Molecular typing of *Salmonella enterica* serotype Worthington isolates from infantile diarrhoea

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**Background & objectives:** *Salmonella* Worthington has been known to be a causative agent for childhood diarrhoea. There is a paucity of information on the molecular relatedness of the strains isolated in various hospitals in India. The present study was carried out to attempt molecular typing of a cluster of *Salmonella* Worthington isolates obtained from cases of infantile diarrhoea during a six month period, from a tertiary care paediatric hospital in Delhi, India.

**Methods:** Nine isolates of *S*. Worthington obtained from faecal samples of infants suffering from diarrhoea during October 2001 to March 2002, were identified by the conventional biochemical methods and by serotyping. The antimicrobial susceptibility was determined by the disk diffusion method. Molecular typing was done by ribotyping.

**Results:** Eight patients were admitted to 3 different wards of the hospital and one was an outpatient. Four patients including the first patient visited the hospital with diarrhoea as the presenting symptom while five developed diarrhoea after admission. Stool microscopy showed no specific findings. *Salmonella* Worthington was isolated from stool cultures of these patients. Repeated cultures of the common drinking water source of the hospital and the milk supplied to children from central kitchen were negative for known pathogens. All *S*. Worthington isolates were resistant to all the beta-lactams tested including third generation cephalosporins. Eight isolates were sensitive to furazolidone and 6 to ciprofloxacin. Molecular characterization by ribotyping revealed four different clones.

**Interpretation & conclusion:** As four different ribotypes of the isolated *Salmonella* Worthington isolates were identified, it was clear that there was no single source of infection.

**Key words** Gastroenteritis - molecular typing - *Salmonella* Worthington

Diarrhoeal diseases continue to be the major cause of childhood morbidity and mortality in developing countries. *Salmonella* is one of the predominant bacteria causing gastroenteritis in infants and children. Diarrhoea due to non-typhoidal salmonella refers to the disease caused by serotypes of *Salmonella* other than *Salmonella enterica* serotype Typhi. The highest incidence rates of non-typhoidal
Salmonella infection occurs in children less than 5 yr of age, especially those less than 1 yr, and in individuals over 70 yr. Transmission of non-typhoidal salmonella to humans occurs by ingestion of contaminated food, water and contact with infected animals or contaminated medical instruments. Though many virulence factors have been identified in Salmonella enterica serotypes Typhimurium and Typhi, the underlying reasons for different host specificities and disease outcome of various serotypes are not fully understood. Salmonella enterica subspecies enterica serotype Worthington (Salmonella Worthington) was isolated for the first time in 1937 from an animal source. Since then it has been reported from various animal and human sources. This serotype has been reported to be causative agent in several outbreaks of septicaemia and meningitis in nurseries and neonatal units. But none of the Indian studies have attempted to determine the molecular relatedness of the isolates. The present study was undertaken to do molecular typing of a cluster of nine isolates of S. Worthington from infants with diarrhoea over a period of 6 months in a paediatric care hospital in north India, to determine their clonality.

Material & Methods

A total of nine isolates of S. Worthington were identified as sole pathogens from culture of stool specimens at the Department of Microbiology, Lady Hardinge Medical College, New Delhi, India. These isolates were from inpatients and outpatients attending the paediatric care hospital (Kalawati Saran Children’s Hospital, New Delhi) during October 2001 to March 2002. Kalawati Saran Children’s Hospital is a 360 bedded paediatric hospital with three wards, a neonatal unit and outpatient department.

Gross and microscopic examination of faecal samples was done followed by inoculation onto MacConkey agar, deoxycholate citrate agar (DCA), xylose lysine desoxycholate agar (XLD) and selenite broth (Hi-Media Laboratories, Mumbai, India) that was subcultured on DCA after 6 h of incubation. Plates were incubated overnight at 37°C. Non-lactose fermenting colonies were identified by standard biochemical reactions. Serotyping was done by the Kauffmann-White Scheme and the isolates were identified as Salmonella group G in the laboratory. National Salmonella and Escherichia Centre, Central Research Institute, Kasauli, India later confirmed them as S. Worthington (1,13,23;1,w,l:z). Rotavirus, though a common pathogen in this age group was not looked for in the stool specimens. Blood and cerebrospinal fluid of the patients were also cultured where clinically indicated.

Antimicrobial susceptibility was determined by the Kirby Bauer disk diffusion method and interpreted according to the National Committee for Clinical Laboratory Standards (NCCLS) guidelines. The isolates were tested for susceptibility to amikacin (30 µg), ampicillin (10 µg), amoxycillin+clavulanic acid (20+10 µg), ampicillin+ sulbactam (10+10 µg), cefotaxime (30 µg), ceftazidime (30 µg), ceftriaxone (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), furazolidone (100 µg), gentamicin (10 µg) and norfloxacin (10 µg) (Hi-Media Laboratories, Mumbai, India).

The medical and laboratory records of all nine patients as well as the laboratory records of other cases of diarrhoea in the similar age group, whose stool specimens were submitted during this period, were reviewed. An effort was made to identify the source. The common drinking water source of the hospital and the milk supplied to children from the central kitchen were subjected to microbiological analysis using standard methods.

All isolates except one, which was lost during storage, were subjected to ribotyping in order to determine their clonality. The total cell DNA from the bacterial isolates was extracted using the method of Boom et al. About 5 µg of the extracted DNA was completely digested with 20U of PvuII following the manufacturer’s recommendations (New England Biolabs, USA). The generated DNA fragments were separated by horizontal agarose gel electrophoresis on 0.8 per cent agarose gel using HE 99X apparatus (Pharmacia, LKB, Sweden). Digoxigenin-labelled DNA molecular weight marker II (Boehringer-Mannheim, Germany) was included in each gel. The gel was depurinated by soaking in 0.25N HCl and...
subsequently in 0.5M NaOH-0.5M NaCl and neutralized with 3M NaCl-1 M Tris-HCl (pH 8.0). DNA fragments were transferred to a nylon membrane (Boehringer Mannheim, Germany) overnight using 20 x SSC as the transfer solution. The transferred DNA was fixed to nylon membrane by UV-cross linking for 5 min using ordinary transilluminator (300 nm, Ultralum, USA). Using commercial 16S and 23S rRNA of *Escherichia coli* (Boehringer-Mannheim, Germany), avian myeloblastosis virus reverse transcriptase and DIG-dUTP, a DIG labeled cDNA probe was synthesized. After hybridization and post-hybridization washes, the hybrids on the membrane were detected by an enzyme immunoassay using alkaline phosphatase-conjugated anti-digoxigenin antibodies and colour substrate mixture of nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP).

**Results**

The age of infants ranged from 20 days to 1 yr. The isolates were confirmed to be serotype Worthington (1,13,23:1, w, i: z) at the National Salmonella and Escherichia Centre, Central Research Institute, Kasauli, India. Eight of these nine isolates were obtained from inpatients and one from an outpatient. Blood and CSF were sterile. The patients were admitted in different wards of the hospital. Five patients developed diarrhoea after 4-15 days of admission while 4 children including the first case were admitted with diarrhoea as the presenting symptom (Table). All children were on bottle feeds. Gross examination revealed the stool specimens to be watery in consistency with no blood. On microscopic examination most of the stool specimens showed presence of mucus, which is a nonspecific finding.

Antimicrobial susceptibility test showed all isolates to be resistant to all the beta-lactams tested i.e., ampicillin, ampicillin+clavulanic acid, ampicillin+sublactam, cefotaxime, cefaizidine, and ceftriaxone. Six isolates were susceptible to ciprofloxacin, 6 to norfloxacine, 2 to chloramphenicol, 2 to gentamicin and 1 to amikacin. All isolates except one were susceptible to furazolidone. Ribotyping could ascribe the isolates to four different ribotypes (Fig.). Isolates in lanes 2, 3, 5 and 8 belonged to ribotype I, 6 and 9 to ribotype II, and 4 and 7 had unique ribotype patterns, type III and IV respectively. Comparison of ribotype patterns and antibiograms of the isolates showed no association.

Review of laboratory records showed that during the study period a total of 130 stool specimens of children less than 5 yr were processed. Of these, 26 were culture positive for bacterial pathogens namely *Salmonella* Worthington (9), *Vibrio cholerae* (9), *Salmonella* Typhimurim (2), *Salmonella*

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age</th>
<th>Sex</th>
<th>Diagnosis at admission</th>
<th>Microscopic observations of faecal samples</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11 months</td>
<td>M</td>
<td>Acute gastroenteritis with bronchopneumonia</td>
<td>Mucus</td>
<td>Discharged</td>
</tr>
<tr>
<td>2</td>
<td>1 month</td>
<td>M</td>
<td>Sepsis with keratomalacia</td>
<td>Mucus</td>
<td>Discharged</td>
</tr>
<tr>
<td>3</td>
<td>28 days</td>
<td>F</td>
<td>Pyogenic meningitis with gastroenteritis</td>
<td>No abnormal findings</td>
<td>Discharged</td>
</tr>
<tr>
<td>4</td>
<td>20 days</td>
<td>M</td>
<td>Pyogenic meningitis</td>
<td>No abnormal findings</td>
<td>Discharged</td>
</tr>
<tr>
<td>5</td>
<td>9 months</td>
<td>F</td>
<td>Diarrhoea</td>
<td>Mucus</td>
<td>Discharged</td>
</tr>
<tr>
<td>6</td>
<td>1 yr</td>
<td>M</td>
<td>Bronchopneumonia with anaemia</td>
<td>Mucus</td>
<td>Discharged</td>
</tr>
<tr>
<td>7</td>
<td>1 month</td>
<td>M</td>
<td>Acute gastroenteritis with sepsis</td>
<td>No abnormal findings</td>
<td>Discharged</td>
</tr>
<tr>
<td>8</td>
<td>4 months</td>
<td>M</td>
<td>Bronchopneumonia</td>
<td>Mucus</td>
<td>Discharged</td>
</tr>
<tr>
<td>9</td>
<td>1 month</td>
<td>M</td>
<td>Subacute intestinal obstruction</td>
<td>Mucus</td>
<td>Expired</td>
</tr>
</tbody>
</table>
Senftenberg (2), Shigella dysentriae (2), Shigella flexneri (1) and Shigella sonnei (1). In addition, 5 specimens showed pathogenic protozoa (Entamoeba histolytica and Giardia lamblia) on microscopy. Results of repeated cultures of drinking water and milk were non contributory.

Discussion

S. Worthington has been reported as a cause of various clinical infections from different parts of the world. In 1976, a study from Hong Kong reported an outbreak of diarrhoea in a nursery transmitted by a delivery room suction apparatus14. S. Worthington has been reported in a study from Iraq as one of the predominant causes of infantile gastroenteritis due to Salmonella1. Diarrhoea due to S. Worthington has been reported among infants in a neonatal unit in a hospital in Pakistan and in children from a boarding school in Mexico3,15. In India, S. Worthington has been implicated in outbreaks of meningitis and septicaemia in Chandigarh and Mumbai, diarrhoea in Kolkata and a splenic abscess in a patient with chronic myeloid leukaemia from Pune2,6,7,16,17.

In this study eight of the 9 children were under 1 yr of age, dietary habits and reduced resistance to infection may be considered as predisposing factors. It has earlier been reported that breast fed babies are far less prone to gastroenteritis as compared to those on bottle feeds1. It is interesting to note that all patients in this study were on bottle feeds, which may get contaminated due to poor handling at different stages.

Multidrug resistant non-typhoidal salmonella have been reported from various parts of the world18,19. Moreover, non-typhoidal salmonellae resistant to the newly introduced antimicrobial agents have also been reported20. In conformity with earlier reports from India and abroad describing infections due to multidrug resistant S. Worthington1,2,6,7,16, a multiresistant antimicrobial pattern was observed in the present study. Most of the isolates were sensitive to furazolidone in vitro. Also, 67 per cent sensitivity to ciprofloxacin was observed. High sensitivity of S. Worthington isolates to ciprofloxacin has been reported previously21. Thus, despite the controversy over use of ciprofloxacin in children, its use in...
treatment of serious Salmonella infections in paediatric patients has been recommended, especially where potential benefits outweigh the risks. In the present study, the patients were treated with rehydration therapy and combination of antibiotics. Quinolones were added to the treatment regimen after the culture and sensitivity reports were released. Eight cases responded to treatment. The death of the one child could not be attributed to S. Worthington infection as this 1 month-old baby was admitted in a critical condition with subacute intestinal obstruction. The isolation of S. Worthington from a limited number of cases of diarrhoea indicate emergence of a Salmonella serotype, which has not been reported from our hospital earlier. In Delhi, the majority of gastroenteritis cases are reported during the summer and monsoon months (between April and September). This could be a possible explanation for the limited number of cases observed in this study.

Ribotyping of the isolates indicated that multiple clones were in circulation in the hospital and in the community. Ribotyping using 16S and 23S rRNA of E. coli as a universal probe has been shown to be highly discriminative and is one of the important molecular techniques for epidemiological typing of many bacterial pathogens. The usefulness of this technique for typing different serotypes of Salmonella has been reported. In the present study, the molecular typing helped in understanding the epidemiology of the infection. The ribotyping was more useful in delineating the epidemiology of what appeared to be an outbreak of acute gastroenteritis caused by S. Worthington as identified by serotyping. Since multiple clones were responsible for causing acute gastroenteritis in infants, it was clear that there was no single source.

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References


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