A STUDY OF PARATYPHOID FEVER IN BULGARIAN CHILDREN

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Abstract. Objective. To study both the molecular discrimination of *D*-tartrate fermenting and non-fermenting strains of *Salmonella enterica* subsp. *enterica* serotype Paratyphi B isolated from patients with paratyphoid fever and the clinical course of this disease. **Materials and methods.** The isolates examined were from children aged 3 months to 9 years. A total of 33 *Salmonella* strains were serotyped as *Salmonella* Paratyphi B, with an antigenic formula based on O- and H- antigens: 1,4, [5], 12: b: 1,2 by Kauffmann–White classification. **Results.** Multiplex PCR analysis confirmed all tested strains as *d*-tartrate fermenting (dT+), also referred to as variant Java. **Discussion.** We found that the most common cause of paratyphoid fever among children in Bulgaria is variant Java *Salmonella* Paratyphi B. Most children had classic symptoms of acute gastroenteritis – fever, watery diarrhea and vomiting.

Key words: *Salmonella* Paratyphi B, *d*-tartrate fermenters, multiplex PCR, paratyphoid fever

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INTRODUCTION

Paratyphoid fever caused by *Salmonella* serotypes Paratyphi A, B and C is an infectious disease prevalent mainly in developing countries, while in developed countries it is most commonly observed in people returning from travel to endemic areas [1, 2]. Paratyphoid fever is a multi-systemic disease characterized by persistent fever, diarrhea or constipation, abdominal pain, chills, immunological complications [3]. Bacteriological confirmation of this disease is based on the isolation of the microorganism from feces or blood [4, 5, 6]. In the last five years,

between 15 and 20 cases of paratyphoid fever have been registered in Bulgaria annually [3].

The subject of many studies is *S. enterica* subsp. *enterica* serovar Paratyphi B and variant *S. Paratyphi* B, previously referred to as *Salmonella* Java. These are biovars of common serotype 1,4, [5], 12: b: 1,2, which makes it impossible to distinguish virulent agents by the use of serotyping methods. *Salmonella enterica* subspecies *enterica* serovar Paratyphi B can be differentiated by the fermentation of dextrorotatory [L(+)]-tartrate (*d*-tartrate), using a laborious process and complex biochemical tests. The main difference between them is that *S. Paratyphi* B are *d*-tartrate-

non-fermenting (dT-) strains and biovar Java are d-tartrate-fermenting (dT+) strains [7, 8, 9].

OBJECTIVE

To apply and describe a fast and reliable PCR-based method for discrimination of *S. Paratyphi B* (dT-) and variant Java *Salmonella* (dT+) strains and to analyze the clinical course of paratyphoid fever among Bulgarian children.

MATERIALS AND METHODS

Bacterial strains and serotyping

The study included 33 *Salmonella* strains, representing *S. enterica* subsp. *enterica* serovar *Paratyphi B* isolated in the period from January 2017 to November 2018 from feces of patients with paratyphoid fever between 3 months and 9 years of age. 15 (15/33) strains from the University Hospital for Infectious Diseases, Sofia, and 18 (18/33) strains from the University Hospital "Sveti Georgi", Plovdiv were submitted to the National Reference Laboratory of Enteric Pathogens at the National Center of Infectious and Parasitic Diseases (NCIPD) for microbiological confirmation.

All strains were confirmed by cultivation on Deoxycholate Citrate Agar, biochemical tests (API 20 E, Biomerieux), OB agglutination: 1,4, [5], 12; H1 phase: b and H2 phase: 1,2-antisera (SSI, Denmark; Sifin, Germany and SSI, Copenhagen, Denmark) according to the White-Kauffmann-Le Minor scheme. The strains were stored at -80°C until further use.

Antimicrobial susceptibility testing

Antimicrobial susceptibility was investigated by Bauer-Kirby disc diffusion method to cefotaxime (30 µg), cefoxitin (30 µg), ceftazidime (30 µg), ampicillin (10 µg), amoxicillin/ clavulanic acid (20/10 µg), amikacin (30 µg), gentamicin (10 µg), tetracycline (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), nalidixic acid (30 µg), trimethoprim/sulfamethoxazole (1,25/23,75 µg) (EUCAST-2018) [10].

PCR-based method for confirmation of S. Paratyphi B

Bacterial DNA was extracted using PrepMan™ (Applied Biosystems, Foster City, CA) in accordance with manufacturer's instructions. The extracted DNA was stored at -20° C until used in the mPCR.

Multiplex PCR was carried out using 2 sets of primers. Primer ST11 (5'-AGC CAA CCA TTG CTA AAT TGG CGCA- 3') and ST15 (5'-GGT AGA AAT TCC CAG CGG GTA CTG-3') - were genus specific for

Salmonella. The resulting PCR product size for these primers was 429 bp. The second primer pair 167 (5' -CAC ATT ATT CGC TCA ATG GAG- 3') and 166 (5'-GTA AGG GTA ATG GGT TCC- 3') were used to detect the presence or absence of ATG start codon for the gene encoding the putative cation transporter in the *S. Paratyphi B*, resulting in a 290-bp PCR fragment if the strain was d-tartratepositive [11].

PCR analysis was optimized to run in a final volume of 25 µl reaction at these primers and the following conditions – 1X PCR buffer with MgCl₂; dNTP 0,2 mM; 0.04 U/µl SuperHotTaq DNA pol.; 0.4 µM of primers 166 and 167, 0.1 µM of primers ST11 and ST151 and 2 µl of the DNA template. A negative control containing the same reaction mixture except the DNA template was included in every experiment and *E. coli* ATCC 25922 as a second one.

An initial denaturation at 95° C for 3 min was followed by 27 cycles of denaturation at 95°C for 30 sec, annealing at 62°C for 1 min and extension at 72°C for 1 min. Finally, an additional extension was achieved for 5 min at 72°C. A 10µl aliquot of each PCR product was electrophoresed on a 1.5% agarose gel and visualized and photographed under UV illumination.

RESULTS AND DISCUSSION

Thirty-three cases of infection with *S. Paratyphi B* were studied in order to analyse paratyphoid fever in the southern and western regions of Bulgaria (city of Sofia and city of Plovdiv). All thirty-three *S. Paratyphi B* isolates showed typical growth and biochemical characteristics and were both OB positive and H:b and H:1.2 positive according to SWARM and Sven Gard methods.

Multiplex PCR was performed according to the protocol described by Norazah Ahmad et al. [11]. This assay provides a specific PCR product for the species *Salmonella*-429bp gene and the gene encoding the putative cation transporter in *S. Paratyphi B* for d-tartrate-positive strains (Java) -290bp. Our study gave the first molecular characterization of the Bulgarian *S. Paratyphi B* strains. After mPCR analysis, the results of capillary gel electrophoresis confirmed all tested strains as d-tartrate fermenting (dT+) *S. Paratyphi B* strains, also called Java (Figure 1). The application of genotypic methods to 108 *S. enterica* serovar *Paratyphi B* strains isolated from diverse sources between 2002 and 2010 in Germany revealed that the serovar is polyphyletic. The three clonal complexes identified and characterized in this study were also recently detected within an international set of *S. enterica* serovar *Paratyphi B* strains based on MLST analysis [12].

We observed the following features during the clinical course of paratyphoid fever in our patients. In most children, watery diarrhea was the leading symptom, with constipation occurring only in 6.6% of the cases. The clinical characteristics are summarized in Table 1. Of all 33 cases of children with paratyphoid gastroenteritis, 30 had active diarrhea. There were only two children with constipation. Twenty-six patients had vomiting. They were all febrile. All children over the age of 5 had abdominal pain complaints. For the younger children, these subjective data are uncertain. Twenty-four of the children had a marked degree of dehydration, which required intravenous infusions as a symptomatic therapy. Eight children had pronounced anemic syndrome and 11 had leukopenia. One child had thrombocytopenia. In 30 patients, the C-reactive protein (CRP) was elevated. No child had rash. In patients from India hallmarks of paratyphoid fever include abdominal pain and high fever, with fever being the main presenting feature (as high as 75% of the cases) in the initial stages. Diarrhoea may be a presenting feature especially in immunocompromised cases and children. Classical clinical features of the disease were comparable among patients under and above 5 years of age but other manifestations, such as diarrhea, hepatomegaly, anemia and complications, are generally more frequent in children up to 5 years of age [13, 14]. In a French study from 1993 to 2015, 50 cases of paratyphoid fever among children and adolescents were identified. Only seven children (14%) had no recent travel history. Illness was characterized by a history of fever combined with diarrhea in 48 patients (96%). Constipation (6%), rose spots (1%), splenomegaly (6%) and relative bradycardia (2%) were not commonly reported [15].

Table 1. Clinical manifestations in 33 children

Symptoms/Laboratory features	N (%) of patients
Fever	33 (100%)
High grade fever (> 40°C)	11 (33.3%)
Diarrhoea	30 (90%)
Blood in stools	21 (70%)
Constipation	2 (6.6%)
Vomiting	26 (78.7%)
Abdominal pain	19 (63.3%)
Dehydration	24 (72.7%)
Anaemia ¹	8 (24.2%)
Leucopenia < 4000/mm ³	11 (33.3%)
Thrombocytopenia < 150.000/mm ³	1 (0.3%)
CRP > 6 mg/l	30 (90%)

¹According to WHO definition of anaemia adjusted to age (<http://www.who.int/vmnis/indicators/haemoglobin.pdf>, accessed [17 July 2016]).

The treatment of the children from our study was symptomatic, with restoration of the loss from dehydration. When etiologically diagnosed, patients were treated with the antibiotics ceftriaxone or ciprofloxacin for a median duration of 7 days. In the French study cited a third-generation cephalosporin was administered as the first-line regimen to 47 (94%) patients, of which 46 (92%) received ceftriaxone for a median duration of 6 days at a median dose of 50 mg/kg/d. The second-line regimen included ciprofloxacin [15].

The European Centre for Disease Prevention and Control in last years reported increased resistance to some antibiotics like ciprofloxacin and nalidixic acid [16]. In our study the standard disk-based susceptibility testing method did not detect salmonella with reduced susceptibility to ciprofloxacin. Susceptibility to all antimicrobial agents used was detected in 30/33 (90%) of the bacterial isolates. Three isolates were characterized by resistance to amikacin and gentamicin.

CONCLUSION

The annual reports of cases of paratyphoid fever among Bulgarian children indicate that a quick and accurate method for microbiological diagnosis of the causative agent is needed. Multiplex PCR allows for quick identification of *Salmonella* species and simultaneous discrimination of *S. enterica* serotype Paratyphi B dT+ and dT- strains. According to the results obtained in our molecular study, we found that the most common cause of paratyphoid fever among children in Bulgaria is variant Java *Salmonella* Para-

Fig. 1. Results of capillary gel electrophoresis. Line 22 negative control *E. coli* ATCC25922

typhi B. Unlike the course in adults, the infection in children occurs mainly with diarrheal syndrome and severe dehydration.

The increase in antibiotic resistance of *S. paratyphi* isolates in travel-associated cases reflects the situation mainly in endemic countries but it is not a leading problem in Bulgaria.

Disclosure summary: *The authors have nothing to disclose.*

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