Acid Exposure Induces Multiplication of *Salmonella enterica* Serovar Typhi

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*Salmonella enterica* serovar Typhi faces several environmental stresses while going through the stomach (acidic pH) to the small intestine (basic pH) and intracellularly in macrophages (acidic pH) in humans. The acidic pH followed by alkaline pH in the small intestine might be responsible for expression of certain stress-induced genes, resulting in not only better survival but also induction of multiplication and invasion of the bacterium in the small intestine. Based on this hypothesis, we developed a process wherein we exposed the blood, urine, and stool specimens from 90 acute typhoid fever patients and 36 chronic typhoid carriers to acidic pH to see the effect on isolation rate of S. Typhi. About 5 g of freshly passed unpreserved stool, a centrifuged deposit of 15 ml of urine, and 5 ml of blood clot were subjected to 5 ml of Luria-Bertani (LB) broth (pH 3.5) for 20 min, followed by enrichment in bile broth-selenite F broth. When the combined isolation from all 3 specimens, i.e., blood, urine, and stool, after acid exposure was considered, a total of 77.7% of the acute typhoid patients were observed to be positive for the isolation of the S. Typhi serotype, compared to 8.8% by the conventional method. Similarly, 42% (15/36) of chronic carriers yielded positive for S. Typhi growth after acid exposure, compared to 5.5% (2/36) by the conventional method. It therefore can be concluded that acid shock triggers the multiplication of the bacteria, resulting in better isolation rates from blood clot, stool, and urine specimens.

The estimated global incidence of typhoid fever is about 26.9 million new cases with 1% mortality annually (1). This seems to be a gross underestimate, due to poor availability and performance of diagnostics in countries where typhoid is endemic (2). The available laboratory methods, i.e., bacterial culture, serological markers, antigen detection, and nucleic acid amplification, are highly unsatisfactory in the field conditions. There is an urgent need for optimization of culture methods because of the absolute specificity of culture isolation. High density (bone marrow) or large volume (>30 ml of peripheral blood) have been reported with satisfactory levels of sensitivity for *Salmonella enterica* serovar Typhi/Paratyphi isolated from acute typhoid fever cases. In patients with acute typhoid fever, the bacterial density has been reported to be 10-fold higher in bone marrow than in peripheral blood (3). However, both of the above options, i.e., higher blood volume or bone marrow aspirations, are not practical, even at the tertiary level of health care settings. Interestingly, a study from Vietnam reported a median count of 0.1 to 1.0 CFU of S. Typhi/ml of blood (range, 0.3 to 387) in cases of acute typhoid fever (4). It means 5 ml of routinely collected blood should have at least 5 bacterial cells sufficient enough to grow in artificial medium that are devoid of all antibacterial factors present in vivo. In routine practice, we hardly exceed 30% of the isolation rate of S. Typhi with the above-mentioned volume of blood. It is worth noting that the bacterium passes through a harsh acidic environment in the stomach, survives for hours, and then traverses to the duodenum, where it invades the mucosa of the small intestine in an alkaline environment. This phenomenon suggests the possibility of a triggering role of such pH variation from the stomach to the small intestine for the induction of S. Typhi multiplication and invasion. Therefore, we decided to assess the effect of initial brief acid exposure followed by inoculation in an alkaline environment on the isolation rate of human-restricted *Salmonella enterica* serotypes on different specimens collected from patients suffering from acute typhoid fever as well as from chronic typhoid carriers.

**MATERIALS AND METHODS**

**Standardization of acid stress tolerance of the S. Typhi bacterium culture on different acidic pH (2.0 to 11.0).** Already-grown reference strain S. Typhi (MTCC 3216) in log phase was added to physiological saline (0.85% NaCl) at a concentration of 3 × 10^7 CFU/ml, corresponding to 1 McFarland turbidity tube. About 10 ml of brain heart infusion (BHI) broth was distributed in 19 different tubes with pH adjusted to 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 10.0, 10.5, and 11.0 using a pH meter. The bacterial suspension (0.5 ml) was added to each of these tubes and exposed for different durations. Subcultures were made at different time intervals, 5, 10, 20, 30, 60, 120, and 180 min, after neutralizing the 500-μl aliquot with the help of phosphate-buffered saline (PBS) (pH 8.0) on MacConkey agar (MA).

**Standardization of different human leukocyte disruption methods (SDS, Triton X-100, CTAB, and brief sonication) and acid stress on culture isolation of S. Typhi.** A total of 10 blood clot samples from the Widal test (TO/TH ≥ 160 [TO, somatic antigen; TH, flagellar antigen])−positive patients were taken. About 5 ml of blood clot was divided in 4 equal parts after mechanical breakdown by using a sterile stick. The first part was exposed to solution containing 1% SDS and 0.1% Triton X-100 and the second to 1% cetyltrimethylammonium bromide (CTAB) for 5 min,
Acid Exposure Enhances Isolation Rate of S. Typhi

TABLE 1 Isolation of Salmonella Typhi from acute typhoid fever cases and chronic carriers by conventional and modified acid stress technique

<table>
<thead>
<tr>
<th>Group</th>
<th>Specimens</th>
<th>Isolation by conventional technique (%)</th>
<th>Isolation after acid stress (%)</th>
<th>Detection of S. Typhi by nested PCR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute typhoid fever (90) (Group A)</td>
<td>Blood clot</td>
<td>8 (8.8)†</td>
<td>48 (53.3)‡</td>
<td>76 (84.4)‡</td>
</tr>
<tr>
<td></td>
<td>Stool</td>
<td>0 (0.0)§</td>
<td>36 (40)§</td>
<td>60 (76.6)§</td>
</tr>
<tr>
<td></td>
<td>Urine</td>
<td>0 (0.0)†</td>
<td>5 (5.5)†</td>
<td>84 (93.3)†</td>
</tr>
<tr>
<td>Chronic typhoid carriers (36) (Group B)</td>
<td>Blood clot</td>
<td>1 (2.7)†</td>
<td>4 (11.1)†</td>
<td>8 (22.2)†</td>
</tr>
<tr>
<td></td>
<td>Stool</td>
<td>2 (5.5)†</td>
<td>15 (42)†</td>
<td>36 (100)†</td>
</tr>
</tbody>
</table>

To test the level of significance between two proportions i.e., isolation vs nested PCR positivity among acute and chronic typhoid carriers by Z-test.

A vs a = Z-6.25, P < 0.001; a vs a* = Z-4.51, P < 0.001; A vs a* = Z-10.0, P < 0.001; b vs b* = Z-3.59, P < 0.001; c vs c* = Z-11.78, P < 0.001; D vs d = Z-1.71, P > 0.05; d vs d* = Z-0.95, P > 0.05; E vs e = Z-3.61, P < 0.001; e vs e* = Z-5.45, P < 0.001.

while the third was subjected to ultrasonication for 1 s for mechanical disruption of human cells. However, the fourth part was exposed to pH 7.2 containing 0.2% sodium polyanethole sulfonate (SPS) and bile (pH 8.0) for incubation at 37°C overnight. Subcultures were made on deoxycholate citrate agar (DCA) and MA plates the next day.

**Study population.** The present study was conducted from January 2011 to July 2013 in Sir Sunderlal Hospital, Institute of Medical Sciences, Banaras Hindu University, Varanasi, located in the eastern part of North India, in collaboration with the Departments of Medicine, General Surgery, and Pediatrics. The study design and protocol were approved by the ethics committee of the university, and well-informed written consent was obtained from each of the participating patients/guardians. A total of 90 patients suspected of suffering from acute typhoid fever were confirmed positive for the presence of S. Typhi by nested PCR in either one or more of the blood, stool, and urine samples. Thirty-six apparently healthy chronic typhoid carriers were included on the basis of high titer against the Vi antigen (≥1:160) and further confirmed for S. Typhi-specific amplicon in their stool specimens by S. Typhi-specific nested primers.

**Collection of specimens from patients suffering from acute typhoid fever.** Approximately 5 to 10 ml of blood, depending on patient age, was collected from each of the 90 patients suffering from acute typhoid fever and from 36 chronic typhoid carriers and allowed to clot at room temperature. The serum was separated and preserved for serological study. The clot was divided in 3 equal parts, i.e., for culture by the conventional method, by modified acid stress techniques, and by nested PCR targeting S. Typhi flagellin gene-specific primers.

**Clot culture.** About 2 to 3 ml of blood clot was transferred to a 10-ml Falcon tube containing 2.5 ml of saponin (2 g/100 ml) in distilled water to break down the clot. The broken-down clot was decanted into a McCartney bottle containing 50 ml bile broth (pH 8.0). About 100 μl of the liquid clot was directly spread on MA and DCA plates for overnight incubation. The second part of the clot (2 to 3 ml) after mechanical breakdown was inoculated in 5 ml of LB broth (pH 3.5) for 20 min, which was further inoculated in 30 ml of bile broth (pH 8.0).

**Stool culture.** About 20 g of fecal specimen was collected in a wide-mouth sterile universal container from 90 patients suffering from acute typhoid fever. About 5 g of feces was directly transferred to the 10 ml of selenite F broth and incubated at 37°C overnight. Two to three loopfuls of fresh stool was inoculated directly on MA and DCA plates. The subcultures were also made after overnight enrichment in selenite F broth.

For acid exposure, about 5 g of the feces was mixed well into 5 ml Luria Bertani broth (pH 3.5) for 20 min followed by centrifugation at 3,000 rpm for 5 min. The deposit was washed twice with physiological saline. About 2 loopfuls of deposit was subcultured directly on MA and DCA plates, and the remaining deposit was transferred to selenite F broth for overnight incubation at 37°C.

**Urine culture.** The urine specimens were collected in a volume of 40 to 50 ml in sterile universal containers. About 15 ml urine was transferred into a 15-ml Falcon tube and centrifuged at 3,000 rpm for 5 min. From the pellet, one loopful of the deposit was inoculated on MA and DCA plates directly, while the remaining part was transferred to the Luria Bertani broth (pH 3.5) to expose for 20 min. This broth was centrifuged at 3,000 rpm for 5 min, followed by being washed with physiological saline. One loopful of the deposit was inoculated on MA and DCA plates. The remaining deposit was subjected to enrichment in bile broth (pH 8.0).

**Collection of specimens from patients suffering from chronic typhoid carriage.** Blood and stool specimens were collected from 36 confirmed cases of chronic typhoid carriage and processed as described above both for acid exposure and the conventional method of culture.

**Identification of isolates.** Following phenotypic and biochemical tests for the identification of the isolates as Salmonella, the isolates were confirmed by serological agglutination test using specific antisera, i.e., poly O, poly H, factor O9, Hd, and Vi antisera (5).

**Extraction of genomic DNA from blood, stool, and urine specimens.** About 2 to 3 ml of blood clot (third part) and a centrifuged deposit of 15 ml urine were subjected to DNA extraction using the phenol-chloroform proteinase K method (6). The stool specimens (3 to 5 g) from each of the study subjects were subjected to genomic DNA extraction using the method described by Van Zwet et al. with slight modification to minimize PCR inhibitors (7). The additional steps included 3 to 5 g of stool added to 10 ml of 10% formal saline (40% [wt/vol] formaldehyde and 0.85% [wt/vol] NaCl) and mixed well to make a suspension. Following this, 3 ml of ether was added and centrifuged at 3,000 rpm for 5 min.

**PCR amplification from blood, urine, and stool specimens targeting the flagellin (fliC) gene of S. Typhi.** Approximately 100 ng of extracted DNA from blood, stool, and urine specimens was subjected to PCR amplification targeting the S. Typhi-specific flagellin (fliC) gene sequence; primers used were designed by Song et al. and further modified by Frankel (8, 9). Forward ST1 (5'-ACTGCTAAAACCACTACT-3') and reverse ST2 (5'-TAAACCGTAAAGAGAG-3') primers for primary PCR amplification and forward ST3 (5'-AGATGTTACTGCGCGTTCGTC-3') and reverse ST4 (5'-TGGAGACTTCCGGTGATAGG-3') primers for nested PCR amplification were used to amplify 495-bp and 364-bp nucleotide sequences, respectively.

**Statistical analysis.** To test the level of significance between two proportions, the Z test was used.

**RESULTS**

The isolation rate from blood clots was 53.3% after brief acid exposure compared to 8.8% by the conventional method in the present study. While none of the stool and urine specimens yielded S. Typhi by the conventional method, acid exposure led to isolation of the bacterium in 40% of stool and 5.5% of the urine samples (Table 1). When the isolation from all 3 specimens after acid exposure was taken together, a total of 77.7% of the acute typhoid patients were observed to be positive for the isolation of the S. Typhi serotype, compared to 8.8% by the conventional method. The difference in isolation is statistically significant (P < 0.05) (Table 2). Further, it is interesting to note that of the 36 chronic typhoid carriers, 42% of stool and 11.1% of blood clot
samples were found positive for *S. Typhi* isolation after acid stress. The isolation rates from these two samples were 5.5% and 2.7% from stool and blood clot samples by the conventional method. The other observation worth mentioning was that 2.2% of urine from stool and blood clot samples by the conventional method. The isolation rates from these two samples were 5.5% and 2.7%.

**Table 2** Isolation of *Salmonella* species from blood clot, stool, and urine specimens after acid shock in Group A subjects (90)

<table>
<thead>
<tr>
<th>No. of patients with typhoid fever yielded positive for S. serotypes</th>
<th>Test result and total no. (%) with a positive result in the respective tests</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blood clot culture</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td>15</td>
<td>+</td>
</tr>
<tr>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>31</td>
<td>+</td>
</tr>
<tr>
<td>19</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td>70</td>
<td>48</td>
</tr>
</tbody>
</table>

**DISCUSSION**

In the present study, we tried to explore the effect of physical conditions mimicking the natural habitat of *S. Typhi*, which enters the stomach through contaminated food or water and multiplies actively in the small intestine. This enteropathogen is constantly under assault by a wide variety of environmental stresses, with acid stress being the most common. It is very interesting to see that after acid shock, >75% of the specimens collected from typhoid fever patients yielded *S. Typhi/Paratyphi* bacteria, in contrast to <10% by conventional culture methods. This observation is being reported for the first time. Our findings become very significant, because reports from South Asian countries have shown the isolation rate of human-restricted *Salmonella* spp. ranging between 9.25 and 21% by using only conventional methods (10–12). Similarly, the specimens collected from chronic typhoid carriers also yielded the bacterium in significantly larger percentages, 42% of cases by acid shock compared to 8.3% by the conventional method (*P* < 0.05). This implies that mild to moderate stress is essential for the induction of multiplication of dormant bacteria, as might be happening *in vivo*.

The observation made in the present study strongly indicates that human bodily defenses, i.e., gastric acid (pH 2.5 to 4.5), bile salt, alkaline pH, and organic acids within the gastrointestinal tract, pose a challenge to the microbes entering the gastrointestinal tract. But the bacteria which have evolved along with humans since time immemorial as facultative intracellular pathogens have adapted themselves not only to survive but to receive signals from these defense systems not only to inhabit, tolerate, and resist but also to divide and express virulence factors reaching the appropriate niche. Although, there are many reports showing resistance to low pH of the stomach mediated through acid tolerance response (ATR), acid resistance (AR), and acid habituation (13–16), the effects of these responses, as well as of the alkaline environment of the duodenum, jejunum, and ileum in multiplication of bacteria, are lacking. The O antigen of the 070 family has also been reported to play a critical role in adaptation and expression of virulence-associated genes among the pathogenic bacteria under stress (17, 18). The *ompR* and *rpoS* genes are known to be responsible for non-acid-inducible and acid-inducible ATRs in stationary and exponential phases. The ATR occurs upon entry into stationary phase as a general stress response (19). It is important to mention here that acid adaptations confer resistance to many other stresses, e.g., heat, salt, H₂O₂, and antibacterial molecules. It means that acid stress is more like a general stress response enabling the bacteria to endure a variety of environmental stresses (20). The details of these mechanisms are yet to be explored. Therefore, there is a strong need of understanding specific metabolic pathways that are upregulated under certain conditions. However, it is a major scientific challenge to identify mechanisms that give signals to bacteria, usually in stationary phase, coming from the harsh environmental conditions of the stomach and then entering the exponential phase inside the small intestine. The products of these stress-responding genes need to be characterized, as they might be playing a role not only in their survival but also in initiation of multiplication and expression of virulence traits (21, 22). Therefore, remodeling of clinical specimens, media, and incubation environment based on detailed information about the full regulon and the effects of different environmental factors on them will definitely result in better isolation of all those bacteria which are presently termed as viable but noncultivable.

**ACKNOWLEDGMENTS**

We gratefully acknowledge the financial support from the Council of Scientific and Industrial Research (CSIR), New Delhi, India, in the form of a Junior Research Fellowship awarded to Chandra Bhan Pratap.

There are no conflicts of interest to report.

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December 2014 Volume 52 Number 12 jcm.asm.org

4333

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