Investigation of *Salmonella enteritidis* outbreak in four kindergartens

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SUMMARY

In June 2018, in the city of Sofia, Bulgaria, 40 children from four different kindergartens suffered from salmonellosis caused by *S. enteritidis*. They were reported to have consumed food prepared and delivered by a private catering service. The patients had fever, diarrhea, and some had vomiting and abdominal pain. Sixteen of them were treated in hospital, and the other 24 received home treatment. Some of the outpatients received antibiotic treatment despite WHO recommendations. All 40 isolates were positive for O: D, H: gm and H: m, and were confirmed to be *Salmonella enteritidis*, respectively. Using conventional and molecular methods, such as serotyping, Multiplex-PCR and PFGE, it was confirmed that the strains were epidemiologically related. Based on molecular genetic methods, we established that the epidemic outbreak had a common origin: contaminated food delivered by a private catering service, which was consumed at all four kindergartens.

Keywords: *Salmonella enteritidis*, outbreak, kindergarten

INTRODUCTION

Diarrheal diseases, including those caused by pathogens in food, are still a serious global health problem. The European Centre for Disease Prevention and Control (ECDC) reported that *Salmonella* is the second most common cause of foodborne outbreaks in the European Union (EU) [1]. Most human salmonellosis cases are associated with consumption of contaminated eggs, milk and milk products [2-5]. The objective of this work is to describe the clinical course in sick children during a salmonellosis epidemic outbreak, by paying attention to the differences in treatment approaches used in hospital and in outpatient setting, and to prove the epidemiologic connection between the cases through molecular methods.

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PATIENTS, MATERIALS AND METHODS

In June 2018, the National Reference Laboratory of Enteric Pathogens at the National Centre of Infectious and Parasitic Diseases confirmed 40 epidemiologically related *S. enteritidis* isolates from stools, using conventional and molecular methods. The strains were obtained from young children (1 to 6 years old) who were attending four different kindergartens; the strains were sent by twenty-three different microbiological laboratories in Sofia. Sixteen of 40 (16/40) patients were hospitalised, and 24 (24/40) received home treatment and care by their general practitioners. We studied the clinical course in both in-patients and out-patients. Clinical data for the patients at the Hospital for Infectious and Parasitic Diseases, Sofia, was collected prospectively during patients' stay therein, and for the patients treated by their general practitioners - through surveys conducted by employees of the Regional Health Inspectorate, Sofia.

Conventional methods
Clinical isolates were incubated at 37-38°C for 24 hours in a selective and differentiating medium MacConkey and Apocholate Citrate Agar (ACA). Next, API 20E biochemical identification was performed. *Salmonella* serotyping is necessary in any epidemiological investigations of foodborne outbreaks. *Salmonella serovars* are identified by slide agglutination tests using O and H antigen-specific antisera, although these procedures are both labour-intensive and time-consuming [6]. Serotyping of the isolates was performed using SerBul-Bio-NCIPD, Ltd (BG) anti-Salmonella sera. Antibiotic susceptibility tests were performed on the isolates using the Kirby Bauer method. Mueller Hinton agar was used as growth medium for performing standard disc diffusion tests [7].

MOLECULAR METHODS

Multiplex-PCR assay
The second confirmation method is multiplex-PCR, which allows detection of *Salmonella* and identification of the strains that belong to the serovar *Enteritidis*. PCR assay allows accurate and rapid serovar identification. Multiplex-PCR was performed using bacterial DNA and five pairs of primers: Three of them targeted the genes hilA, spvA and invA which encode virulence-associated factors, the fourth primer pair amplified a fragment of a unique sequence within *S. enteritidis* genome, and the fifth pair was used as an internal amplification control. The primers used in the assay are listed in Table 1 [8].

PFGE typing
PFGE is the current "gold standard" of fingerprinting method, which allows rapid detection of related cases of foodborne infections in such situations [9-12].
Because of the high probability of epidemic outbreaks in other kindergartens, we did not perform PFGE assay on all 40 strains with view of saving resources of the only reference laboratory in our country. Of all strains, eleven strains obtained from children attending kindergartens A, B, C and D were selected for analysis by performing Standard Operating Procedure for PulseNet PFGE of *Salmonella* serotypes.

RESULTS

Clinical course
The total number of children in all four kindergartens was 211, and the total number of ill children was 40 (40/211). Patients had complaints of fever (38°-41°C), profuse diarrhea, and frequent vomiting episodes requiring hospitalization (n=16) or long-term home treatment (n=24). All affected children had consumed food prepared and delivered by a private catering service, servicing the affected kindergartens. According to the catering employees, in the days before the epidemic, products of animal origin which were used were: hen eggs, cow's milk and pork. Unfortunately, the products were destroyed before the start of the epidemiological investigation, which is a gross violation of hygiene rules.
Among the 16 hospitalised children, all had watery diarrhea and 62.5% (10/16) had blood in the faeces. 43.7% (7/16) children had at least one episode of unmotivated vomiting. Two children had consultation with a surgeon because of severe abdominal pain. All children were febrile. Among

Table 1 - Primers used in the multiplex PCR for *Salmonella* detection.

Primer	*Sequence (5'–3')*	*Target gene*	*Amplicon size (bp)*	*Reference (year)*
invF	ACAGTGCTCGTTTACGACCTGAAT	*invA*	244	E.A. Trafny et al. (2006)
invR	AGACGACTGGTACTGATCGATAAT			
sdfF	TGTGTTTTATCTGATGCAAGAGG	*A unique sequence*	333	
sdfR	CGTTCTTCTGGTACTTACGATGAC			
spvF	ACTCCTTGCACAACCAAATGCGGA	*spvA*	571	
spvR	TGTCTTCTGCATTTCGCCACCATC			
hilAF	CGGAAGCTTATTTGCGCCATGCTGAGGTAG	*hilA within SPI 1*	854	
hilAR	GCATGGATCCCCGCCGGCGAGATTGTG			
univF	CCAGCAGCCGCGGTAATACG	*Escherichia coli 16S rRNA*	996	

the 24 children treated in outpatient setting, 25% (6/24) had bloody stools. Only 2 of them reported vomiting. Oral rehydration was performed at home with good results. According to children's parents, 83.3% (20/24) of the children who received home treatment were febrile. The majority of the hospitalised children were in a lower age group - 68.75% (11/16) were younger than 2 years of age (Table 2). All hospitalised children were treated symptomatically, and antibiotics were not administered. 37.5% (9/24) of the children who received outpatient treatment received oral antibiotics, mainly cephalosporins.

Table 2 - Demographic Characteristics and Symptoms of Epidemic Outbreak.

Sex	*n*	*%*
Male	18	45
Female	22	55
Kindergarten		
Total number of children	sick children	
A 48 (22.74%)	10	25
B 71 (33.64%)	21	52.5
C 25 (11.84%)	3	12
D 67 (31.75%)	6	8.95
Symptoms		
Diarrhea	40	100
Bloody diarrhea	10	25
Vomiting	7	17.5
Abdominal pain	23	57.5
Fever	36	90
Hospitalised	16	40
Hospitalised, between 1-2 years of age	11	68.75
Hospitalised, between 3-6 years of age	5	31.25
Outpatient	24	60

Conventional methods

According to the serotype, all 40 isolates were positive for O: D, H: gm and H: m. All of them were phenotypically confirmed to be *Salmonella enteritidis.* Antibiograms showed that the isolates were highly susceptible to standard antimicrobial agents - ampicillin, trimethoprim/sulfamethoxazole, ceftriaxone, nalidixic acid, ciprofloxacin and chloramphenicol.

Molecular methods

After performing gel electrophoresis, the primers produced up to five different amplicons with lengths corresponding to the serovar of the *Salmonella* strain. Serovar *Enteritidis* produced an amplification of five DNA fragments. The *Salmonella* strains belonging to other serovars produced three or two bands. The presence of

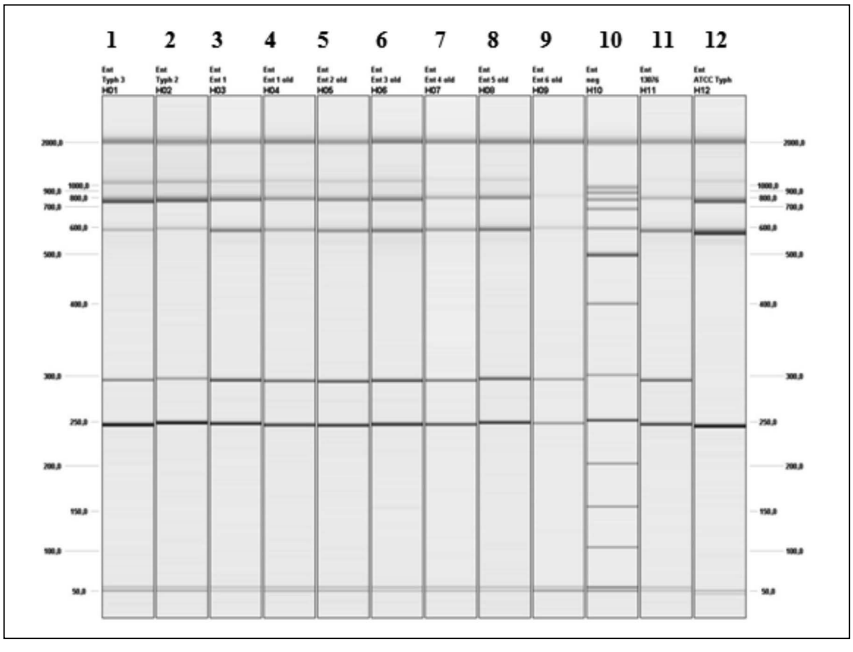

Figure 1 - Multiplex PCR assay for S. enteritidis detection. Line 1 - line 9: *S. enteritidis* isolates (*hilA, spvA* и *invA, univ, sdf*); line 10 - mm; line 12 - ATCC S. Typhimurium (*hilA, spvA* и *invA, univ*); line 11- ATCC S. enteritidis (*hilA, spvA, invA, univ, sdf*), positive control for *S. enteritidis.*

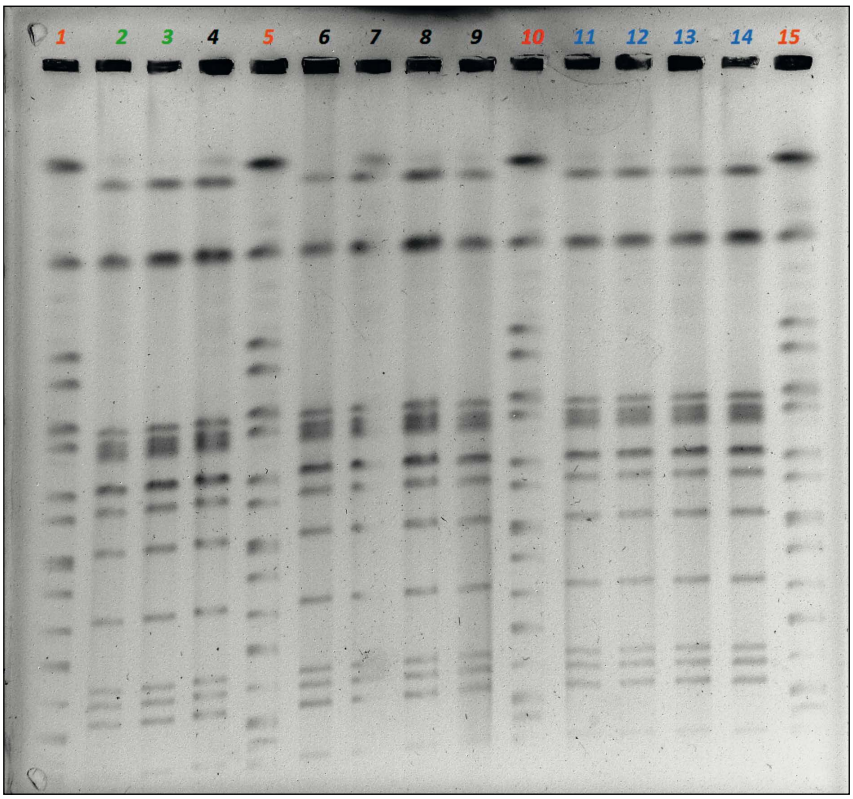

Figure 2 - Line 1, 5, 10, 15: S. Braenderup, MM. Line: 2, 3- isolates *S. enteritidis* (kindergarten A); line: 4, 6, 7- isolates *S. enteritidis* (kindergarten B); line: 8 and 9- isolates *S. enteritidis* (kindergarten C); line: 11, 12, 13, 14- isolates *S. enteritidis* (kindergarten D).

a band of an internal amplification control is an indication that the PCR was not inhibited in the reaction mixture. Through genotyping, it was confirmed that all isolates were *S. enteritidis* (Figure 1).

Pulsed-field gel electrophoresis was performed at the National Reference Laboratory of Enteric Pathogens as a molecular method for typing of *S. enteritidis* strains in order to confirm their epidemiologic connection. The PFGE XbaI analysis profile showed epidemiologic connection between all eleven *S. enteritidis* isolates included in this study (Figure 2).

DISCUSSION

Clinical data analysis showed that the children with more severe infection were hospitalised. The main concerns of the parents who brought the children to the hospital were the bloody stools and vomiting. At the hospital, children were treated according to the WHO standards, without the use of antibiotics. However, a large number of general practitioners in Bulgaria still treat *Salmonella* gastroenteritis with an antibiotic.

Through pheno- and genotyping performed at the National Reference Laboratory of Enteric Pathogens at the National Centre of Infectious and Parasitic Diseases, 40 *Salmonella enteritidis* isolates were identified and confirmed to be serovar *Enteritidis*. All isolates were obtained from children at the age of 1 to 6 years, with gastroenteritis; 16/40 children were hospitalised. Predominantly, hospitalised children were young, less than 2 years of age, which confirms the fact that the course of this disease is more severe in younger children [5, 13]. Epidemiologic data showed that the consumed food was prepared and delivered by a private catering service, which usually prepares and supplies food to 27 other kindergartens. However, there are reports of confirmed salmonellosis from only four kindergartens. Contact points, the staff of the kindergartens and of the catering service providing the food were examined, and 3 (3/124) were found to be positive for *Salmonella enteritidis.* The samples taken from surfaces, utensils and

foods from the private catering service were negative for sanitary indicative and pathogenic microorganisms. The analysis was performed by the microbiological laboratory at the Regional Health Inspectorate, city of Sofia. PFGE, which is the "gold standard" of fingerprinting method, was performed at the National Reference Laboratory in order to confirm epidemiologic connection between the isolates, The PFGE XbaI profile demonstrated that the isolates tested were completely identical. This undoubtedly proved that the isolates were epidemiologically related and that the Salmonella infection was most likely related to contaminated food consumed at all four kindergartens. The fact that the isolates were susceptible to all standard antibacterial agents shows that this was a highly virulent strain. Similar strains were described by Mijovic et al. in 2005 and 2010, in Montenegro [14]. A severe epidemic outburst caused by a similar strain was described by Mandilara et al., with a food source - a church ritual in Greece. 56 people fell ill, some of them children, but unfortunately, as in our case, the food product was not definitely established. More than half of the sick, predominantly young children, were hospitalised [15]. Typically, such highly virulent strains are reported to be the cause of epidemic outbreaks and epidemics. The case described by us is another proof that salmonellosis is one of the most common foodborne infections in Europe, and that preventive measures must be taken in food processing, especially in the services provided to children groups [16].

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Conflict of interest
None declared.

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