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# Immunoblot analysis of sera in uncomplicated typhoid fever & with typhoid ileal perforation

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***Background & objectives*: Ileal perforation is a serious complication of typhoid fever. The exact reasons for the development of perforation in only a few of those infected with *Salmonella* Typhi is unknown, and it is likely that immunological factors are involved. Therefore we undertook this study to compare the antibody profile in patients with uncomplicated typhoid fever with those having ileal perforation by immunoblotting.**

***Methods*: Two groups of patients were included in the study. Group II comprised patients with uncomplicated typhoid fever (n=47), and group I with typhoid ileal perforation (n=33). The flagellar (H), lipopolysaccharide (LPS) and outer membrane protein (OMP) antigens of *Salmonella* Typhi were extracted and used to test patient sera for antibodies by immunoblotting**

***Results*: Immunoblotting using *S.* Typhi antigens enabled the detection of *S.* Typhi antibodies in the two groups of patients. A significant difference was seen in the response of these two groups of patients with respect to antibodies to flagella, lipopolysaccharide and outer membrane proteins. Antibodies to flagella were more pronounced among patients with uncomplicated typhoid fever, while anti-OMP antibodies were significantly associated with typhoid ileal perforation.**

***Interpretation & conclusions*: A comparison of antibodies in patients with uncomplicated typhoid fever and with ileal perforation revealed the differences in the antibody profiles of the two groups. Our study suggests that the difference in antibody response may in some way play a role in the pathogenesis of typhoid ileal perforation which can also potentially be exploited to develop suitable diagnostic tests.**

**Key words** Immunoblotting - *Salmonella* Typhi - typhoid ileal perforation

The global burden of typhoid fever was estimated by the WHO at nearly 22 million cases per year, with a fatal outcome in about 210,000 of them[1]. The majority of these cases occur in the developing world, where health infrastructure is often inadequate and cases progress to complications like ileal perforation or encephalitis[1,2]. Without prompt medical intervention, the prognosis is poor. Despite several attempts, the mechanism of perforation is unknown. Either bacterial or host factors may be responsible. In a study analyzing bacterial factors, isolates of *Salmonella* Typhi from fatal cases of typhoid fever have been reported to show less diversity than those from non fatal cases[3].

A few studies have also looked into the immunological aspects. A Schwartzman like phenomenon has been proposed, and there is some evidence to support this hypothesis[4]. Although cell mediated immunity plays an important role in clearing *S*. Typhi infection, there is a paucity of information on the role of humoral immunity in typhoid perforation. In this study we attempted to assess the differences in antibody levels against certain antigens of *S. typhi* between patients with uncomplicated typhoid fever and typhoid perforation.

## Material & Methods

*Selection of subjects*: This study was carried out between January 2002 and June 2004, at the Jawaharlal Institute of Postgraduate Medical Education & Research (JIPMER), Puducherry. The study protocol was reviewed and approved by the Institute Research Council. Informed written consent was taken from the subjects or their guardians, as applicable. Subjects were selected consecutively over the study period, from Surgery and Medicine OPDs and in the case of the typhoid ileal perforation group, from among surgery inpatients as well. Two groups of subjects were included:

The first group (Group I) comprised 33 patients who had a clinical diagnosis of ileal perforation and had at least one test positive for typhoid fever. They were included in this group if they satisfied the following inclusion criteria: (*i*) Clinical features suggestive of typhoid fever and perforation-fever for more than 10 days, diarrhoea/constipation, acute abdominal pain of sudden onset, *etc*. and (*ii*) One or more microbiological tests positive culture, Widal agglutination, or Typhidot ELISA or, (*iii*) Histopathological evidence of typhoid at the site of perforation.

The exclusion criteria for this group included perforation secondary to obstruction, or due to trauma or other infections like TB.

Since microbiological evidence was difficult to obtain for this entity, a battery of tests was performed to determine the cause of perforation *viz.* culture of blood, bone marrow, stool and ileal biopsy samples. Blood and bone marrow cultures were performed using a biphasic brain heart infusion (BHI) medium, which were incubated for 7 days or till growth was seen in the bottles; these were subcultured onto blood and MacConkeys agar and the colonies identified by standard techniques[5]. The ileal biopsy sample obtained intraoperatively was transported in sterile saline. Initial enrichment was done in Selenite F broth, and BHI broth was also used. All media, chemicals and reagents for culture and immunoblotting were sourced from Hi-Media, Bangalore, unless otherwise specified. Serological tests, namely the Widal agglutination test and a commercially available ELISA, (Typhidot ®, MBDR, Malaysia) were performed. Histopathological examination of ileal biopsy was also done. All patients were resuscitated preoperatively using IV fluids and antibiotics. Wedge resection with anastomosis or segmental resection with anastomosis was performed at the discretion of the operating surgeon. Perioperative antibiotics given to the patients included combinations of ampicillin, metronidazole, gentamicin and ceftriaxone.

The 2nd group (Group II) included 47 patients with typhoid fever confirmed by blood culture and who did not manifest any complications. A Widal agglutination test was also performed for all cases.

Immunoblotting was performed for detecting antibodies to *S*. Typhi antigens- the flagella (H), lipopolysaccharide-LPS (O) and the outer membrane proteins (OMP) of *S*. Typhi O901, a non motile strain and its motile variant H901 both strains maintained in our laboratory[6]. Prior to testing patients' sera, the immunoblotting procedure was first standardized using serum from healthy controls and culture confirmed cases of typhoid fever to determine the optimal concentration of patient serum and reagents to be used.

*Extraction of flagellar antigen (H)*: The H antigen was extracted by heating a suspension of *S*. Typhi H901 cells at 60 ºC followed by centrifugation at 11269 *g* for 5 min. The flagellar antigens produced a single band of 51 kDa on SDS-PAGE which was performed on a minigel apparatus (Bangalore Genei, Bangalore)[7]. This band was of the appropriate molecular weight, and was absent in extracts of *S*. Typhi O901.

*Extraction of O antigen (LPS)*: The LPS was extracted from the O901 strain by the proteinase K method as described by Chart *et al*[7]. SDS-PAGE of LPS resulted in a ladder like separation of its components. The uppermost bands represent the larger O antigen bearing molecules and the lower bands represent the molecules with progressively shorter side chains and the core polysaccharides. Interpretation of the blotting results was derived using a grading 0-2[8]. A grade of 0 indicated the absence of reactivity to any of the bands;

grade 1, reactivity to the uppermost bands; grade 2 was applied to samples reacting to all the bands.

*Extraction of outer membrane protein (OMP)*: The outer membrane proteins were extracted from the O901 strain using the method of Santiviago *et al*. Six bands could be seen on SDS-PAGE namely a single band of 60 kDa, 3 bands of 37, 35 and 33 kDa which migrated closely together and 2 bands of 18 and 12 kDa. The migration pattern of 37, 35 and 33 kDa bands matched that of the porins OmpC, OmpF and OmpA respectively; the molecular weights were also in the appropriate range.

*Immunoblotting*: The extracted antigens were separated by SDS-PAGE, and then transferred onto nitrocellulose membrane (0.2 μm pore, BioTrace®NT membrane, Pall Gelman laboratories, USA) Transfer was performed using an Amersham Pharmacia Biotech (SF Corp, USA) unit at 100V for 1 h for the H and OMP samples, and at 45V for 45 min for LPS[10].

Serum samples from each patient were incubated with strips of blotted membranes at a dilution of 1:100 for 1 h for the H and OMP blots, and 1.5 h for the LPS blots. The strips were then washed and incubated with anti-human IgG-horse radish peroxidase conjugate for 1 h. Following washing, the strips were incubated with diaminobenzidine substrate for 5-10 min. The reaction was stopped using distilled water[10].

*Statistical analysis*: Data were analysed using the SPSS (version 10.0) and EpiInfo (2002) software. The odds ratio was calculated for quantifying the strength of the association and its significance was calculated using the Chi square test for calculating the *P* value for the categorical variables. Significance was taken as $P<0.05$.

## Results

In Group I (n=33, 4 females 29 males) the mean age of the patients was 30.5 ± 12.12 yr, and the average duration of illness was 8.9 days ± 3.9 days. *S*. Typhi was isolated from ileal biopsy of 6 patients. Three of these were multidrug resistant (MDR), and all were nalidixic acid resistant (NAR). All 6 isolates were indistinguishable from those of patients with uncomplicated typhoid fever with respect to the antibiogram and phage type (E1) previously reported by us[11]. Stool culture yielded *S*. Typhi in only one patient, and this isolate was also MDR and NAR. Blood and bone marrow cultures were negative in all cases.

In 22 patients either Widal and/or dot ELISA for anti-OMP antibodies was positive (19 IgM anti-OMP positive and 3 only IgG anti-OMP positive; and 9 Widal positive). Paired serum samples could be collected from 13 cases, but no rise in Widal titre was demonstrable, probably because the patients had been put on broad spectrum antibiotics. In 4 patients in absence of microbiological evidence, the gross and histopathological picture of ileal biopsy was taken as a marker of infection.

Group II comprised 47 patients; 42 of whom had significant Widal titres (≥1:160). Twenty three patients were female. The average age of these patients was 25.8 ± 13.91 yr, and the average duration of illness was 12.08 ± 7.8 days (Table I).

Eleven patients (33%) of group I and 33 (70%) in group II had antibodies to H antigen. Anti-H antibodies were significantly more in group II, *i.e.* uncomplicated typhoid fever as compared to group I [OR=0.21 (CI 0.07 to 0.61), $P<0.0001$].

In both groups I and II, the anti-LPS antibody response was pronounced in contrast to the anti-H antibodies. Overall, 24 (73 %) of group I and 37 (79 %) of group II were positive for antibodies to LPS (Table II).

The occurrence of different grades of reaction was compared for the 2 groups. A grade 1 reaction was significantly more common in group II than group I

**Table I.** Some clinical and laboratory characteristics of the two groups in this study

| Parameter | Uncomplicated typhoid (n=47) | Typhoid perforation (n=33) |
|---|---|---|
| Average age (yrs) | 25.8 | 30.5 |
| Duration of fever (days) | 12.08 ±7.8 | 8.9 ± 3.9 |
| Diarrhoea (days) | 1.7 ± 2.6 | ---- |
| Constipation (days) | ---- | 1.5 ± 2 |
| Vomiting (days) | 3.2 ± 4.4 | 1.7 ± 1.1 |
| Systolic blood pressure (mmHg) | 105.45 ± 12.13 | 99.7 ± 15.5 |
| Diastolic blood pressure (mmHg) | 70.45 ± 9.6 | 67.5 ± 7.5 |
| Pulse rate (beats/min) | 99 ± 20 | 102 ± 12 |
| Total WBC count (cells/cu mm) | 7523 ± 3421 | 12960 ± 1590 |
| Neutrophils (%) | 69.8 ± 9.8 | 67.1 ± 7 |
| Lymphocytes (%) | 26.3 ± 9.7 | 24.1 ±6.7 |
| Hemoglobin (g/dL) | 10.6 ±2.7 | 11.03 ± 1.6 |

Values are the mean ±SD for the groups

**Table II.** Detection of antibodies to LPS in patients' serum samples by immunoblotting

| Grade | Number of serum samples reacting in | |
|---|---|---|
| | Group 1 (n=33) | Group II (n=47) |
| 0 | 9 (27) | 8 (17) |
| 1 | 16 (48) | 37 (79) |
| 2 | 8 (24) | 2 (5) |

(Numbers in bracket indicate percentages)
Group I- Patients with typhoid ileal perforation
Group II- Patients with uncomplicated typhoid fever

**Table III.** Detection of antibodies to outer membrane proteins (OMP) in patients' serum samples by immunoblotting

| OMP (kDa) | Number of serum samples reacting in | |
|---|---|---|
| | Group I (n=33) | Group II (n=47) |
| 60 | 7 (21) | 20 (43) |
| 37 | 28 (85) | 19 (40) |
| 35 | 15 (45) | 13 (28) |
| 33 | 31 (94) | 45 (96) |

(Numbers in bracket indicate percentages)
Group I- Patients with typhoid ileal perforation
Group II- Patients with uncomplicated typhoid fever

**Table IV.** Detection of antibodies to outer membrane proteins (OMP) in patients' serum samples by immunoblotting- antibodies in combination

| OMP (kDa) | Number of serum samples reacting in | |
|---|---|---|
| | Group I (n=33) | Group II (n=47) |
| 60+37 | 6 (18) | 10 (21) |
| 37+35 | 15 (45) | 10 (21) |
| 37+33 | 27 (82) | 18 (38) |
| 35+33 | 14 (42) | 13 (28) |
| 37+35+33 | 14 (42) | 10 (21) |

(Numbers in bracket indicate percentages)
Group I- Patients with typhoid ileal perforation
Group II- Patients with uncomplicated typhoid fever

[OR=0.25 (CI 0.08 to 0.75), *P*=0.01] whereas Grade 2 reactivity was significantly more common among group I than group II [OR=7.2 (CI 1.26 to 53.57), *P*=0.01].

Anti-OMP antibodies were more common in group I than group II. With respect to the individual OMPs, it was seen that although antibodies were more common in group I than group II, the differences were not significant, with the exception of antibodies to the 37 kDa OMP [OR=8.25 (CI 2.44 to 29.72), *P*<0.01]. However, antibodies to both the 37 and 35 kDa [OR=3.08 (CI 1.40 to 9.25), *P*=0.04] and 37 and 33 kDa [OR=7.25 (CI 2.26 to 24.35), *P*<0.01] were significantly more common among group I patients than in group II. Antibodies to all 3 of these OMPs was also significantly associated with group I [OR= 2.73 (CI 0.92 to 8.20), *P*=0.038] than group II (Table III & IV).

Antibodies to the 18 and 12 kDa OMPs were seen in only 2 samples of group I. Due to the low positivity, tests of significance were not performed.

## Discussion

Perforation is a serious but poorly understood complication of typhoid fever the worldwide incidence about 0.5-15 per cent[12]. As with other studies, we noticed a male preponderance in our study population[4,13,14]. Patients with ileal perforation in our study were, on average, older than those with typhoid fever (median age 31 versus 26 yr). This was in concordance with a previous study from this region, where most patients were in the second and third decades of life[13]. In other studies, the median age ranged from 19-36 yr[4,15,16].

Although commonest after the 2nd week of illness, there have been reports of patients presenting with perforation in the first week of illness[4,13,17]. In our study the median length of illness was just under 9 days in the perforation group, while, interestingly, the patients with uncomplicated typhoid fever had a longer mean duration of illness. Perhaps in a previously sensitized individual in endemic areas, the infection may not follow the classical course, and it is possible that perforation may occur in the first week itself; *i.e.,* in patients with typhoid perforation, there is a state of hyper-responsiveness probably induced by repeated subclinical exposure to *S.* Typhi.

In this study, one interesting feature was the low number of positive cultures in group I. Other studies have also reported poor yields from blood or bone marrow ranging from 11-27 per cent[4,13,17]; stool recovery even reported negative in some studies[18]. The novel idea of culturing ileal biopsies was adopted in this study for the purpose of increasing the recovery of *S.* Typhi.

Antibodies in the 2 groups of patients, differed from each other qualitatively (and quantitatively with respect to the Widal test). In the majority of the group II cases, the Widal test was positive, suggesting that this test can be a reliable indicator of infection with *S.* Typhi in uncomplicated cases if interpreted with caution. The Widal test positivity was low in

the patients with typhoid perforations in this study. Other studies have commented on the low positivity, and also that titres are generally lower among typhoid perforation cases[19,20]. This reflects that dependence on culture or Widal test alone is insufficient to confirm typhoid perforation.

The significant association of anti-H antibodies with uncomplicated typhoid fever seen here suggests that these may protect against ileal perforation. Studies using typhoid vaccines have previously shown that these antibodies are protective[21]. The paucity of antibodies to flagella among typhoid perforation patients points to its possible role in modulating the severity of the infection.

LPS molecules have been shown to be heterogenous, even within a single strain[22]. In this study, uncomplicated typhoid fever the antibodies are directed to the O antigen in its entirety, while in patients with ileal perforation there was a significant reaction to the molecules bearing smaller side chains and core polysaccharides as well. It would appear that antibodies against the larger LPS molecules are more effective in clearing the bacteria, presumably because these are the antigens that are most exposed on the surface of the bacteria. It is possible that subsequent exposure to *S*. Typhi in a previously sensitized individual results in an exaggerated antibody response to the smaller LPS molecules as well.

Antibodies to the OMP of estimated molecular weight 60 kDa were more prominent among patients with typhoid fever, whereas those directed against the 37 kDa OMP were more common among patients with perforation. In patients with ileal perforation, antibodies to 3 OMPs namely, 37, 35 and 33 kDa, either together or in combinations of 2 (37 and 35 kDa, 37 and 33 kDa) were more pronounced. There was a poor humoral response to relatively high molecular weight proteins like the H antigen among patients with perforation as compared to patients with typhoid fever. The flagellar antigen is strongly immunogenic[22] and it is intriguing that patients with perforation do not possess antibodies to it. The absence of antibodies could have been attributed to the short duration of illness; however, antibodies to OMPs were detectable at the same time. These findings suggest that ileal perforation patients show a selective reactivity.

Though, ileal biopsy cultures were positive in some cases, we suggest that the bacteria may not have a direct role in the pathogenesis of the disease. Further evidence for this hypothesis can be obtained from the studies of Massi *et al*[23], who reported that there were no differences among isolates from cases of ileal perforation or uncomplicated typhoid fever. It has been previously suggested that a Schwartzman like phenomenon may be responsible[24]. This study did not involve an analysis of this aspect. We put forward the hypothesis that more than the bacteria, the antibody response to some of the bacterial antigens may play a role in perforation, which may be the outcome of an exaggerated immune response. The short duration of the illness lends credibility to this hypothesis. In an endemic area, subclinical exposure to *S*. Typhi is very common, and could account for the initial encounter of *S*. Typhi antigens. On subsequent exposure, the immune response would be mounted rapidly, and there would be a quicker onset of symptoms. It has been previously reported that immune complexes are more common in patients with ileal perforation than with uncomplicated typhoid fever[25]. Therefore, it is also possible that a combination of hyper-responsiveness to LPS and OMP antigens could contribute to the formation of immune complexes at the Peyer's patches, thereby inducing tissue destruction and ultimately perforation.

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