy). High rates of survival were observed when mice were infected 12 weeks postimmunization, indicating that the vaccines elicited long-lived protective immunity. Whereas CVD 1931 did not protect against *S.* Enteritidis R11, CVD 1944 did mediate protection against *S.* Typhimurium D65 (81% efficacy). These findings suggest that a bivalent (*S.* Typhimurium and *S.* Enteritidis) vaccine would provide broad protection against the majority of invasive NTS infections in sub-Saharan Africa.

Non-typhoidal *Salmonella* (NTS) is a leading cause of bacterial bloodstream infections in febrile children and immunocompromised individuals in sub-Saharan Africa and has been associated with a high case fatality rate of 20 to 25% (1). Although severe malarial anemia and human immunodeficiency virus (HIV) are important risk factors for invasive NTS infection, the disease is also common in low-HIV-prevalence areas (1–4).

There are >2,500 *Salmonella* serovars that can be differentiated on the basis of the O polysaccharide (OPS) antigens of their lipopolysaccharide (LPS) and their H flagellum antigens, using the Kauffman-White typing scheme (5). For example, *Salmonella enterica* serovar Typhimurium has O antigens 1, 4, 12, and occasionally 5. Epitope 12 is formed by trisaccharide repeats of mannose, rhamnose, and galactose; glucosylation of the galactose residue forms epitope 1. An abequose linked to each mannose defines it as a serovar within group B and constitutes the immunodominant O4 epitope; epitope 5 results from a phage conversion that introduces an O-acetyl moiety on the abequose. *S.* Typhimurium also has separate H antigens, i (phase 1) and 1 and 2 (phase 2), expressed alternatively via a process called phase variation. In contrast, *Salmonella enterica* serovar Enteritidis has O antigen epitope 9, which identifies it as a member of group D. Epitope 9 is formed by a tyvelose residue that is linked to the mannose of the same trisaccharide OPS backbone as group B strains, which is also glucosylated at galactose. *S.* Enteritidis produces only phase 1 H antigens g and m (i.e., it does not make phase 2 flagella).

In sub-Saharan Africa, *S.* Typhimurium and *S.* Enteritidis account for 75 to 90% of all the NTS strains isolated from blood and other normally sterile compartments (6–14). In blood culture surveillance studies conducted by our group in febrile pediatric patients in Bamako, Mali, since 2002, we found that *S.* Typhimurium and its variants were the NTS bacteria most commonly isolated from blood (69%), followed by *Salmonella enterica* serovar Dublin (group D, 11%), *S.* Enteritidis (10%), and *Salmonella enterica* serovar Stanleyville (group B, 8%) (15, 16). The remaining 2% of NTS strains belonged to other serovars. However, other African sites have reported the isolation of rare serovars, such as the group C1 serovars *Salmonella enterica* serovar Isangi in South Africa (17) and *Salmonella enterica* serovar Concord in Ethiopia (18).

A novel genotype of *S.* Typhimurium, sequence type 313 (ST313), was identified in Malawi and Kenya by multilocus sequence typing (MLST) and associated with invasive disease (19, 20). In contrast, *S.* Typhimurium strains associated with gastroenteritis and commonly found throughout the world are ST19. There are multiple sequence types of *S.* Typhimurium circulating in Africa that can cause invasive disease, though ST313 seems to be the most common. In comparison to *S.* Typhimurium ST19 strains, the prototypic ST313 strain D23580 exhibits many pseudogenes and gene deletions (19). We and others have recently

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TABLE 1 Bacterial strains used in this study

O group	O antigens	H antigens	Serovar	Strain	Source and characteristics	Reference
B	1, 4, [5], 12[a]	i; 1, 2	Typhimurium	D65	Clinical isolate from blood culture, Mali; ST313; antibiotic sensitive[b]	15
				CVD 1930	*S.* Typhimurium D65 Δ*guaBA*	This work
				CVD 1931	*S.* Typhimurium D65 Δ*guaBA* Δ*clpX*	This work
	1, 4, [5], 12, [27]	z4, z23; [1, 2]	Stanleyville	J65	Clinical isolate from blood culture, Mali	15
	1, 4, [5], 12	e, h; 1, 5	Reading	26A	Clinical isolate from blood culture, Chile	CVD collection
	1, 4, [5], 12	b; 1, 2	Paratyphi B var. Java	S78	Clinical isolate from blood culture, Mali	15
D	1, 9, 12	g, m; −[c]	Enteritidis	R11	Clinical isolate from blood culture, Mali; antibiotic sensitive[b]	15
				CVD 1940	*S.* Enteritidis R11 Δ*guaBA*	26
				CVD 1944	*S.* Enteritidis R11 Δ*guaBA* Δ*clpX*	26
	1, 9, 12, [Vi]	g, p; −	Dublin	R17	Clinical isolate from blood culture, Mali	15
				P10	Clinical isolate from blood culture, Mali	15
	1, 9, 12	l, v; 1, 5	Panama	107	Clinical isolate from blood culture, Chile	CVD collection

[a] The brackets indicate that the O or H factor may be present or absent.
[b] Sensitive to ampicillin, ceftriaxone, chloramphenicol, gentamicin, ciprofloxacin, and trimethoprim-sulfamethoxazole.
[c] The minus symbol indicates that phase 2 H antigens are absent.

shown that *S.* Typhimurium ST313 is also phenotypically different from ST19 (21–24). Despite observable differences between *S.* Typhimurium ST19 and ST313 isolates, it is also feasible that the morbidity and mortality associated with these strains in sub-Saharan Africa is due to other factors, such as comorbidities (e.g., malnutrition, HIV, and malaria), antibiotic resistance, or genetic predisposition (25).

We previously developed live oral *S.* Typhimurium and *S.* Enteritidis vaccines with deletion mutations in *guaBA* and *clpP*. Mutation of *guaBA* alone increased the oral 50% lethal dose (LD_{50}) in BALB/c mice by ~5 log units (26). The *clpPX* genes encode a protease that normally degrades the master flagellum regulator FlhDC (27, 28). In the absence of ClpPX, FlhDC accumulates, resulting in increased FliC production. Deletion of *clpP*, an independently attenuating mutation, resulted in increased expression of flagella (26). Our prototypic *S.* Typhimurium and *S.* Enteritidis vaccine strains, CVD 1921 and CVD 1941, with mutations in *guaBA* and *clpP*, were immunogenic in BALB/c mice and protected the mice against homologous oral challenge with 100 LD_{50} of wild-type *S.* Typhimurium or *S.* Enteritidis, respectively (26). Importantly, the *S.* Typhimurium vaccine CVD 1921 was well tolerated by orally immunized, simian immunodeficiency virus (SIV)-infected rhesus macaques (29). Murine and macaque antibodies induced by these vaccine strains were functional *in vitro*, mediating bactericidal and opsonophagocytic activities (26, 29). Robust enhancement of opsonophagocytic uptake was noted for other invasive serovars of homologous serogroups, but the uptake was variable and generally less robust for strains from heterologous serogroups (26). These data suggest that serogroup-specific cross-protection may be possible and that NTS live attenuated vaccines may offer partial protection against heterologous serogroups. A live *Salmonella enterica* serovar Paratyphi A vaccine, CVD 1902, with deletions in *guaBA* and *clpX*, was tested in a phase 1 clinical trial and found to be safe in volunteers immunized orally with up to 10^{10} CFU (K. K. Kotloff and D. A. Shirley, personal communication).

Here, we describe refinements of the prototypic live attenuated NTS vaccines and direct assessment of cross-protection mediated by these advanced vaccine candidates against homologous and heterologous serogroups.

MATERIALS AND METHODS

Bacterial strains, medium, and chemicals. The bacterial strains used in this study (shown in Table 1) were grown in HS bacteriological medium (5 g sodium chloride, 10 g soytone [Teknova, Hollister, CA], 5 g Hy yest [Sigma-Aldrich, St. Louis, MO] in 1 liter distilled water) at 37°C. All *guaBA* mutants were grown on media containing 0.005% (wt/vol) guanine. When required, antibiotics were used at a final concentration of 50 μg/ml carbenicillin, 50 μg/ml kanamycin, or 20 μg/ml chloramphenicol. Chemically defined medium was prepared as described previously (26). NTS serovars were verified by agglutination of bacteria with O-grouping and H-typing antisera (Denka Seiken Co. Ltd., Japan). Phase switching was performed by preparing swarm agar (nutrient broth containing 0.5% agar) and dropping H:i or H:2 antiserum on the surface, followed by stab inoculation of the center of the medium. Following incubation at 37°C for 20 h, the bacteria were agglutinated with H-typing antiserum.

DNA methods. Plasmid extraction and gel purification of DNA fragments were performed using Wizard (Promega, Madison, WI, USA) and QIAquick Gel Extraction (Qiagen, Valencia, CA, USA) kits, respectively, as directed by the manufacturer. Restriction enzymes were purchased from New England BioLabs (Ipswich, MA, USA). PCR amplifications were routinely performed with 1 to 2.5 U *Taq* DNA polymerase (Genscript, Piscataway, NJ, USA) and 1× PCR buffer containing 1.5 mM $MgCl_2$, 200 μM each deoxynucleoside triphosphate (dNTP), and 1 μM each primer in a reaction volume of 20 to 50 μl in an Eppendorf Mastercycler. For PCRs using long primers (>25 bp), the amount of $MgCl_2$ was increased as necessary. When error-free and/or blunt-end PCR products were required, Vent DNA polymerase (New England BioLabs) was used according to the manufacturer's instructions.

Construction of attenuated *Salmonella* strains. Deletion of *guaBA* and *clpX* in *S.* Typhimurium D65 was achieved by Lambda Red-mediated mutagenesis as described previously (26). Plasmids pKD46, pKD13, and pCP20 were used for chromosomal deletions (30). Plasmid pCR-Blunt II-TOPO (Invitrogen, Carlsbad, CA) was used to clone blunt-ended PCR products. The deletions were verified genotypically by PCR using primers external to the deletion and by sequencing at least 500 bp both upstream and downstream of the deletion.

Challenge experiments. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Maryland School of Medicine. BALB/c mice were acclimated for 7 days after arrival before starting the experiments. For LD_{50} determinations, both attenuated and wild-type *Salmonella* strains were grown by incubation at 37°C in HS medium for 20 h without shaking. Bacteria were pelleted by centrifugation and resuspended in phosphate-buffered saline (PBS) at the

TABLE 2 Vaccine efficacy of live attenuated *S.* Typhimurium CVD 1931 (*S.* Typhimurium D65 Δ*guaBA* Δ*clpX*) and CVD 1944 (*S.* Enteritidis R11 Δ*guaBA* Δ*clpX*)[a]

Immunization	Challenge	Challenge dose (LD_{50})	Mortality rate	Vaccine efficacy (%)	*P* value[b]
PBS	*S.* Typhimurium D65	~10,000	12/12		
CVD 1931			0/12	100	<0.001
PBS	*S.* Enteritidis R11	~10,000	12/12		
CVD 1944			2/12	83	<0.001

[a] Mice were immunized orally with 10^9 CFU three times, with 1 month between immunizations, and orally challenged 1 month later with 2.6×10^8 CFU of *S.* Typhimurium D65 and 1.5×10^8 CFU of *S.* Enteritidis R11. The mice were monitored daily for 29 days.
[b] Fisher's exact test (two tailed).

appropriate concentration. Tenfold dilutions (generally 10^3 to 10^8 CFU) of wild-type and attenuated NTS strains were administered to five 7-week-old female BALB/c mice (Charles River Laboratories, Wilmington, MA, USA). The mice were infected orally with 200 μl of bacterial suspension using a 1.5-in curved gavage needle with a 2.25-mm ball (Braintree Scientific, Braintree, MA, USA) or intraperitoneally (i.p.) using a 25-gauge needle. The exact number of organisms administered was determined by viable counts. Mice were weighed and monitored daily after infection for 14 (i.p.) or 28 (oral) days. Any mouse that lost >20% of its body weight (compared to its weight at the time of challenge) or that showed signs of extreme morbidity (e.g., shallow breathing or hunched posture) was euthanized and scored as a death. The 50% lethal dose of each strain was calculated by linear regression analysis. For challenge with additional serovars, mice were infected with one of the following strains: 3.2×10^7 CFU *Salmonella enterica* serovar Reading 26A, 6×10^8 CFU *S.* Stanleyville J65, 9.2×10^8 CFU *Salmonella enterica* serovar Java S78, 4.6×10^7 CFU *Salmonella enterica* serovar Panama Chile 107, 3.6×10^8 CFU *S.* Dublin Mali R17, or 3.6×10^8 CFU *S.* Dublin Mali P10.

Immunization, serological analysis, and protection against challenge. Female BALB/c mice 6 to 8 weeks of age were immunized via oral gavage with 200 μl PBS containing 10^9 CFU of live attenuated *Salmonella* or PBS alone. The immunization schedule consisted of three spaced doses administered on days 0, 28, and 56. Serum was obtained on days −1, 27, 55, and 83, and anti-LPS and anti-FliC serum IgG titers were determined as described previously (26). The homologous LPS or flagella (i.e., *S.* Typhimurium LPS and FliC for mice immunized with CVD 1931 and *S.* Enteritidis LPS and FliC for mice immunized with CVD 1944) were used as coating antigens in the enzyme-linked immunosorbent assays (ELISAs), as previously described (31). On day 83, the mice were challenged orally or i.p. with wild-type *Salmonella*, as described above.

Statistical methods. Data were analyzed using Fisher's exact test. A *P* value of ≤0.05 (two tail) was considered significant.

RESULTS

Construction of refined live attenuated invasive NTS vaccines. The first-generation *S.* Typhimurium vaccine, CVD 1921 (Δ*guaBA* Δ*clpP*), was made using *S.* Typhimurium I77 as the parental strain, which is from ST19, a minor endemic genotype in sub-Saharan Africa (26). Here, we describe attenuation of an *S.* Typhimurium strain of the ST313 genotype, the predominant circulating sequence type in the sub-Saharan region, which is responsible for a high burden of disease (19, 20). Our second refinement was in the secondary attenuating mutation within *clpPX*. Instead of deleting *clpP*, we deleted the second gene in the operon, *clpX*, as this combination of mutations resulted in a live attenuated *S.* Paratyphi A vaccine that was well tolerated in humans. We constructed the live oral *S.* Typhimurium vaccine strain CVD 1931 (*S.* Typhimurium D65 Δ*guaBA* Δ*clpX*). We confirmed the phenotypes of CVD 1931 and the previously constructed *S.* Enteritidis vaccine strain CVD 1944 (Δ*guaBA* Δ*clpX*) (26) and showed that both were unable to grow on minimal medium lacking guanine and were more motile than the parental strains (reference 26 and data not shown).

Determination of the LD_{50} for invasive *Salmonella* serovars. The oral LD_{50} of *S.* Typhimurium ST313 strain D65 was found to be 2×10^4 CFU. This is the same oral LD_{50} that was determined for the ST19 strain *S.* Typhimurium I77 (26). In preparation for cross-protection studies, we assessed the LD_{50} of several additional *Salmonella* serovars isolated from blood or other normally sterile sites. Mice (3/group) were infected orally with $>10^7$ CFU of one of the following group B or D *Salmonella* strains: *S.* Reading Chile 26A (group B), *S.* Stanleyville Mali J65 (group B), *S.* Java Mali S78 (group B), *S.* Panama Chile 107 (group D), or *S.* Dublin Mali R17 or Mali P10 (group D). Only *S.* Dublin Mali R17 and *S.* Stanleyville Mali J65 showed lethality—100% and 67%, respectively. We then performed LD_{50} experiments by infecting BALB/c mice with six 10-fold dilutions of bacteria. Linear regression was used to calculate the LD_{50}.The oral LD_{50} for *S.* Dublin R17 was 9.1×10^4 CFU. The oral LD_{50} for *S.* Stanleyville J65 was $>10^9$ CFU. For the last strain, since a dose of ~100 LD_{50} would require ~10^{11} CFU and cannot be practically given, we elected to pursue i.p. infection; the i.p. LD_{50} of *S.* Stanleyville J65 was experimentally determined to be $\sim 1.4 \times 10^5$ CFU. Intraperitoneal LD_{50} were not determined for the other strains.

***S.* Typhimurium CVD 1931 and *S.* Enteritidis CVD 1944 protect against a highly lethal homologous challenge.** We immunized BALB/c mice orally three times at 28-day intervals with 10^9 CFU of CVD 1931 or CVD 1944 and challenged them orally with ~10,000 LD_{50} of *S.* Typhimurium D65 or *S.* Enteritidis R11 1 month after the last immunization. BALB/c mice, which are NRamp negative and cannot control disseminated *Salmonella*, were chosen as an infection model because they can be vaccinated orally, and a lethal infection is induced when they are challenged with wild-type organisms by the same route, thus providing a model to assess protective efficacy (32). Likewise, oral immunization and infection routes are relevant for humans. All control mice that received PBS and were challenged with virulent *S.* Typhimurium D65 succumbed to the infection, whereas none of the CVD 1931-immunized mice died (Table 2 and Fig. 1). For mice challenged with *S.* Enteritidis, we observed 83% vaccine efficacy against a highly fatal infection that killed all the control mice (Table 2 and Fig. 1). High anti-LPS and anti-FliC serum IgG titers were elicited after three immunizations (Fig. 2). CVD 1931 attained 100% anti-LPS seroconversion after only two doses, whereas CVD 1944 achieved 92% anti-LPS seroconversion after three immunizations.

Abilities of live oral vaccines CVD 1931 and CVD 1944 to offer cross-serovar protection against other group B and D *Salmonella* serovars circulating in sub-Saharan Africa. We investi-

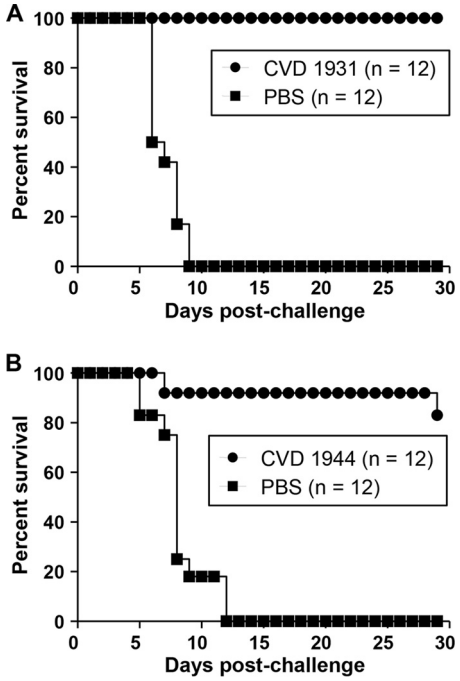
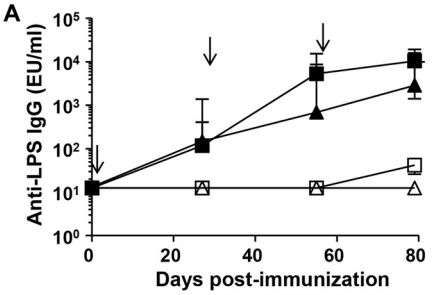
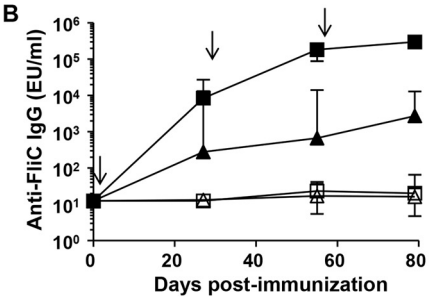


FIG 1 Percent survival of mice immunized with live attenuated NTS strains and challenged with the homologous serovar. The mice were immunized orally with 10^9 CFU of CVD 1931 (*S.* Typhimurium D65 Δ*guaBA* Δ*clpX*), CVD 1944 (*S.* Enteritidis R11 Δ*guaBA* Δ*clpX*), or PBS three times, with 1 month between immunizations. One month after the last immunization, they were orally challenged with 10,000 LD_{50} of *S.* Typhimurium D65 (A) or *S.* Enteritidis R11 (B).



	% seroconversion			
	Pre	Dose 1	Dose 2	Dose 3
CVD 1931	0	58	100	100
CVD 1944	0	58	67	92



	% seroconversion			
	Pre	Dose 1	Dose 2	Dose 3
CVD 1931	0	83	100	100
CVD 1944	0	50	58	92

FIG 2 Seroconversion and anti-LPS and anti-FliC IgG geometric mean titers (GMTs) elicited by live oral vaccines CVD 1931 and CVD 1944. Mice ($n = 12$) were immunized orally with 10^9 CFU in a 200-μl volume three times (on days 0, 28, and 56 [indicated by arrows]), with 1 month between immunizations. Anti-LPS (A) and anti-FliC (B) serum IgG titers were measured by ELISA pre- and postimmunization (squares, CVD 1931; triangles, CVD 1944; solid symbols, immunized; open symbols, PBS). Titers are expressed as GMTs ± standard errors of the mean (SEM). The percent seroconversion (4-fold increase in titer versus preimmune) is shown. EU, ELISA units.

gated *in vivo* whether our live oral vaccines would protect against *Salmonella* strains representative of heterologous group B and D serovars. For this, mice were immunized orally, on three occasions 1 month apart, with *S.* Typhimurium vaccine CVD 1931 and challenged i.p. with 3 LD_{50} of *S.* Stanleyville J65. This challenge dose was selected to optimize the kinetics of infection, as we found that higher i.p. challenge doses resulted in ~100% mortality after 24 h, presumably due to overwhelming endotoxemia. CVD 1931 exhibited 91% vaccine efficacy in preventing death from an *S.* Stanleyville J65 i.p. challenge that led to the deaths of all the PBS control mice (Fig. 3A).

We also determined the ability of the *S.* Enteritidis vaccine CVD 1944 to protect mice against the group D serovar *S.* Dublin (H:g, p). Mice were similarly orally immunized and challenged with 800 LD_{50} of *S.* Dublin R17. As shown in Fig. 3B, CVD 1944 manifested 85% vaccine efficacy in the face of a virulent *S.* Dublin challenge that caused 100% mortality in the PBS control mice. These data clearly show that both our *S.* Typhimurium CVD 1931 and *S.* Enteritidis CVD 1944 vaccines can protect against other group B and D serovars (Table 3).

Live oral vaccines CVD 1931 and CVD 1944 mediate cross-protection. First, we tested the ability of the live attenuated *S.* Typhimurium vaccine CVD 1931 (group B) to mediate protection against the heterologous group D serovar *S.* Enteritidis. Mice were immunized orally with each of the vaccine strains three times, with 1 month between immunizations, and challenged orally with 50 LD_{50} of *S.* Enteritidis R11 or with 200 LD_{50} of *S.* Typhimurium D65. When CVD 1931-immunized mice were challenged with *S.* Enteritidis R11, we observed a vaccine efficacy of 50.7% (36% mortality for vaccinated mice and 73% mortality for the PBS control [Fig. 3C]). In contrast, 81.3% vaccine efficacy was observed for CVD 1944 against challenge with virulent *S.* Typhimurium D65 (15% of immunized mice and 80% of PBS control mice succumbed [Fig. 3D]). These data indicate that while the *S.* Typhimurium live vaccine CVD 1931 was not effective in cross-protecting against a lethal *S.* Enteritidis infection (Table 4), the *S.* Enteritidis vaccine CVD 1944 (group D) afforded cross-protection against the group B serovar *S.* Typhimurium (Table 4).

Live oral vaccines CVD 1931 and CVD 1944 elicit long-lasting protective immunity. To confirm that the live NTS vaccines CVD 1931 and CVD 1944 can elicit long-lasting immunity, we repeated the immunization experiment but challenged the mice 12 weeks after the last immunization. BALB/c mice were immunized orally with *S.* Typhimurium CVD 1931 and challenged orally with 5,000 LD_{50} of *S.* Typhimurium D65 or i.p. with 6 LD_{50} of *S.* Stanleyville J65. CVD 1931 exhibited vaccine efficacies of 60% and 90% against *S.* Typhimurium and *S.* Stanleyville, respec-

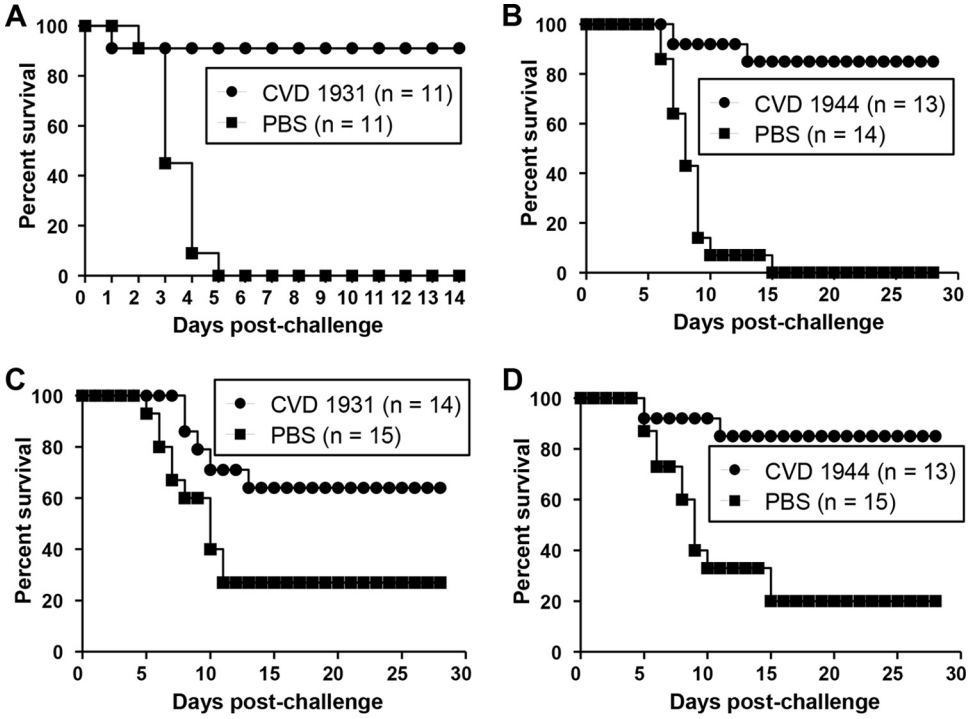


FIG 3 Percent survival of mice immunized with live attenuated NTS strains and challenged with other group B and D serovars. Mice were immunized orally with 10^9 CFU of CVD 1931 (*S.* Typhimurium D65 Δ*guaBA* Δ*clpX*), CVD 1944 (*S.* Enteritidis R11 Δ*guaBA* Δ*clpX*), or PBS three times, with 1 month between immunizations, and challenged 1 month after the last immunization. (A) CVD 1931-immunized mice were intraperitoneally challenged with 3 LD_{50} of *S.* Stanleyville J65. (B) CVD 1944-immunized mice were orally challenged with 800 LD_{50} of *S.* Dublin R17. (C) CVD 1931-immunized mice were orally challenged with 50 LD_{50} of *S.* Enteritidis R11. (D) CVD 1944-immunized mice were orally challenged with 200 LD_{50} of *S.* Typhimurium D65.

tively (Table 5 and Fig. 4). Likewise, BALB/c mice were immunized orally with *S.* Enteritidis CVD 1944 and challenged orally with 5,000 LD_{50} of *S.* Enteritidis R11 or 1,000 LD_{50} of *S.* Dublin R17. CVD 1944 exhibited vaccine efficacies of 78% and 56% against *S.* Enteritidis and *S.* Dublin, respectively (Table 5 and Fig. 4). As shown in Fig. 5, anti-LPS serum IgG titers remained elevated after the last immunization, with 95 to 100% seroconversion.

DISCUSSION

Two subunit parenteral vaccines are in development to prevent invasive NTS infections among young children in sub-Saharan Africa, including a conjugate consisting of core plus O polysaccharide linked to phase 1 flagellin subunits of the homologous serovar and another consisting of outer membrane particles, called Generalized Module for Membrane Antigens (GMMA) (Gendrivax [http://www.gendrivax.org]) (31, 33). Administered parenterally, these vaccines are expected to protect by eliciting functional (likely IgG) antibodies that intercept the invading bacteria during primary bacteremia. Here, we propose an alternative approach to prevent invasive NTS disease in Africa with live oral vaccines that in an optimistic view offer theoretical advantages over the parenteral vaccines. First, oral vaccines elicit secretory IgA mucosal antibodies, thereby providing barrier protection against mucosal invasion, in addition to stimulating serum antibodies and a panoply of cell-mediated immune mechanisms. Second, consequent to mucosal immune responses, the oral vaccines may confer protection against NTS gastroenteritis. It is not known whether *Salmonella* parenteral vaccines would protect against mucosal colonization. However, IgG induced by parenteral vaccination with *Streptococcus pneumoniae* capsular polysaccharides protects against invasive infections (34), and it is conceivable that parenteral *Salmonella* vaccines could have a similar effect. Anti-*Salmonella* IgG antibodies induced in rabbits by heat-killed vaccines have been

TABLE 3 Vaccine efficacies of live attenuated *S.* Typhimurium CVD 1931 (*S.* Typhimurium D65 Δ*guaBA* Δ*clpX*) and CVD 1944 (*S.* Enteritidis R11 Δ*guaBA* Δ*clpX*) against other group B or D serovars[a]

Immunization	Challenge	Challenge dose (LD_{50})	Mortality rate	Vaccine efficacy (%)	*P* value[b]
PBS	*S.* Stanleyville J65	~3	11/11		
CVD 1931			1/11	91	<0.001
PBS	*S.* Dublin R17	~800	14/14		
CVD 1944			2/13	85	<0.001

[a] Mice were immunized orally with 10^9 CFU three times, with 1 month between immunizations, and challenged 1 month later with 4.5×10^5 CFU of *S.* Stanleyville J65 (i.p.) or 7.9×10^7 CFU of *S.* Dublin R17 (orally). The mice were monitored daily for 14 (*S.* Stanleyville infection) or 28 (*S.* Dublin infection) days.
[b] Fisher's exact test (two tailed).

TABLE 4 Vaccine efficacies of live attenuated *S.* Typhimurium CVD 1931 (*S.* Typhimurium D65 Δ*guaBA* Δ*clpX*) and CVD 1944 (*S.* Enteritidis R11 Δ*guaBA* Δ*clpX*) against heterologous serogroups[a]

Immunization	Challenge	Challenge dose (LD_{50})	Mortality rate	Vaccine efficacy (%)	*P* value[b]
PBS	*S.* Enteritidis R11	~50	11/15		
CVD 1931			5/14	51	0.07
PBS	*S.* Typhimurium D65	~200	12/15		
CVD 1944			2/13	81	<0.001

[a] Mice were immunized orally with 10^9 CFU three times, with 1 month between immunizations, and orally challenged 1 month later with 1.2×10^6 CFU of *S.* Enteritidis R11 or 3.9×10^6 CFU of *S.* Typhimurium D65. The mice were monitored daily for 28 days.
[b] Fisher's exact test (two tailed).

shown to accumulate in the gastrointestinal lumen by passive transudation through the mucosal epithelium (35) and to retain functional bactericidal capacity. In addition to functionally active systemic IgG, live vaccines would have the advantage of inducing strong mucosal IgA. Other advantages include their lower cost and easier implementation. Finally, as demonstrated here, the live vaccines stimulate impressive cross-protection against other serovars within the same group and, for the *S.* Enteritidis vaccine, serovars from another serogroup.

We have previously reported the development of prototypic live attenuated NTS vaccines harboring deletions in *guaBA* and *clpP*. Here, we have described the development of live NTS vaccines with deletions in *guaBA* and *clpX*. Furthermore, the *S.* Typhimurium vaccine is derived from a clinically relevant genotype that has been associated with invasive disease and is prevalent in sub-Saharan Africa. There are multiple genetic and phenotypic differences (22, 23) between ST19 and ST313 strains, and a vaccine derived from a genotype that is circulating in the target population is likely to be most effective by affording cross-protection via shared antigens. However, one of the main reasons for developing an ST313-based vaccine is that these strains produce less enteropathogenicity than ST19 strains, which we anticipate will result in less inflammation and possibly reduced shedding (22). One of the barriers for developing live attenuated *S.* Typhimurium vaccines to date has been their propensity to cause bacterial shedding for long periods in human volunteer studies (36).

The immunogenic capacities of our NTS vaccines *S.* Typhimurium CVD 1931 (*S.* Typhimurium D65 Δ*guaBA* Δ*clpX*) and *S.* Enteritidis CVD 1944 (*S.* Enteritidis R11 Δ*guaBA* Δ*clpX*) in mice were comparable with those of our previous prototypic vaccines, CVD 1921 (*S.* Typhimurium I77 Δ*guaBA* Δ*clpP*) and CVD 1941 (*S.* Enteritidis R11 Δ*guaBA* Δ*clpP*) (26) (Fig. 2). The *S.* Typhimurium CVD 1921 and CVD 1931 vaccines elicited 100% seroconversion for anti-LPS and anti-FliC IgG. The *S.* Enteritidis vaccine CVD 1941 elicited anti-LPS serum IgG seroconversion in 93% (13/14) of mice and anti-FliC serum IgG seroconversion in 100% (14/14) of mice. Similarly, CVD 1944 elicited anti-LPS and anti-FliC seroconversion in 92% (11/12) of mice. Importantly, the live oral vaccines CVD 1931 and CVD 1944 were able to protect mice against a highly lethal challenge dose of *S.* Typhimurium and *S.* Enteritidis strains recently isolated from the blood of febrile children in Mali, indicating that these vaccines can protect against clinically relevant virulent strains. Furthermore, protection against these high challenge doses was observed when mice were infected 12 weeks after the last immunization, indicating that the vaccines can elicit long-lived specific immunity.

We also screened several *Salmonella* serovars in mice in an attempt to identify the ones that were pathogenic for use in cross-protection experiments. However, only a few serovars were found to cause lethal infection in mice (see Table S1 in the supplemental material), in agreement with previous studies (37–39). It is interesting that despite the existence of scores (if not hundreds) of *Salmonella* serovars that have been linked to human disease, less than a handful are pathogenic in mice (40). We confirmed that some strains of *S.* Dublin are lethal and identified the group B serovar *S.* Stanleyville as another lethal serovar. To our knowledge, this is the first report that *S.* Stanleyville is pathogenic in a mouse model. *S.* Stanleyville has been isolated sporadically from clinical specimens, including those from Senegal and Cameroon (41, 42). Interestingly, a recent publication described a case of a urinary tract infection due to *S.* Stanleyville following enteritis in a healthy child (43). The 26 *S.* Stanleyville strains that we previously isolated from the blood of children in Mali, West Africa, is the largest known outbreak of invasive *S.* Stanleyville to date (15).

TABLE 5 Vaccine efficacies of live attenuated *S.* Typhimurium CVD 1931 (*S.* Typhimurium D65 Δ*guaBA* Δ*clpX*) and CVD 1944 (*S.* Enteritidis R11 Δ*guaBA* Δ*clpX*) against lethal challenge 12 weeks after the last immunization[a]

Immunization	Challenge	Challenge dose (LD_{50})	Mortality rate	Vaccine efficacy (%)	*P* value[b]
PBS	*S.* Typhimurium D65	~5,000	12/12		
CVD 1931			4/10	60	0.003
PBS	*S.* Stanleyville J65	~6	10/12		
CVD 1931			1/12	90	<0.001
PBS	*S.* Enteritidis R11	~5,000	12/12		
CVD 1944			2/9	78	<0.001
PBS	*S.* Dublin R17	~1,000	12/12		
CVD 1944			4/9	56	0.006

[a] Mice were immunized orally with 10^9 CFU three times, with 1 month between immunizations, and challenged 3 months later with 9.3×10^7 CFU of *S.* Typhimurium D65 (oral), 1.19×10^8 CFU of *S.* Enteritidis R11 (oral), 8.2×10^5 CFU of *S.* Stanleyville J65 (i.p.), or 8.0×10^7 CFU of *S.* Dublin R17 (oral). The mice were monitored daily for 14 days (i.p.) or 28 days (oral).
[b] Fisher's exact test (two tailed).

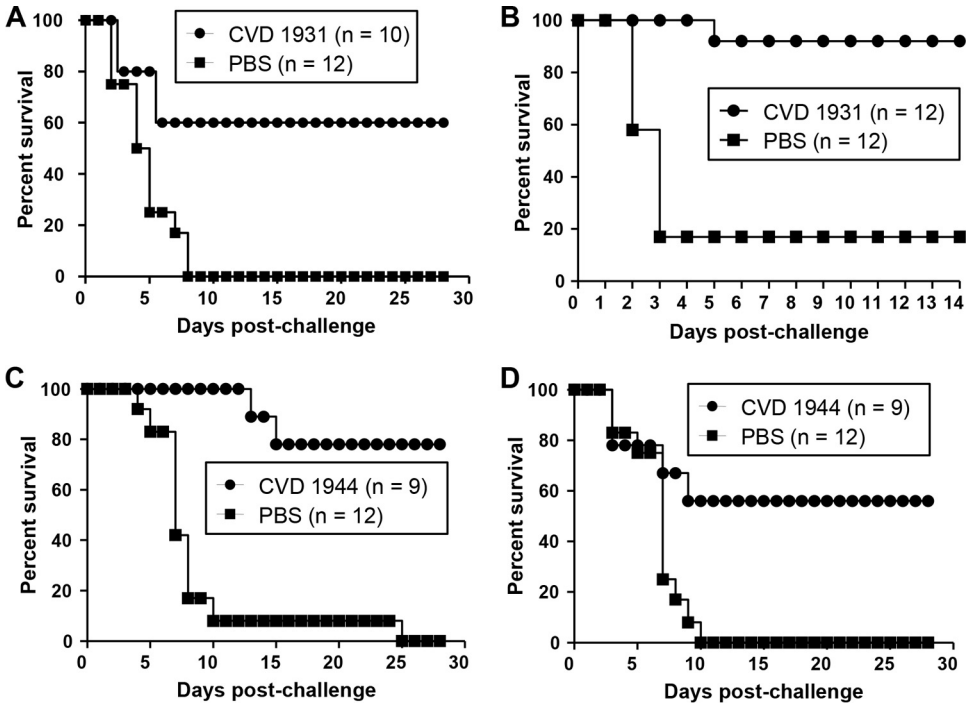
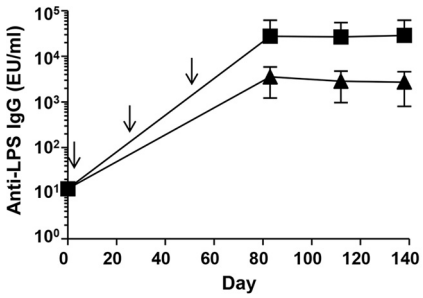


FIG 4 Percent survival of mice immunized with live attenuated NTS strains and challenged with other group B and D serovars. The mice were immunized orally with 10^9 CFU of CVD 1931 (*S.* Typhimurium D65 Δ*guaBA* Δ*clpX*), CVD 1944 (*S.* Enteritidis R11 Δ*guaBA* Δ*clpX*), or PBS three times, with 1 month between immunizations, and challenged 3 months after the last immunization. (A) CVD 1931-immunized mice were orally challenged with 5,000 LD_{50} of *S.* Typhimurium D65. (B) CVD 1931-immunized mice were intraperitoneally challenged with 6 LD_{50} of *S.* Stanleyville J65. (C) CVD 1944-immunized mice were orally challenged with 5,000 LD_{50} of *S.* Enteritidis R11. (D) CVD 1944-immunized mice were orally challenged with 1,000 LD_{50} of *S.* Dublin R17.

The ability of live attenuated *S.* Typhimurium and *S.* Enteritidis vaccines to confer cross-protection against a heterologous serovar or other *Salmonella* serovars has been reported. For instance, NTS DNA adenine methylase (Dam) mutants have been evaluated as vaccines in several animal models, including murine, avian, and bovine models (44–47). An *S.* Enteritidis Dam$^-$ mutant protected mice against challenge with *S.* Dublin or *S.* Typhimurium at 10,000 LD_{50} (44). An *S.* Typhimurium Dam$^-$ mutant protected mice against *S.* Dublin at 10,000 LD_{50} or *S.* Enteritidis at 1,000 LD_{50}. More recently, Matulova et al. (48) showed that *S.* Typhimurium and *S.* Enteritidis live vaccines with SPI-1 mutations can protect chickens against challenge with either serovar. However, our results are congruent with those of Hormaeche et al. (49), who showed that the *S.* Typhimurium *aroA* vaccine SL3261 conferred protection against 10,000 LD_{50} of *S.* Typhimurium but no protection against *S.* Enteritidis. Taken together, the cross-protection data suggest that live attenuated *S.* Typhimurium vaccines can protect against challenge with *S.* Typhimurium but that protection against other serovars is variable. On the other hand, live attenuated *S.* Enteritidis vaccines are highly immunogenic and can protect against homologous and heterologous serogroup challenge.



	% seroconversion			
	Pre	Day 83	Day 112	Day 138
CVD 1931	0	100	95	95
CVD 1944	0	100	100	100

FIG 5 Seroconversion and anti-LPS IgG GMTs elicited by live oral vaccines CVD 1931 and CVD 1944 up to 12 weeks after the last immunization. Mice ($n = 24$) were immunized orally with 10^9 CFU in a 200-μl volume three times (on days 0, 28, and 56 [indicated by arrows]), with 1 month between immunizations. Anti-LPS serum IgG titers were measured by ELISA pre- and postimmunization (squares, CVD 1931; triangles, CVD 1944). Titers are expressed as GMTs ± SEM. The percent seroconversion (4-fold increase in titer versus preimmune) is shown.

Our data suggest that an *S.* Enteritidis-based live attenuated vaccine may be sufficient to mediate protection against *S.* Enteritidis, *S.* Dublin, and *S.* Typhimurium, which is particularly important considering their concomitant prevalence, as demonstrated in West Africa. We speculate that our *S.* Enteritidis vaccine CVD 1944 elicits immunity against an antigen common to *S.* Enteritidis, *S.* Dublin, and *S.* Typhimurium. Cross-protection may be due to humoral immune response cross-reactivity among LPS epitopes or common outer membrane proteins, such as OmpD, which has been shown to be a key target of protective antibodies (50). However, the protection can also be due to cross-reacting cell-mediated immune responses, which live oral *Salmonella* vaccines potently stimulate (51, 52). There is evidence for cross-pro-

tection in humans between certain typhoidal *Salmonella* serovars. Besides protecting against typhoid fever, live oral *Salmonella enterica* serovar Typhi vaccine strain Ty21a confers significant cross-protection against *S.* Paratyphi B disease but not against *S.* Paratyphi A (53, 54).

Based on our results, we believe that the most effective vaccine strategy to combat invasive NTS would be to implement a bivalent (*S.* Typhimurium and *S.* Enteritidis) NTS vaccine in target pediatric populations. If the results of mouse studies can predict the protective activities of these vaccines in humans, not only would the vaccines target the most common invasive NTS serovars, but they would also protect children against other group B and D *Salmonella* serovars, such as *S.* Stanleyville and *S.* Dublin. In the future, one might wish to combine these NTS vaccines with typhoid and paratyphoid vaccines, as well as *Salmonella* group C vaccines, to target the predominant causes of invasive *Salmonella* disease.

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