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Identification of *In Vivo*-Induced Bacterial Proteins during Human Infection with *Salmonella enterica* Serotype Paratyphi A

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*Salmonella enterica* serotype Paratyphi A is a human-restricted pathogen and the cause of paratyphoid A fever. Using a high-throughput immunoscreening technique, *in vivo*-induced antigen technology (IVIAT), we identified 20 immunogenic bacterial proteins expressed in humans who were bacteremic with *S. Paratyphi A* but not those expressed in *S. Paratyphi A* grown under standard laboratory conditions. The majority of these proteins have known or potential roles in the pathogenesis of *S. enterica*. These include proteins implicated in cell adhesion, fimbrial structure, adaptation to atypical conditions, oxidoreductase activity, proteolysis, antimicrobial resistance, and ion transport. Of particular interest among these *in vivo*-expressed proteins were *S. Paratyphi A* (SPA)2397, SPA2612, and SPA1604. SPA2397 and SPA2612 are prophage related, and SPA1604 is in *S. enterica* serovar Paratyphi A2397, SPA2612, and SPA1604. SPA1604 is in *Salmonella* pathogenicity island 11 (SPI-11). Using real-time quantitative PCR (RT-qPCR), we confirmed increased levels of mRNA expressed by genes identified by IVIAT in a comparison of mRNA levels in organisms in the blood of bacteremic patients to those in *in vitro* cultures. Comparing *in vivo*- to *in vitro* samples, we also detected a significant increase in the reaction of *in vivo*-expressed proteins with two proteins identified by IVIAT: SPA2397 and SPA0489. SPA2397 is a phage-related lysozyme, Gp19, and SPA0489 encodes a protein containing NlpCP60 and cysteine, histidine-dependent amidohydrolase/peptidase (CHAP) domains. In a previous study utilizing a different approach, we found that transcripts for 11 and 7 of the genes identified by IVIAT were detectable in organisms in the blood of bacteremic with *S. Paratyphi A* and *Salmonella enterica* serovar Typhi, respectively. *S. Paratyphi A* antigens identified by IVIAT warrant further evaluation for their contributions to pathogenesis and might have diagnostic, therapeutic, or preventive relevance.
Stock Center, Calgary, Alberta, Canada) was used to construct an inducible genomic expression library in an *Escherichia coli* BL21(DE3) host strain (New England BioLabs, Ipswich, MA). All strains were grown in Luria-Bertani (LB) broth at 37°C with aeration. Clones containing pET30c constructs (New England BioLabs) were grown in LB broth and solid agar containing 50 μg/mL kanamycin. Glycerol stocks were maintained at −80°C in LB medium supplemented with 15% glycerol (Sigma-Aldrich, St. Louis, MO).

**Patient and control serum.** Paired acute- (days 0 to 2) and convalescent-phase (days 14 to 28) serum samples were obtained from eight individuals with *S. Paratyphi* A bacteremic infection presenting to the Mirpur or Kamalapur field sites of the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B) in Dhaka, Bangladesh. In this study, control serum samples were also collected from cholera patients in Bangladesh at the acute and convalescent phases of illness. This study was approved by the institutional review boards at Massachusetts General Hospital, Boston, MA, and the ICDDR,B, Dhaka, Bangladesh.

**Adsorption of serum.** Convalescent-phase serum samples from eight individuals who were bacteremic with *S. Paratyphi* A were pooled. The serum samples were adsorbed extensively with *in vitro*-grown *S. Paratyphi* A strain ATCC 9150. The organisms were grown to late log phase under standard laboratory conditions (*in vitro*) in LB broth (37°C, aeration shaker); the cells were pelleted and resuspended in phosphate-buffered saline (PBS) with 0.05% sodium azide. To produce cell lysates, in *vitro*-grown cells were lysed with 0.1-mm zirconia-silica beads in a Mini-Beadbeater (BioSpec, Bartlesville, OK) per the manufacturer’s instructions. Heat-denatured cell lysates were generated by boiling the cell lysates. Whole-cell *S. Paratyphi* A, nondenatured cell lysates, and heat-denatured cell lysates were separately immobilized on 0.5-μm polystyrene beads (Bangs Laboratories, Inc., Fishers, IN), and pooled convalescent-phase sera were serially adsorbed with whole-cell *S. Paratyphi* A and these beads. Adsorbed serum was aliquoted and stored at −80°C.

**Construction of inducible genomic expression library of *S. Paratyphi* A.** Genomic DNA was extracted from *S. Paratyphi* A ATCC 9150 using a Qiagen genomic-tip 100/G and genomic buffer set (Qiagen, Valencia, CA). The DNA was sheared using a Covaris M220 AFA focused-ultrasonicator (Covaris, Woburn, MA) and settings were optimized to yield 500- to 1,500-bp fragments. The resulting fragments were gel purified and extracted using a QIAquick PCR purification kit (Qiagen, Valencia, CA). Blunt ends were created on the *S. Paratyphi* A fragments using the End-It DNA end-repair kit (Epicentre, Madison, WI), and the fragments were inserted into pET30c treated with EcoRV (New England BioLabs) and calf intestinal alkaline phosphatase (New England BioLabs). These products were electroporated into *E. coli* DH5α (Invitrogen, Carlsbad, CA) to generate libraries with all three possible open reading frames (ORFs). The resulting plasmids were pooled, and the library mixture was transformed into *E. coli* BL21(DE3) (New England BioLabs); we confirmed that >80% of the library contained inserts of 500- and 1,500-bp insertion sizes.

**Screening the inducible genomic expression library for antigenic proteins expressed during *S. Paratyphi* A infection.** In the primary immunoscreening, aliquots of the genomic library were plated on LB plates with kanamycin overnight at 37°C to obtain approximately 500 colonies per plate. Colonies were transferred onto nitrocellulose membranes (Invitrogen, Carlsbad, CA), and the membranes were incubated on LB plates containing 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h at 37°C. Adherent colonies were lysed by placing the nitrocellulose membranes on chloroform-soaked blotting paper for 10 s. The membranes were air dried for 5 min and blocked with phosphate-buffered saline (PBS) containing 5% nonfat milk. The membranes were then incubated with adsorbed serum in PBS-0.25% Tween at a dilution of 1:10,000 overnight at 4°C. Clones reacting with adsorbed serum were detected using peroxidase-conjugated goat anti-human IgG antibody (MP Biomedicals Cappel, Aurora, OH) at a dilution of 1:3,000 in PBS-0.25% Tween. The immunoblots were developed using enhanced chemiluminescence (ECL) Western blotting detection reagents (GE Healthcare, Piscataway, NJ). Immunoreactive clones were recovered and glycerol stocks were frozen at −80°C.

A whole-colony immunoblot assay was used to confirm reactive clones by comparing their immunoreactivities to that of the control strain *E. coli* BL21(DE3) containing pET30 vector with no insert. Plasmids were purified from reactive clones using a QIAprep spin miniprep kit (Qiagen, Valencia, CA) and the inserts were sequenced with predesigned forward and reverse primers for the T7 promoter and termination sites. Since inserts might contain multiple open reading frames and fragments of genes, each identified gene was separately PCR amplified and cloned into pET30c as Ndel and NotI fragments. Whole-colony immunoblot assay was used in tertiary screening to confirm the immunoreactivities of individually cloned gene products by comparing their immunoreactivities to that of *E. coli* qBL21(DE3) containing pET30 with no insert.

**Prediction of function of antigens identified by IVIAT.** The functional classification of antigens identified by IVIAT was predicted using published studies of identified proteins in *S. enterica and using the J. Craig Venter Institute (JCVI) Comprehensive Microbial Resource (http://cmr.jcvi.org/tigr-scripts/CMR/CmrHomePage.cgi).

**Confirmation of *S. Paratyphi* A gene expression in patients with *S. Paratyphi* A bacteremia by real-time quantitative PCR (RT-qPCR).** Bacterial mRNA expression levels of the genes encoding proteins identified by IVIAT were quantified to evaluate whether they were upregulated in the blood of infected humans compared to *in vitro* cultures. Previously collected blood samples were used that had been immediately stored in TRI-zol LS (Invitrogen, Grand Island, NY) at the time of clinical presentation in febrile patients in Dhaka, Bangladesh. Samples from patients (n = 3) who were subsequently confirmed to have been bacteremic with *S. Paratyphi A* at the time of presentation were used. *S. Paratyphi* A cultures were grown to mid-logarithmic phase in LB at 37°C to measure mRNA expression levels in *in vitro*-grown bacteria. RNA was treated with an Ambion DNA-free kit (Austin, TX) and generated cDNA using random decamers and an Ambion RETROscript kit (Austin, TX), per the manufacturer’s two-step RT-qPCR protocol. Real-time quantitative PCR analysis was performed using Bio-Rad iQ SYBR green supermix reagent, an MJ Research Chromo4 thermocycler (Bio-Rad), Bio-Rad High Shell 96-well BLK/WHT PCR plates, and Bio-Rad Microseal “B” film optical plate covers (Bio-Rad, Hercules, CA). Primers were designed with Beacon Design (Premier Biosoft, Palo Alto, CA), primer3, and BLAST software to optimize reaction thermodynamics. Primers were designed to produce PCR fragments of 150 to 225 bases, and each quantification was performed at least twice. The calculated threshold cycle (Ct) was set in the lower/linear portion of product curves. MJ Opticon Monitor software version 3.1 (Bio-Rad, Hercules, CA) was used to quantify gene copy numbers against the concentration curve of pET30 tertiary-screened clone plasmids. Gene copy numbers were normalized against cDNA copies of 16S rRNA.

**Comparison of acute- to convalescent-phase immunoreactivities of proteins identified by IVIAT.** To compare the immunoreactivities of *S. Paratyphi* A proteins identified by IVIAT in acute versus convalescent-phase serum samples of patients with bacteremia, *in vitro* transcription-translation-based protein expression and enzyme-linked immunosorbent assay (ELISA) methods were used. *S. Paratyphi* A proteins were expressed using the Promega *E. coli* T7 S30 extract system (Promega, Madison, WI), and the resulting products were bound to 96-well ELISA plates (Nunc, Rochester, NY). Wells were immune probed with pooled acute- or convalescent-phase serum from four *S. Paratyphi* A bacteremic patients (1:150 dilution in PBS with 5% milk) that was distinct from serum in the screening pool. Pooled acute (day 2) or convalescent (day 21) sera were also used from four Bangladeshi patients with cholera caused by *Vibrio cholerae* O1 who presented to the same care facility in Bangladesh as did the paratyphoid patients. Immunoreactivity was detected with peroxidase-conjugated goat anti-human immunoglobulin (IgG) antibody (MP Biomedicals Cappel, Aurora, OH) at a dilution of 1:1,000 in PBS with 5% milk and was
TABLE 1 Proteins identified by applying IVIAT to S. enterica serotype Paratyphi A

| Functional category                                      | SPA locus* | SPA gene* | Description                                      |
|----------------------------------------------------------|------------|-----------|--------------------------------------------------|
| Cell envelope and adhesion                               | SPA0021    | bcIA      | Fimbrial subunit                                 |
|                                                           | SPA0181    | stkE      | Putative fimbrial protein                        |
|                                                           | SPA0201    | stfA      | Putative fimbrial subunit                        |
|                                                           | SPA0703    | stcD      | Putative fimbrial protein                        |
|                                                           | SPA1533    | osmE      | Osmotically inducible lipoprotein E precursor    |
|                                                           | SPA1604    | envE      | Putative lipoprotein                             |
|                                                           | SPA1645    | ycfL      | Putative lipoprotein                             |
|                                                           | SPA3787    | yjeI      | Putative lipoprotein                             |
|                                                           | SPA4148    | yjeI      | Putative membrane protein                        |

Cell wall and antimicrobial resistance

Energy metabolism, nutrient acquisition, cellular processes

Mobile and extrachromosomal element functions

* SPA, S. Paratyphi A.

developed using SuperSignal West Femto maximum sensitivity substrate (Thermo Scientific, Rockford, IL). Reactions were read using a SpectraMax Gemini XPS (Molecular Devices, Sunnyvale, CA), and results were expressed as fold-change (sample protein reactivity to empty vector reactivity) that equilibrated cholera immunoreactivity to 1.

Statistical analyses. Comparisons of gene expression levels between in vivo and in vitro conditions were tested for significance using one-way analysis of variance (ANOVA) (Holm-Šídák). When we compared the differences in the fold-increase of immune responses, we used unpaired t tests. Analyses were performed using GraphPad Prism 5.0 and SigmaStat 3.1.

RESULTS

Identification of S. Paratyphi A antigens by IVIAT. We screened 100,000 clones from the S. Paratyphi A inducible genomic expression library and identified 505 immunoreactive clones in our primary screening. Forty-seven of these clones had clear and consistent immunoreactivity on repetitive (secondary) screening. Plasmids of these 47 clones contained 87 whole or partial S. Paratyphi A open reading frame fragments. We subcloned each identified open reading frame (ORF) in its entirety into pET30, electroporated resultant plasmids into E. coli BL21(DE3), and confirmed consistent immunoreactivity for 20 resultant clones (Table 1).

Functional classification of antigens identified by IVIAT. We were able to assign a functional classification to the 20 proteins expressed from genes identified by IVIAT (Table 1). The majority of the proteins have known or potential roles in the pathogenesis of S. enterica infection. These included proteins that are implicated in fimbrial structure (S. Paratyphi A [SPA0021, SPA0181, SPA0201, and SPA0703], the cell envelope and membrane (SPA1533, SPA1604, SPA1645, SPA3787, and SPA4148), energy metabolism and cellular processes (SPA0489, SPA0608, SPA0652, SPA1239, SPA1734, SPA2362, SPA2948, and SPA4117), and anti-

microbial resistance (SPA1644). Two genes were phage related (SPA2397 and SPA2612) (10).

Comparison of S. Paratyphi A genes identified by IVIAT to homologs in other Enterobacteriaceae. Homologs of genes encoding 10 of the 20 S. Paratyphi A proteins identified by IVIAT are not found in E. coli (SPA0021, SPA0181, SPA0201, SPA0489, SPA0703, SPA1604, SPA1734, SPA2397, SPA2362, and SPA2612). Two of these genes, SPA2397 and SPA2612, reside in the prophage regions of the S. Paratyphi A genome designated SPA-1 and SPA-3-P2, respectively. Ten of the genes (SPA0021, SPA0201, SPA0489, SPA1239, SPA1533, SPA1644, SPA1645, SPA2948, SPA3787, SPA4117, and SPA4148) have homologs in E. coli as well as in various Salmonella spp., including S. Typhi, S. Paratyphi B and C, and nontyphoidal Salmonella spp. Homologs of 5 of the 20 S. Paratyphi A genes identified by IVIAT to homologs in other Enterobacteriaceae, for example, the stkE (SPA0181) gene that encodes a putative fimbrial protein, is specific to S. Paratyphi A and S. enterica serotype Heidelberg, and it is not found in other bacteria.

Real-time quantitative PCR analysis of expression of IVIAT-identified antigens. To confirm the IVIAT results, we quantified the expression of six of the identified S. Paratyphi A genes (SPA0181, SPA0608, SPA1239, SPA1644, SPA1645, SPA2362, and SPA2612) in the blood of all three S. Paratyphi A-infected patients. We were unable to detect a sufficient mRNA signal for the remaining genes in TRizol-preserved in vivo samples, perhaps reflecting the low organism CFU/ml load that is common in enteric fever (11). For the genes whose mRNA we were able to detect in vivo, we compared the expression levels in bacteremic humans to those in in vitro-grown bacterial samples, normalizing by 16S rRNA. We also assessed in vivo versus in vitro expression levels for the S. Paratyphi A housekeeping gene SPA3294 (rplE) that encodes the 50S ribosomal protein L5 (12). We found that the genes
whose proteins were identified by IVIAT were upregulated in the blood of bacteremic patients compared to the in vitro-grown cultures (Fig. 1). The fold-changes of the expression of these genes between in vivo and in vitro samples were 125- to 2,103-fold.

**In vitro transcription-translation-based ELISA.** Comparing convalescent- to acute-phase serum samples, we detected a significant increase in the reaction of convalescent-phase IgG antibodies with two genes identified by IVIAT, SPA0489 and SPA2397.
(P \leq 0.05; Fig. 2), while control patients with cholera did not have any significant changes in immunoreactivity between samples.

SPAS2937 encodes Gp19, a lysozyme, and SPAS0489 encodes a protein that contains NlpC/P60 and cysteine, histidine-dependent amidohydrolase/peptidase (CHAP) domains.

**DISCUSSION**

In this study, we applied the IVIAT technique to S. Paratyphi A and identified 20 immunogenic proteins expressed uniquely in vivo. IVIAT is a technique that identifies bacterial antigens expressed in infected humans and not in bacteria grown under standard laboratory conditions. Identifying such a subgroup of antigens can assist in focusing analysis efforts, since antigens expressed uniquely in humans might play significant or critical roles in in vivo survival or pathogenesis. For 11 of the S. Paratyphi A genes encoding proteins identified by IVIAT, we have previously demonstrated by a different capture and amplification transcriptional profiling technique, selective capture of transcribed sequences (SCOTS), mRNA expression in the blood of humans in Bangladesh who were bacteremic with S. Paratyphi A (12). Using SCOTS, we have similarly identified seven of the corresponding 20 genes in the blood of humans who were bacteremic with S. Typhi in Bangladesh (13). We identified mRNA for four of the genes in both S. Paratyphi A- and S. Typhi-infected patients (12, 13). Our identification by transcriptional analysis of the majority of the genes detected by IVIAT suggests the complementary nature of these approaches and might support the potential importance of these genes in infected humans.

The 20 genes identified in this IVIAT study can be categorized into several functional groups. These include proteins involved in cell adhesion, fimbral structure, adaptation to atypical conditions, energy metabolism, oxoreductase activity, proteolysis, antimicrobial resistance, and ion transport. Our results suggest that S. Paratyphi A expresses genes in vivo that might be involved in recognizing and responding to the in vivo environment, including genes involved in energy metabolism, use of alternate energy sources, and membrane attachment and signaling. These observations are in agreement with those from our previous transcriptional-based analyses (12, 13).

Previous studies have shown that the fimbrial proteins of S. Typhi (5) and S. Typhimurium (14) are upregulated under in vivo compared to in vitro conditions. In this study, we also identified a number of fimbrial genes that were upregulated, including bcfA, stfA, stkE, and stcD, and whose proteins were immunogenic. BcfA and StfA are detected on the surfaces of bacterial cells after oral infection of mice with S. Typhimurium and are not expressed when the bacteria are grown in vitro (14, 15). Additionally, infected mice that were seroconverted to these fimbrial proteins (14), and mice immunized with BcfA-glutathione S-transferase (GST) and StfA-GST fusion proteins had reduced fecal shedding after challenge with S. Typhimurium (14). Homologs of bcfA and stfA are found in nontyphoidal Salmonella spp. and S. Paratyphi B and C, but not in E. coli. S. Typhi has a homolog of bcfA, but not stfA. stkE and stcD were also identified in our screen, as both are putative fimbrial genes involved in cell adherence. In mice infected with S. Typhimurium, StcD appears to be involved in gastrointestinal and long-term systemic infections (16, 17). Homologs of stcD are found in nontyphoidal Salmonella, but not in S. Typhi, S. Paratyphi B or C, or E. coli. The stk operon was reported at first to be specific for S. Paratyphi A (18, 19); however, a homolog of stkE appears to be present in the S. enterica serotype Heidelberg strain. Using RT-qPCR, we confirmed the increased in vivo expression of the transcript of stkE in the blood of patients with S. Paratyphi A bacteremia, suggesting that StkE may play some role in adherence in infected humans.

In addition to fimbrial antigens, we identified a number of cell envelope proteins in our analyses. Yjel, a putative membrane protein, has homologs in S. Typhi, S. Typhimurium, and E. coli. In E. coli, yjel is upregulated by external acidification (20). In our study, we confirmed upregulation of yjel in the blood of patients infected with S. Paratyphi A. YifL is a putative lipoprotein with homologs in various organisms, including S. Typhi, nontyphoidal Salmonella spp., E. coli, and also S. Paratyphi B and C. EnvE is a putative lipoprotein expressed by envE (SPA1604) located within Salmonella pathogenicity island 11 (SPI-11). SPI-11 contains a number of genes involved in S. enterica pathogenesis, including pagC, whose expression is regulated in vivo by the PhoP/Q regulon (21, 22). The PhoP/Q regulatory system controls a network of S. enterica genes involved in virulence and survival within macrophages (21, 22). Of note, envE is present in a variety of Salmonella spp. and is transcribed in the opposite direction of pagC. osmE is also found in S. Typhi, S. Typhimurium, and E. coli, and encodes an outer membrane protein. OsmE is induced by elevated osmolality and might be useful for adaptation to atypical conditions that typically lead to poor bacterial growth (23, 24). In a previous study, the gene expression profiles of S. Typhi-infected macrophages demonstrated upregulation of osmE compared to in vitro conditions.
We confirmed convalescent-phase reactivity to SPA0489 in human protein with NlpC/P60 and CHAP domains (40, 41). NlpC/P60 is a family of cell wall peptidases found in several bacterial lineages (40), with CHAP domains being associated with proteins that control catecholate-mediated iron uptake in E. coli (26). Homologs of both ycfL and ycfM are present in a wide variety of organisms, including both tiphoidal and nontiphoidal Salmonella spp. and E. coli.

Using IVIAT, we identified a number of S. Paratyphi A genes that encode proteins involved in energy metabolism, nutrient acquisition, and transport and binding (melB, napB, ldhA, scsD, cirA, yggG, SPA0489, and SPA2362), and we confirmed the increased expression of three of these genes (melB, napB, and ldhA) by RT-qPCR in the blood of S. Paratyphi A-infected patients. melB is present in a variety of organisms, including E. coli and different Salmonella species, and encodes a melibiose permease (27). In S. Typhimurium, melibiose permease is a symporter that couples melibiose transport with the transport of Na⁺, Li⁺, or H⁺ cations (28). scsD encodes the secreted protein ScsD, a suppressor of copper sensitivity that has homologs in a number of bacteria (29). S. Typhimurium ScsD restores copper tolerance in copper-sensitive E. coli mutants (29). Another metabolism- and nutrient-related gene identified by IVIAT is cirA, which encodes a colicin I receptor. During infection, S. enterica requires iron for survival, metabolism, and pathogenesis (10). CirA is an outer membrane receptor that controls catecholate-mediated iron uptake in Salmonella (30). CirA is vital for norepinephrine-enhanced growth and motility (31, 32), and cirA mutants colonize mice poorly. napB is a cytochrome c-type protein involved in anaerobic respiration in S. Typhimurium (33), suggesting a potential role in the survival of S. Paratyphi A in the in vivo environment.

The ldhA gene, encoding α-lactate dehydrogenase, is involved in pyruvate metabolism. In E. coli, this enzyme converts pyruvate to α-lactate under anaerobic and low-pH conditions (34). In an acidic pH environment, E. coli upregulates the expression of this enzyme by 10-fold (34), and the deletion of ldhA in E. coli results in reduced glucose consumption and bacterial growth (35). Active transport of metabolites via membrane-bound vesicles is energetically coupled to α-lactate dehydrogenase activity in E. coli (36).

YggG is a zinc-dependent metalloprotease with homologs in various pathogenic organisms, including S. Typhi, nontiphoidal Salmonella spp., and E. coli (37, 38). YggG is a membrane-associated heat shock protein that binds to the Era protein in stress response (38). Era is a membrane-associated GTPase and is important for the viability of E. coli (39). In E. coli under normal conditions, the expression of yggG is upregulated by heat shock and UV irradiation (39).

Using IVIAT, we also identified SPA0489; this gene encodes a protein with NlpC/P60 and CHAP domains (40, 41). NlpC/P60 proteins are a family of cell wall peptidases found in several bacterial lineages (40), with CHAP domains being associated with amidase function (41). SPA0489 is only present in Salmonella spp. We confirmed convalescent-phase reactivity to SPA0489 in humans recovering from paratyphoid fever, supporting the expression of this protein in vivo.

The S. Paratyphi A genome harbors three prophages, SPA-1, -2, and -3 (10). SPA-1 is a lambda prophage and encodes 47 genes (SPA2385 to SPA2431). SPA-2 and -3 are P2-type prophages designated SPA-2-SopE (SPA2554 to SPA2600) and SPA-3-P2 (SPA2601 to SPA2625) (10). We identified Gp19 (SPA2397), which is encoded in SPA-1, and SPA2612, which is encoded in SPA-3-P2, in our analysis. We confirmed convalescent-phase reactivity to Gp19 in humans recovering from paratyphoid fever, supporting the expression of Gp19 in vivo.

Our study has a number of limitations. IVIAT does not identify antigens expressed both in vitro and in vivo that might play important roles in pathogenesis and host-pathogen interactions. IVIAT also only identifies antigens that stimulate humoral immunity and does not identify antigens that induce only innate or cellular responses (5). Also, IVIAT does not predict whether any of the identified immune responses are involved in clearing infection or mediating host protection. It simply uses a comparative immunoscreening technique to identify antigens expressed in vivo that might warrant further evaluation. During IVIAT, we also adsorbed convalescent-phase serum using bacteria grown in LB broth at 37°C with aeration. It is very possible that protein expression profiles might vary under a range of in vitro conditions, but once again, the purpose of IVIAT is to identify antigens that might warrant additional analysis. Also, in our study, we were only able to confirm the specific upregulation of gene expression for 6 of the 20 identified genes using RT-qPCR. We suspect that this might reflect the very low median copy number of S. enterica organisms in the blood of infected humans, <1 CFU/ml, a reality that impedes mRNA profiling and quantification in human tiphoidal and paratyphoid fever (42).

In summary, we have identified 20 S. Paratyphi A proteins expressed in vivo during human infection that are immunogenic for humoral immunity. Some identified antigens are only present in Salmonella strains producing enteric fever and are not found in other Enterobacteriaceae. We confirmed the upregulated expression of IVIAT-identified genes in the blood of patients with S. Paratyphi A bacteremia. IVIAT-identified antigens warrant a focused evaluation to further understand their roles in S. Paratyphi A pathogenesis and potentially in diagnosis and disease control.

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