Salmonella species in a neonate with necrotizing enterocolitis

R. Chaudhry¹, N. Sharma¹, S. Bhushan², V.K. Paul², M. Singh², P. Panigrahi³

INTRODUCTION

Necrotizing enterocolitis (NEC) is a disorder that occurs in newborns of low birth weight babies (< 1200 gm) particularly when they have suffered from asphyxia, apnoeic attacks, hyaline membrane disease or hypothermia. These stress conditions give rise to intestinal ischaemia with devitalization, infection and necrosis. Association of NEC is well known with E. coli and with other enteric pathogens. Salmonella infection in premature baby with NEC have been reported earlier with single serotype namely Salmonella i.e. S. thompson. We are reporting here a unique case of necrotizing enterocolitis in a newborn baby where more than one serotype of Salmonella sp. were isolated from stool along with E. coli, thus highlighting polymicrobial etiology of NEC in premature baby.

CASE REPORT

A male baby was born on 29 Dec 1996 by emergency caesarean and was admitted to the nursery. He was a preterm baby of 31+wks. gestation weighing 1.249 kg. Indication for emergency LSCS was imminent eclampsia, baby had apgar score at 1min of 7/10 and at 5 min of 9/10. He was lethargic from day 1 and improved with nelozone. He developed hyperbilirubinemia on day 5 (serum bilirubin level 12.9 mg/dl) for which phototherapy was given for 48 hrs.

On day 8 he had abdominal distension, persistent metabolic acidosis and apnoea. His stool for occult
blood was also positive. The baby was diagnosed as having NEC and antibiotic therapy was started with Cefotaxime, Amikacin, Vancomycin and Metronidazole. However, despite these he developed perforation on day 11, patient was managed conservatively. A left side drain was complicated by fecal fistula. He was also having congenital cardiac anomaly in the form of PDA on day 16, chest infecstion developed on day 48 and infective diarrhea on day 60th. The baby was started on injection of Cefotaxime and Amikacin for infective diarrhea and he gradually improved.

MATERIALS and METHODS

Quantitative method for isolation of stool flora

Stool samples were collected on day 9, 19 and 30. Aseptically collected stool sample weighing 0.1 gm in sterile vial and 1 ml of sterile normal saline was added with 2-4 sterile glass beads and then vortexed. Ten fold dilution (10-1 to 10-8) were made and plated 10 ml of each dilution to 5% sheep blood agar and MacConkey agar for aerobic organism and Brain heart infusion agar for anaerobic organisms. Both aerobic and anaerobic organism were identified by using conventional, biochemical test and rapid automated tests such as AP120 E test, API staph/strep and Rapid ANA II system (bioMérieux Vitel, Inc).

PCR for stool and blood

i) Isolation and detection of DNA from stool and blood samples

The DNA was isolated from 200 µl of diluted stool sample and 200 µl of blood using QIA amp blood kit, according to manufacturer instructions. To the sample 25 µl of QIAGEN protease and 200 µl buffer AL were added, vortexed and incubated in water bath at 70°C for 10 min. 210 µl of isopropanol or ethanol was added to the sample and mixed again by vortexing. A QIA amp spin column was placed in a 2 ml collection tube. The above mixture was applied on QIA amp spin column and centrifuged at 8000 rpm for 1 min. The QIA amp spin column was placed in a clean 2 ml collection tube and washed with 500 µl of buffer AW. It was then centrifuged for 1 min at 8000 rpm. The QIA amp column was placed in a clean 2 ml collection tube, again 500 µl of buffer AW was added and centrifuged for 2 minutes at 8000 rpm. Then the QIA amp spin column was placed in a clean 1.5 ml microfuge tube, DNA was eluted with 50 µl of buffer AE (Pre heated to 70°C). And incubated at room temp. for 1 min and centrifuged for 2 minutes at 8000 rpm. The eluent contained DNA and was used for PCR.

ii) PCR for S.typhi

PCR was performed according to methods described before by us6. The reaction mixture for the first round of PCR contained 5 µl of DNA, 25 picomoles of RK1 and RK2, primers, 200 µl of (each) all four Deoxyribonucleoside Triphosphates, 0.75 units of Taq polymerase (Bangalore Genie, India ) and standard PCR buffer (100 mM Tris-HCl, 1.5 mM MgCl2 , 50 mM KCl, 0.1. Gelatin, pH 8.3) in a final volume of 25 µl. Amplification in an automated DNA thermal cycler (MJ. Research Inc. US) consisting 40 cycles at 94°C for 1 min (Denaturation ) 57°C for 1 min 15 sec. (an-nealing) and 72°C for 3 min (Polymerization ). After the reaction, 7.5 µl of amplified products of the first PCR was transferred to a IIth reaction mixture (17.5 µl), containing 50 picomoles (each) of RK3 and RK4 primers for the nested PCR7. The nested PCR was performed for 35 cycles at 94°C for 1 min, 68°C for 1 min and 15 sec and 72°C for 3 min.

iii) Detection of PCR products

The DNA fragments of the flagellin gene of S.typhi amplified by the PCR were identified by agarose gel electrophoresis, 8 µl of the amplified products from the both rounds of PCR were electrophoresed on a 1% gel for 60 min at constant 100 volts with TBE buffer (90 mM Tris borate, 2mM EDTA) molecular sized marker (lambda Hind III Bangalore, Genie, India) was run concurrently. The gels stained with ethidium bromide were examined under U.V. illumination for the presence of 486 or 342 bp bands.

RESULTS

1) Isolation of organism from stool

Three samples of stool which were cultured on different dates yielded E.coli and S.gallinarum on day 9, coagulate negative Staphylococcus and S.typhi on day 19 and only S.senftenberg on day 30. Isolated Salmonella sp. were further confirmed by API 20E (Table 1).

2) PCR for stool

All the three stool samples were processed by PCR for S.typhi. A specific band of 486 bp was detected only in the second sample (day 19) which was also positive for S.typhi isolation, while first and third samples were negative.
3) PCR for blood
Blood sample was negative for first round of PCR with RK1 and RK2 primers, whereas nested PCR using RK3/RK4 primers yielded 342 bp band on gel electrophoresis (Table 2 and 3).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Day of culture</th>
<th>Isolated organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Stool</td>
<td>9</td>
<td>E.coli, S.gallinarum</td>
</tr>
<tr>
<td>2. Stool</td>
<td>19</td>
<td>Staph coag-ve, S.typhi</td>
</tr>
<tr>
<td>3. Stool</td>
<td>30</td>
<td>S. senftenberg</td>
</tr>
<tr>
<td>4. Blood</td>
<td>19</td>
<td>No organism isolated</td>
</tr>
</tbody>
</table>

Table 1. Isolated organism from samples

4) PCR for isolated organism
All the isolated organisms including three Salmonella sp., E.coli and Staphylococcus were again subjected to PCR for specificity and sensitivity. Only S.typhi gave band of 486 bp while all other strains were negative for PCR. (Table 4).

<table>
<thead>
<tr>
<th>Isolated organism</th>
<th>PCR with specific primer for S.typhi</th>
</tr>
</thead>
<tbody>
<tr>
<td>E.coli</td>
<td>Negative</td>
</tr>
<tr>
<td>S.gallinarum</td>
<td>Negative</td>
</tr>
<tr>
<td>Staph coag-ve</td>
<td>Negative</td>
</tr>
<tr>
<td>S.typhi</td>
<td>Positive</td>
</tr>
<tr>
<td>S.senftenberg</td>
<td>Negative</td>
</tr>
</tbody>
</table>

Table 4. PCR for isolated organism

Blood culture was found to negative for S.typhi isolation as well as for nested PCR. Widal test for both baby and mother was also negative in a titre < 1:40 for TO and TH antigens.

6) To trace the source of S.typhi in this baby, samples of all the medical and paramedical staffs were screened for S.typhi by Widal test culture and PCR. The stool sample collected from one of the paramedical staff working that time in the neonatal intensive unit was positive by PCR for S.typhi where as stool culture remained to be negative for S.typhi.

DISCUSSION

Necrotizing enterocolitis is complicated by bacteremia or peritonitis5. Early reports implicating some bacteria were tempered when recovered organism were those that typically colonize the intestinal tracts of premature infants. Such colonizing organisms including aerobes (coagulase negative staphylococcus, facultative anaerobes (Enterobacteriaceae and entero- cocci) and strict anaerobes (Bacteroides, Clostridium sp). An enrichment of certain colonizing bacteria in cohorts of symptomatic infants has been reported8-10 in NEC, but considerable overlap with the flora content of unaffected infants usually exist11. Overgrowth of a single predominant stool organism has been implicated in NEC development12,13.

This is remarkable that NEC has been associated with most well known enteric pathogens14. In a few instances in which enteropathogens such as Salmonella sp. and enteroxinogenic E.coli were identified in outbreaks among neonates, presenting symptoms were diarrhea5,15. Whereas this patients did not present with diarrhoea initially.

In this case of NEC Salmonella sp. were isolated thrice from stool samples. However the unique finding was that all Salmonella sp. were of different serotypes, identified by rapid system of biochemical (BioMérieux Vitek, Inc.). Isolated S.typhi from fecal samples were further confirmed by PCR. In order to trace the source of S.typhi, mother’s blood/stool processed for culture and PCR were reported to be negative. Samples (stool and blood) of all the medical and para medical staffs were also subjected to culture and PCR for S.typhi. Out of which one of the para medical staff was found to be positive for S.typhi by PCR in both stool/ blood whereas cultures remained negative. Her Widal test was also positive for TO and TH antibodies in hightitres (> 360).

5) Mother’s blood for isolation of S.typhi was collected to trace the source of infection with S.typhi in baby.
The transient existence of *S. gallinarum* and *S. typhi* in stool is amazing, both of these serotypes were later replaced by third type of *Salmonella* sp. i.e. *S. senftenberg* (Gp-E). It appears that multiple serotypes of *Salmonella* sp. were circulating in the environment thus competing with each other for their localisation in the gut of premature infants. One of these is able to colonize in the intestinal flora depending on availability of suitable nidus for their existence in gut mucosa. Polymicrobial/multifactorial aetiology of necrotizing enterocolitis in premature baby is highlighted in the etiopathogenesis of this disease. Use of rapid molecular techniques such as PCR for identification of etiological agents is indicated in tracing the source of infection and for specific management of necrotizing enterocolitis.

In addition to stool culture, PCR for amplification of *Salmonella* sp. specific DNA sequences using specific primers may emerge as a rapid diagnostic tool for identification of etiological agents in NEC. Thus helping to provide specific and appropriate rapid management of premature babies.

REFERENCES