Development of a Live Attenuated Bivalent Oral Vaccine Against *Shigella sonnei* Shigellosis and Typhoid Fever

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*Shigella sonnei* and *Salmonella* Typhi cause significant morbidity and mortality. We exploited the safety record of the oral, attenuated *S. Typhi* vaccine (Ty21a) by using it as a vector to develop a bivalent oral vaccine to protect against *S. sonnei* shigellosis and typhoid fever. We recombine the *S. sonnei* form I O-antigen gene cluster into the Ty21a chromosome to create Ty21a-Ss, which stably expresses *S. sonnei* form I O antigen. To enhance survivability in the acid environment of the stomach, we created an acid-resistant strain, Ty21a-AR-Ss, by inserting *Shigella* glutaminase–glutamate decarboxylase systems coexpressed with *S. sonnei* form I O-antigen gene. Mice immunized intranasally with Ty21a-AR-Ss produced antibodies against *S. sonnei* and *S. Typhi*, and survived lethal intranasal *S. sonnei* challenge. This paves the way for proposed good manufacturing practices manufacture and clinical trials intended to test the clinical effectiveness of Ty21a-AR-Ss in protecting against *S. sonnei* shigellosis and typhoid fever, as compared with the current Ty21a vaccine.

**Keywords.** shigellosis; Ty21a; *Shigella sonnei*; vaccine; typhoid fever; acid resistance.

Shigellosis (bacterial dysentery) is caused by *Shigella* spp. bacteria. There were 188 million human *Shigella* infections in 2010 [1], affecting mainly children and causing 65,000 deaths. There are >40 serotypes of *Shigella*, but only a few responsible for the majority of shigellosis. In developing countries, *Shigella flexneri* accounts for most cases in children >5 years old, and *S. sonnei* is the second leading cause, at approximately 24% [2]. In developed countries *S. sonnei* is the leading cause of shigellosis, with 75% of the estimated approximately 500,000 annual US cases caused by *S. sonnei* [3, 4]. Moreover, drug-resistant *S. sonnei* infections associated with international travel have been increasingly reported [5, 6]. There is no safe and efficacious licensed vaccine against shigellosis [7].

Protection against shigellosis is believed to be mediated primarily by antibodies to O polysaccharide (O antigen) [8]. Genes encoding *S. sonnei* O antigen are uniquely located on the virulence plasmid so that only cells that express O antigen (form I) are virulent [9]. Our strategy is to generate a bivalent vaccine against typhoid fever and *S. sonnei* shigellosis by administering the *Salmonella Typhi* TyphTy21a vaccine expressing the *S. sonnei* form I O antigen (Ss-fl-Oag).

The typhoid vaccine Ty21a (Vivotif) [10] administered orally during 1 week, affords sustained protection for 7 years, with efficacies ranging from 42% to 96% in Indonesian, Chilean, and Egyptian field trials [11–13], and has had an excellent safety record in >200 million recipients [10, 14–17]. Ty21a is nonpathogenic even when given at 100 times the standard dose [12].

Ty21a expressing Ss-fl-Oag from a plasmid was shown to be protective in clinical trials [18]. However, because plasmid was unstable [19] and associated with lot-to-lot variation [18, 20], development of this Ty21a-based shigellosis vaccine was halted.

The genetic instability associated with plasmid-borne immunogens has now been overcome. A recombinant Ty21a strain with a genome-integrated Ss-fl-Oag gene cluster induced serum antibodies against Ss-fl-Oag and *Salmonella* O 9,12 antigens and protected mice against lethal challenge [21]. However, the immunization and infection routes were through intraperitoneal injection, which is not the natural oral, mucosal route for Ty21a immunization and *S. Typhi* and *S. sonnei* infection. It is unknown whether the vaccine strain will be effective when immunization and challenge are administered through a mucosal route.

To induce protective immune responses, Ty21a must pass from the mouth to the ileum, where Ty21a invades M cells. The major barrier for a live, oral *S. Typhi* vaccine is the low pH in the stomach. *Salmonella* does not survive well at pH <3. Most *Escherichia coli* strains and *Shigella* spp. remain viable at low pH for several hours [22, 23]. This difference probably explains why only 10–100 *Shigella* cells are sufficient to cause infection, compared with approximately 10⁵ colony-forming units (CFUs) of *Salmonella*. To facilitate the journey from mouth to ileum without being eliminated in the stomach, Ty21a is placed in enteric-coated capsules meant to withstand gastric low pH.

The ability of *E. coli* and *Shigella* to withstand pH <2.5 primarily relies on an acid-resistant (AR) system known as the
glutamate-dependent AR pathway [22, 24] consisting of the enzyme glutamate decarboxylase (GAD) and a membrane-bound antiporter. GAD consumes an intracellular proton to decarboxylate glutamate to produce γ-amino butyric acid (GABA) [25–29]. There are 2 isoforms of GAD, encoded by gadA and gadB, which are 98.7% identical in amino acid sequence and functionally redundant. The antiporter, encoded by gadC, pumps glutamate and GABA in and out of the cell [27].

Recently, a newly discovered glutamine-dependent AR system was reported [30]. A previously uncharacterized bacterial glutaminase, encoded by ybaS, converts glutamine to glutamate in acidic conditions, releasing an ammonium that neutralizes an intracellular proton [30]. The antiporter for transporting substrate and product across cell membrane is also GadC [30]. Because the product of glutaminase is the substrate for GAD, we hypothesized that the glutaminase-GadC and GAD-GadC systems work in concert to convert a glutamine molecule into GABA, neutralizing 2 protons in the cell (Supplementary Figure 1 and [30]). This concerted AR systems should function more efficiently than the GAD-GadC system alone [31] to enhance Ty21a viability in an acid environment.

We report the construction and characterization of Ty21a strains expressing Ss-f1-Oag alone or with the concerted glutaminase-GAD AR systems. Expression of the AR genes rendered our vaccine construct AR in vitro. The strains induced antibodies to Shigella and Salmonella O antigens when administered intranasally to mice, and the immunized mice survived intranasal lethal challenge with S. sonnei. These results provide the foundation for a cost-effective and easy-to-administer vaccine against shigellosis and typhoid fever.

METHODS

Bacterial Strains and Media

Bacterial strains used or generated are listed in Supplementary Table 1. Ty21a (Vivotif; Crucell Vaccines) was purchased. Seed banks were made in CY medium (1.2% yeast extract, 2% HyCase, 1.2% pepticase, 0.125% monosodium phosphate, 0.33% sodium chloride (pH 7.2), with 0.2% glucose and 0.005% galactate glutamate) which is used for Vivotif production [10]. Case, 1.2% pepticase, 0.125% monosodium phosphate, 0.33% sodium chloride (pH 7.2), with 0.2% glucose and 0.005% galactose), which is used for Vivitif production [10]. S. sonnei 53G was a gift from Dennis Kopecko [9]. Form I S. sonnei 53G were distinguished from form II based on morphological characteristic. Unless indicated, Ty21a and derivatives were grown in CY medium or CY medium supplemented with 1% trehalose and 0.75% arabinose (AR activated) were harvested and resuspended in phosphate-buffered saline to a target concentration of 1 × 10^11 CFUs/mL. Mice anesthetized by isoflurane were intranasally inoculated with 5 µL of bacterial suspension at the anterior of each naris. Serum samples were collected a day before the first immunization, and 2 weeks after each immunization.

S. sonnei Challenge

First, 3–6 single colonies of form I S. sonnei 53G were pooled to inoculate into TSB and allowed to grow until mid–log phase (optical density at 600 nm [OD600], approximately 1.0). Cells were resuspended in phosphate-buffered saline to a targeted concentration of 1 × 10^11 CFUs/mL, and 10 µL was used to inoculate each mouse intranasally. Mice were monitored daily for 14 days after infection.

Statistical Analyses

All tests were 2 sided and performed using R software (version 3.2.3) with stat and survival (2.38–3) packages.

RESULTS

Stable Integration and Expression From Ty21a Chromosome

We constructed a recombinant Ty21a with stable chromosomally integrated Ss-f1-Oag gene cluster between the tvID and vexA open reading frames (ORFs) (Figure 1A) [21, 32, 33]. This construct, though similar to what has been previously described [21], is different in that the gene cluster was amplified from genomic DNA of S. sonnei 53G form I and initiated from the conserved 5’ border of O-antigen clusters [35] and downstream primer VexA-1066R (5’-AGAAAAAGATT AGTGCCCGGG-3’) and integrated in Ty21a harboring pKD46 [21, 32]. The KanR selectable marker was deleted from the chromosomal integrants by transformation with pCP20 and selection for KanR transformants, as described elsewhere [21, 33]. Chromosomal integration and selection marker eviction were confirmed using genomic PCR analysis and antibiotics sensitivity tests.

Plasmids

Plasmids are listed in Supplementary Table 1, and details of plasmid construction using standard techniques and enzymes (New England BioLabs) are described in the Supplementary data. The integrity of all plasmids generated in this study was confirmed by DNA sequencing.

Assays

Methods for assays used in this study are described in detail in the Supplementary data.

Animal Immunization

Mice (4–8-week old female BALB/c mice; Jackson Laboratory) were maintained at Bioqual. They were immunized and assessed according to a protocol [34] approved by the Bioqual Laboratory Animal Care and Use Committee. On the day of immunization, overnight cultures of bacterial strains grown in CY medium or CY medium supplemented with 1% trehalose and 0.75% arabinose (AR activated) were harvested and resuspended in phosphate-buffered saline to a target concentration of 1 × 10^11 CFUs/mL. Mice anesthetized by isoflurane were intranasally inoculated with 5 µL of bacterial suspension at the anterior of each naris. Serum samples were collected a day before the first immunization, and 2 weeks after each immunization.
Because the arabinose-controlled AR genes in Ty21a-AR-Ss are regulated by Para, which repression is not fully understood, we put these genes under the regulation of the arabinose-controlled promoter, AraC, which activates robust gene transcription. We used PCR to amplify \textit{Shigella} ybaS coding sequences, fused them, and placed them under \textit{araC} to create a polycistronic \textit{AraC}-\textit{ara}-\textit{YbaS}-\textit{GadBC} expression cassette. This cassette was integrated into the Ty21a chromosome to replace the \textit{tvIE} ORF. The resulting strain was then used for stable integration of the \textit{Ss}-\textit{f1-Oag} expression cassette, using \textit{gadC} and \textit{vexA} as homologous sequences for recombination (Figure 1C), and the KanR marker was evicted. The resulting final, markerless, strain was designated Ty21a-AR-Ss.

Cell Surface Expression

We examined \textit{Ss}-\textit{f1-Oag} expression by means of Western blots (Figure 2A and 2B) and immunofluorescence assays (Figure 2C). Because the arabinose-controlled AR genes in Ty21a-AR-Ss are located 5' to the \textit{Ss}-\textit{f1-Oag} gene cluster, we also examined form I O-antigen expression in the presence of 0.75% arabinose in the culture medium, mimicking AR-activating conditions. \textit{Ss}-\textit{f1-Oag} in \textit{S. sonnei} 53G is expressed as a ladder of 2-sugar repeats that is lipopolysaccharide (LPS) core linked, and a smear at higher molecular weight, which is linked to group 4 capsule (Figure 2A). Ty21a-Ss expressed \textit{Ss}-\textit{f1-Oag}, and Ty21a did not (Figure 2A and 2C). As described elsewhere [21, 36], most \textit{Ss}-\textit{f1-Oag} was expressed in the form that is capsule linked in Ty21a-Ss. Ty21a-Ss expressed the \textit{Ss}-\textit{f1-Oag} uniformly on the cell surface, similar to the native form expressed on \textit{S. sonnei} 53G; we believe they arose from natural variation between laboratory isolates.

We hypothesized that expression of the AR genes in Ty21a would make a better vaccine. Because regulation of AR gene expression is not fully understood, we put these genes under the regulation of the arabinose-controlled promoter, \textit{araC}, which responds quickly to the arabinose-bound transcription factor, AraC, which activates robust gene transcription. We used PCR to amplify \textit{Shigella} \textit{ybaS} and the \textit{gadBC} coding sequences, fused them, and placed them under \textit{araC} to create a polycistronic \textit{AraC}-\textit{ara}-\textit{YbaS}-\textit{GadBC} expression cassette. This cassette was integrated into the Ty21a chromosome to replace the \textit{tvIE} ORF. The resulting strain was then used for stable integration of the \textit{Ss}-\textit{f1-Oag} expression cassette, using \textit{gadC} and \textit{vexA} as homologous sequences for recombination (Figure 1C), and the KanR marker was evicted. The resulting final, markerless, strain was designated Ty21a-AR-Ss.

**Acid Resistance**

To confirm that the integrated AR genes expressed on induction were enzymatically active, we subjected Ty21a-AR-Ss to glutaminase and GAD assays [37], with slight modifications. Triton X-100 was omitted from the original GAD and glutaminase reagents. Therefore, only when a functional GadC transporter was present could glutamine or glutamate be transported into the cell for enzyme utilization. Bromocresol green was the pH indicator. Ty21a and Ty21a-Ss were unable to use glutamine or glutamate to increase pH in the test reagent; the reactions remained yellow after incubation at 37°C for 30 minutes (Figure 3A, lanes 1 and 2). Reactions containing Ty21a-AR-Ss grew in the absence of arabinose turned slightly bluish-green, indicating a low-level leaky expression of the AR enzymes (Figure 3A, lane 3). When grown in the presence of arabinose, Ty21a-AR-Ss...
turned the assay reagents strongly bluish-green, indicating robust expression of enzymatically active AR genes (Figure 3A, lane 4); Ty21a-AR-Ss expressed functional AR genes in a transcriptionally controlled manner.

We next tested the ability of Ty21a-AR-Ss to survive at pH 2.5. Because Salmonella needs acidic culture conditions to induce acid tolerance response, a prerequisite for AR [38], 1% trehalose was included for acid fermentation in addition to 0.75% arabinose. Bacterial strains were grown to stationary phase, and cultures diluted 1:20 in acid medium at pH 2.5 in the presence of 1.5 mmol/L glutamine. Reactions were incubated at 37°C with agitation, and viability was examined at the indicated time points (Figure 3B). S. sonnei 53G form II maintained approximately 100% viability after 1-hour incubation at pH 2.5. In contrast, Ty21a survived poorly at this condition: viability was reduced by $>10^2$-fold at 15 minutes and $>10^7$-fold by 30 minutes.

Cell viability was further reduced in Ty21a-Ss, with no viable colonies recovered at 15 minutes, equivalent to a $>10^8$-fold reduction. Ty21a-AR, the parental strain for Ty21a-AR-Ss, maintained $>50\%$ viability at 30 minutes and approximately 10% at 45 minutes. Ty21a-AR-Ss maintained approximately 50% viability at 30 minutes. However, viability quickly deteriorated, with approximately 1% viability at 45 minutes. Increasing glutamine concentration to 6 mmol/L improved survival for Ty21a-AR-Ss; cell survival at 45 minutes improved to approximately 50%. Ty21a recovered from the acid exposure was slow growing and lost expression of the Salmonella O antigen, whereas Ty21a-AR and Ty21a-AR-Ss maintained levels of O-antigen expression comparable to levels before acid exposure (Figure 3C). Expression of the concerted glutaminase-GAD AR systems improved AR and cell viability of recombinant Ty21a strains.

**Stability**

We generated genetic seedbanks for Ty21a-Ss and Ty21a-AR-Ss. All microbiological, biochemical, immunological, genetic,
and molecular properties were as expected (Figure 4A). Cells were grown under nonselective conditions for 200 generations, and 100 colonies from each culture were subjected to colony immunoblot analysis for Ss-f1-Oag expression. All 100 colonies of Ty21a-Ss (Figure 4B) and Ty21a-AR-Ss (Figure 4C) tested retained Ss-f1-Oag expression, demonstrating 100% stability of the chromosomally integrated genes after 200 generations of growth.

Immunogenicity
To assess whether Ty21a-Ss and Ty21a-AR-Ss were immunogenic when administered via the mucosal route, we immunized...
4 groups of 10 mice intranasally with 4 doses (1 × 10⁹ CFUs) of Ty21a, Ty21a-Ss, Ty21a-AR-Ss (AR gene not expressed), and Ty21a-AR-Ss grown in the presence of trehalose and arabinose (AR genes activated; referred to as Ty21a-AR-Ss + ARA) at 2-week intervals. Serum immunoglobulin (Ig) G antibody responses against Ss-f1-Oag at 2 weeks after doses 2, 3, and 4 were assessed with enzyme-linked immunosorbent assay. Results are reported as the geometric mean (GM) OD 1.0 (serum dilution at which the OD was 1.0; Figure 5A). Mice immunized with Ty21a-Ss, Ty21a-AR-Ss, and Ty21a-AR-Ss + ARA produced higher serum IgG antibodies against Ss-f1-Oag than the Ty21a control or naive mice, and antibodies increased with increased numbers of immunizations (P < .05; Wilcoxon rank test). At 2 weeks after dose 4, the GM OD 1.0 to Ss-f1-Oag of mice immunized with Ty21a-Ss, Ty21a-AR-Ss, and Ty21a-AR-Ss + ARA was 7909.5 (range, 2560–21 700), 7202.4 (3363–15 910), and 23 020.8 (8521–71 575), respectively.

We also assessed antibodies to Salmonella groups O 9,12-antigens, the native O antigens expressed on Ty21a surface that induce protective immunity against typhoid fever (Figure 5B). For mice immunized with Ty21a, the GM OD 1.0 of serum IgG antibodies to Salmonella O9,12-antigens was 53.9 (range, 1–864), 54.5 (1–545), and 328.3 (72–2295) at 2 weeks after doses 2, 3, 4, respectively. Ty21a-Ss, Ty21a-AR-Ss, and Ty21a-AR-Ss + ARA induced anti-Salmonella O9,12-antigen serum IgG at comparable levels. At 2 weeks after dose 4, the GM OD 1.0 was 421.6 (range 98–1241), 182.3 (range (74–1030), and 426.8 (range 78–2539) for Ty21a-Ss, Ty21a-AR-Ss, and Ty21a-AR-Ss + ARA. Naive mice had a GM OD 1.0 of 13.6 (range, 1–38).

**Protective Efficacy**

We challenged the immunized mice 6 weeks after dose 4 with S. sonnei 53G form I, using intranasal instillation. Only 3 of 9 control mice immunized with Ty21a survived the challenge, but 7 of 10, 9 of 10, and 9 of 10 mice immunized with Ty21a-Ss, Ty21a-AR-Ss, and Ty21a-AR-Ss + ARA, respectively, survived and remained healthy throughout the 14-day monitoring.
period (Figure 6). The protective efficacies were 55.2%, 85.1%, and 85.1%, respectively, for Ty21a-Ss, Ty21a-AR-Ss, and Ty21a-AR-Ss + ARA. For the 2 groups that received the Ty21a-AR-Ss constructs, the difference between Ty21a control and immunized mice was significant ($P = .02$; 2-tailed Fisher exact test).

**DISCUSSION**

Oral immunization with Ty21a expressing Ss-f1-Oag from a plasmid protected humans against *S. sonnei* diarrhea and dysentery [18]. However, the plasmid-based construct was unstable with DNA rearrangements and deletions of plasmid material probably causing lot-to-lot variation [19]. Chromosomal

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**Figure 5.** Ty21a-Ss and Ty21a-AR-Ss immunized through a mucosal route generated high antibody responses to *Shigella sonnei* form I Ty21a O antigens. Mice were immunized intranasally with $1 \times 10^9$ colony-forming units of Ty21a (black), Ty21a-Ss (blue), Ty21a-AR-Ss (red), or Ty21a-AR-Ss + ARA (green) 4 times at 2-week interval. Serum samples were collected 2 weeks after doses 2, 3, and 4, and antibody responses to extracted *S. sonnei* lipopolysaccharide (LPS) (A) or Ty21a LPS (Salmonella groups 9,12 O antigens) (B) were measured using enzyme-linked immunosorbent assay (ELISA) to determine the serum dilution at which the optical density was 1.0 (OD 1.0). Data were collected using Softmax 5.0 software and were fit to a 4-parameter logistic curve. Serum samples from 5 unimmunized, naive mice (gray) of the same age were also collected and measured as negative controls. Each point represents an individual mouse, and the intensity of the point indicates whether the mouse was protected (black) or unprotected (gray) from a lethal *S. sonnei*53G form I infection. The geometric mean for each group is indicated by a horizontal bar. Abbreviations: ARA, arabinose; IgG, immunoglobulin G.
Ty21a was reduced by >10^2-fold at 15 minutes. The half-life for gastric emptying time in humans is about 26 minutes. Ty21a quickly lost viability and O-antigen expression on exposure to acid, but Ty21a-AR-Ss maintained the same levels of O-antigen expression (Figure 3C). Moreover, we observed slightly better antibody responses and higher protective efficacy in mice immunized with Ty21a-AR-Ss than in those immunized with Ty21a-Ss, especially when the AR genes were activated (Ty21a-AR-Ss + ARA). This was probably because culture conditions for AR gene induction favored cell viability. Even though we immunized mice with similar amounts of bacterial cells (determined by OD_{600}), the viable counts for the Ty21a-AR-Ss + ARA were always higher, but no more than 2-fold higher than that of the other 3 groups. By rendering Ty21a AR, we expect increased bioavailability, which should increase immunogenicity and protective efficacy.

Humans become infected with S. Typhi by ingestion of the bacteria. Ty21a is an orally administered vaccine. However, it does not infect mice, making it difficult to assess immunization by oral administration. Thus, testing of immunogenicity and protective efficacy in mice after intraperitoneal administration was undertaken [21]. This could be misleading. LPS is highly immunogenic when administered intraperitoneally but not when administered by mucosal routes [42]. Furthermore, deaths from intraperitoneal inoculation of gram-negative bacteria are related to the toxic effects of endotoxin [43], which may be distinct from the effects of invasive Shigella infection in human intestinal epithelium.

The route for immunization and challenge of a Ty21a-based vaccine is ideally oral. However, because Ty21a, S. Typhi, [44], and Shigella spp. do not infect normal mice when administered orally, an intranasal route has been established in mice to evaluate mucosal immune responses to live attenuated bacterial vaccines [34, 45]. The serum antibody responses in mice were about 10-fold lower using the intranasal compared with the intraperitoneal route (unpublished data). Nevertheless, using this approach, we demonstrated that Ty21a-Ss and Ty21a-AR-Ss stimulated antibodies against both S. sonneti and Salmonella LPS. Most importantly, 9 of 10 mice immunized intranasally with Ty21a-AR-Ss or Ty21a-AR-Ss + ARA vaccine, compared with 3 of 9 immunized with Ty21a alone, survived a lethal intranasal infection with S. sonneti 53G (protective efficacy for each, 85.1%).

Ty21a protects for up to 7 years [12]. We expect similar protection with the typhoid-shigellosis combination vaccine. Ty21a can be foam dried, providing for temperature stabilization and a potential shelf life of 5–10 years [46], which will facilitate storage and distribution. Most importantly, Ty21a is extremely safe. To our knowledge, there have been no reports of bacteremia or Ty21a-associated postvaccination reactive arthritis, a potential problem with other live attenuated vectors, including nontyphoid Salmonella, Shigella, and Yersinia. We do not foresee safety concerns with inclusion of the AR genes. These genes are present and functional in avirulent strains of E. coli and Shigella spp., such as S. sonneti 53G form II (Figure 3B). Therefore, they are insufficient to cause disease on their own. The AR genes are stably integrated into the Ty21a genome and placed under an artificially inducible promoter. These properties make horizontal transfer to and gene activation in another bacterium extremely unlikely. Finally, except for the expression of the AR and Ss-fl-Oag transgenes, Ty21a-AR-Ss displays the

![Figure 6.](image-url)
microbiological, biochemical, immunological, and genetic properties of Ty21a (Figure 4), demonstrating that expression of the AR genes has no effect on Ty21a attenuation. This finding is similar to a recently reported study with the GAD-GadC AR system, but not the glutaminase-GadC system we also included [31]. We cannot directly compare our results because the viability of the Ty21a control after acid challenge was lower in our experiments.

The recent Global Enteric Multicenter Study (GEMS) field study of 1130 Shigella spp. concluded, “A quadrivalent vaccine with O-antigens from S. sonnei, S. flexneri 2a, S. flexneri 3a, and S. flexneri 6 can provide broad direct coverage against these most common serotypes and indirect coverage...through shared S. flexneri group antigens...” (up to 88% coverage) [2]. Our goal is to develop such a quadrivalent vaccine.

However, S. sonnei is responsible for 75% of all cases of shigellosis in the United States [4] and most cases in Israel [47], Thailand [48], and Southern Vietnam [49]. While developing the 3 other constructs for the quadrivalent vaccine, we plan to move forward in parallel to good manufacturing practices manufacture of our AR Ty21-AR-Ss vaccine (TyOraSs). The next step will be to prove that it is safe, immunogenic, and protective against shigellosis and typhoid in humans.

**Supplementary Data**

Supplementary materials are available at http://jid.oxfordjournals.org. Consisting of data provided by the author to benefit the reader, the posted materials are not copied and are the sole responsibility of the author, so questions or comments should be addressed to the author.

**Notes**

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**References**


