Salmonella species in a neonate with necrotizing enterocolitis

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Abstrak

Dilaporkan di sini, bayi laki-laki, preterm, kehamilan 31 minggu, berat 1,249 kg, masuk ke unit perawatan intensif neonatus (NICU) di AIIMS, New Delhi. Bayi ini mengalami kembung, asidosis metabolik, serangan apnoe, tinja lunak/encer dan tes darah samar positif, berdasarkan gambaran klinis tersebut bayi itu didiagnosis sebagai enterokolitis nekrotikans (Bell stage 1). *Tiga sampel tinja (hari 9, 19 dan 30) dan satu sampel darah (hari 19) diperiksa. Ditemukan 3 serotip* Salmonella *sp. yang berbeda dari ketiga sampel tinja tersebut* (S. gallinarum *pada hari 9,* S. typhi *hari 19, dan* S. senftenberg *hari 30) bersama-sama dengan* E. coli *dan* Staphylococcus *koagulase negatif menggunakan pemeriksaan biokimiawi konvensional dan kit identifikasi cepat (BioMérieux, Vitek, Inc). Reaksi rantai polimerasi (PCR) untuk deteksi gen flagelin spesifik* S. typhi *dilakukan pada sampel tinja dan darah. Sampel tinja memberikan hasil positif pada PCR satu tahap sementara sampel darah positif pada* nested PCR. *Walaupun demikian, sampel darah ibu memberikan hasil negatif* S. typhi *pada PCR. Bayi tersebut memberikan respon pada pengobatan suportif, pemberian antibiotika sefotaksim dan amikasin, dan dipulangkan setelah 2 bulan. Laporan ini menekankan penyebab mikroba multipel dan infektif dari NEC serta penggunaan teknik molekuler cepat seperti PCR untuk mengidentifikasi etiologi, yang mungkin akan memberikan pandangan baru tentang patogenesis NEC.*

Abstract

We report, a male preterm baby of 31 weeks gestation weighing 1.249 kg, admitted to the neonatal intensive care unit (NICU) at AIIMS, New Delhi. He had abdominal distension, metabolic acidosis, apnoic attacks and loose stools that was positive for occult blood, on the basis of above clinical features the baby was diagnosed to have Necrotizing enterocolitis (Bell stage I). Three samples of stool (day 9, 19, 30) and one sample of blood (day 19) were processed. Three serotype of Salmonella *sp. (*S. gallinarum *day 9,* S. typhi *day 19 and* S. senftenberg *day 30) were isolated at different occasions from stool samples along with* E. coli *and coagulase negative* Staphylococci *by using conventional biochemical and rapid identifications kits (BioMérieux Vitek, Inc). Polymerase chain reaction (PCR) for detection of specific flagellin gene sequence of* S.typhi *was applied on stools and blood samples of baby. Stool was positive for* S. typhi *by one round of PCR while blood was positive by nested PCR. However mother's blood was found to be negative by PCR for* S. typhi. *The baby responded to supportive treatment, antibiotic therapy with cefotaxime and amikacin and was discharged after a period of two months. Polymicrobial and infectious etiology of NEC and the use of rapid molecular techniques such as PCR for identification of etiological agents is highlighted which may provide newer insights into the pathogenesis of Necrotizing enterocolitis (NEC).*

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[1,2]. Association of NEC is well known with *E.coli* and with other enteric pathogens[3]. Salmonella infection in premature baby with NEC have been reported earlier with single serotype namely Salmonella i.e. *S. thompson*[4,5].

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We are reporting here a unique case of necrotizing enterocolitis in a newborne 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 neloxone. He developed hyperbilirubinemia on day 5 (serum billirubin 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 feacal fistula. He was also having congenital cardiac anomaly in the form of PDA on day 16, chest infection developed on day 48 and infective diarrhoea on day 60th. The baby was started on injection of Cefotaxime and Amikacin for infective diarrhoea 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 Vitek, 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 us[6]. 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 (Bangelore Genei, India) and standard PCR buffer (100 mM Tris-HCl, 1.5 mM $MgCl_2$, 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.(annealing) and 72°C for 3 min (Polymerization). After the reaction, 7.5 µl of amplified products of the first PCR was transferred to a IInd reaction mixture (17.5 µl), containing 50 picomoles (each) of RK3 and RK4 primers for the nested PCR[7]. 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 Banglore, Genei, 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, coagulase 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.

Table 1. Isolated organism from samples

Sample	Day of culture	Isolated organism
1. Stool	9	*E.coli, S.gallinarum*
2. Stool	19	Staph coag-ve, *S.typhi*
3. Stool	30	*S. senftenberg*
4. Blood	19	No organism isolated

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 2. PCR for *S.typhi* from stool

Sample	PCR with specific primer for *S.typhi*
Stool	Positive
Blood	Negative

Table 3. Nested PCR for *S.typhi* (Rk1, Rk2, Rk3 and Rk4) from blood

Sample	Culture	Widal	Nested PCR
Blood	Negative	Negative	Positive

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 4. PCR for isolated organism

Isolatated organism	PCR with specific primer for *S.typhi* Rk1 and Rk2
1. *E.coli*	Negative
2. *S.galinarum*	Negative
3. Staph coag-ve	Negative
4. *S.typhi*	Positive
5. *S.senftenberg*	Negative

5) Mother's blood for isolation of *S.typhi* was collected to trace the source of infection with *S.typhi* in baby.

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 peritonitis[3]. Early reports implicating some bacteria were tempered when recovered organism were those that normally, colonize the intestinal tracts of premature infants. Such colonizing organisms including aerobes (coagulase negative staphylococcus, facultative anaerobes (Enterobacteriacae and entrococci) and strict anaerobes (Bacteroides, Clostridium sp). An enrichment of certain colonizing bacteria in cohorts of symptomatic infants has been reported[8-10] in NEC, but considerable overleap with the flora content of unaffected infants usually exist[11]. Overgrowth of a single predominant stool organism has been implicated in NEC development[12,13].

This is remarkable that NEC has been associated with most well known enteric pathogens[14]. In a few instances in which enteropathogens such as *Salmonella* sp. and enterotoxigenic *E.coli* were identified in outbreaks among neonates, presenting symptoms were diarrhoea[5,15]. 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).

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 nectrotizing 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 nectrolizing 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.

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