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Conventional and molecular investigation of *Shigella* isolates in relation to an outbreak in the area of Isfahan, Iran

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ABSTRACT

Background and Objective: Over 165 million cases of shigellosis occur in the world each year, mostly in developing countries. Outbreaks of shigellosis are associated with poor sanitation, natural calamities, contaminated food and crowded living conditions. In late summer 2006, during the final stage of an outbreak of shigellosis at a vast region of Isfahan province, Naein & Ardestan, our laboratory was assigned to investigate the outbreak in order to determine the causative agent.

Materials and Methods: A total of 146 rectal swabs which had been collected from the patients by local laboratories on separate days were screened using a battery of conventional and molecular tests.

Results: Thirteen specimens tested positive for *Shigella* spp. They were identified as *S. sonnei* (6, 46.1%), *S. dysenteriae* (4, 30.8%), *S. flexneri* (2, 15.4%) and *Shigella* spp (1, 7.7%) by conventional and molecular microbiological tests. According to ribotyping results the isolates were grouped into 3 distinct clusters encompassing the majority of isolates and a single line of descent representing isolate S122 which was nonreactive with any *Shigella* polyvalent antisera.

Conclusion: This diarrheal outbreak appeared to be the result of shigellosis. Despite the fact that *Shigella sonnei* was the predominant organism isolated from patients, the causative agent of outbreak diarrhea remains obscure, since other *Shigella* species were also involved. The serologic testing supports this conclusion, as do the molecular patterns of the *Shigella* isolates. Having considered the time of investigation which was in the late stage of the outbreak, it was very likely that a collection of endemic and epidemic clinical samples was screened resulting in isolation of various *Shigella* species.

Keywords: Outbreak, *Shigella,* Ribotyping

INTRODUCTION

Shigellosis is a bacterial infection affecting the intestinal tract caused by *Shigella* species, i.e., *Shigella dysenteriae, Shigella flexneri, Shigella sonnei* and *Shigella boydii*. Dysentery has been known to man throughout recorded history (1).The *Shigella* organisms are highly contagious, causing bacillary dysentery after ingestion of as few as 100-200 organisms (2). It is a fairly common disease; over 165 million cases occur in the world each year, mostly in developing countries causing over 1 million deaths in the world, including approximately 880,000 in Asia (3). Most cases are seen in the summer and early fall and occur as single cases or outbreaks. Outbreaks of shigellosis are associated with poor sanitation, natural calamities (e.g., earthquakes, floods), contaminated food and water and crowded living conditions (1-3).

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In summer 2006, an outbreak of gastroenteritis occurred at a vast region of Isfahan province, Naein & Ardestan. During a period of two months, hundreds of people became ill with the disease. At the late stage of outbreak our research laboratory was assigned to look into this problem in order to determine the causative agent and pattern of outbreak using a battery of conventional and molecular tests.

MATERIALS AND METHODS

A total of 146 rectal swabs taken from patients affected by the outbreak were included in the current study. The rectal swabs were placed in a Stuart's medium transport tube and sent by local health authorities and practising doctors to our research laboratory at Infectious Diseases Research Center, Isfahan (IDRC). The microbiological screening methods were applied to the rectal swabs for isolation, definitive identification and molecular typing of causative agents.

Conventional identification. For recovery and identification, the rectal swabs were inoculated into 50 ml Luria Bertani (LB**)** broth and enriched overnight at 37°C. They were then plated to several different types of media including selenite broth, MacConkey (MCK) agar and Salmonella-Shigella (SS) agar. The plates were incubated for 18-24 hours at 36°C. Non- lactose fermenting *Shigella*-like colonies were first isolated on MCK agar plates and then inoculated on Triple Sugar Iron agar (TSI) medium to test for glucose and lactose fermentation, sulfide and gas production. They were further examined by the biochemical tests including citrate utilization. indole, lysine and ornithine decarboxylase production, 2-Nitrophenyl β-D-galactopyranoside (ONPG) production, Methyl Red-Voges-Proskauer (MR-VP) reaction, xylose and mannitol fermentation. They were tentatively identified as *Shigella* species according to standard methods (4).

After preliminary identification the final confirmatory identification of *Shigella* like isolates was carried out by slide agglutination using polyvalent antisera (Volkaleks, Mast, England).

Antibiotic susceptibility testing. Antimicrobial susceptibility testing and the interpretation of zone of inhibition were performed for all *Shigella* isolates by standard disk diffusion method, following NCCLS guideline (5). Tests were performed on Mueller Hinton agar plates using antimicrobial disks (Mast Diagnostics, UK): Amoxicillin-clavulanic acid (AMC 30 μg), ceftriaxone (CRO 30 μg), chloramphenicol (CHL 30 μg), ciprofloxacin (CIP 5 μg), tetracycline (TET 30 μg), and trimethoprim-sulfamethoxazole (SXT 1, 25/23, 75 μg).

Molecular identification DNA extraction. Chromosomal DNA from *Shigella* isolates was extracted using the guanidium thiocyanate method of Pitcher *et al.* (6). The concentration and purity of DNA preparations were determined by spectrophotometry and the quality of the DNA samples was assessed by agarose gel electrophoresis.

Ribotyping. Ribotyping was carried out following a protocol recommended by Regnault *et al.* (7). In brief 5 μg of extracted whole genomic DNA was digested at 37ºC by restriction endonuclease *Mlu*I (Roche Diagnostics GmbH, Mannheim, Germany). Digested DNA was electrophoresed at 50 V for 4.5 h on horizontal 0.8 % (W/V) agarose gel (Fermentas, Germany). DNA was denatured and transferred onto positively charged nylon membrane using vacuum transfer system (Biometra, Germany). Hybridization was performed in hybridization buffer at 53°C using a mixture of digoxigenin (DIG) labeled oligonucleotides (18-23 mer) (Oligomix-5; MWG, Germany) as a probe. Final detection was performed using anti-digoxigenin antibody conjugated to alkaline phosphatase (Roche Diagnostic GmbH, Mannheim, Germany).

Data analysis. The identity of ribotyping patterns was defined on the basis of exact numbers and matching positions of major bands. Only major bands were considered and band intensity was not used as a criterion. The levels of similarity of these digitized patterns then were calculated with the Pearson product moment correlation coefficient of the Gel Compar program (Applied Maths, Belgium). The unweighted pair group method using arithmetic averages (UPGMAs) was used for cluster analysis and led to establishment of normalized drafts and dendrogram.

RESULTS

Out of 146 stool specimens, 13 isolates showed microbiologic characteristics consistent with *Shigella*

Table 1. Antibiotic susceptibility testing of Iranian *Shigella* isolates.

Antibiotics	*Shigella sonnei* isolates			*Shigella flexneri* isolates		*Shigella dysenteriae* isolates		non-agglutinable *Shigella* isolates (NAG)		All *Shigella* Spp
	S	I	R	S	R	S	R	S	R	Resistance rate (%)
Amc [1]	-	S603	S33, S143, S144, S201, S601	-	S146, S203	S121	S123	S91, S122	S41	69.23%
SXT [2]	-	S601, S603	S33, S143, S144, S201	-	S146, S203	-	S121, S123	S122	S41, S91	76.92 %
CP [3]	S33, S143, S144, S201, S601, S603	-	-	S146, S203	-	S121, S123	-	S41 ,S91, S122	-	0
CRO [4]	S33, S143, S144, S201, S601, S603	-	-	S146, S203	-	S121, S123	-	S41 ,S91, S122	-	0
Tet [5]	-	S143, S201, S601, S603	S33, S144	-	S146, S203	-	S121, S123	S122	S41, S91	61.53%
CHL [6]	S33, S143, S201, S601, S603	-	S144	-	S146, S203	-	S121, S123	S122	S41, S91	53.84%

[1]Amoxiclav (Amc): zone of inhibition; <13; Resistant (R); 14-17: Intermediate (I); >18 Susceptible(S).
[2]Cotrimoxazole (SXT): zone of inhibition ;< 10: Resistant(R); 11-15: Intermediate (I); >16: Susceptible (S).
[3]Cephteriaxon (CP): zone of inhibition; <15: Resistant(R); 16-20: Intermediate (I); >21: Susceptible(S).
[4]Ciproflaxacin (CRO): zone of inhibition; <13: Resistant(R); 14-20: Intermediate (I); >21: Susceptible(S).
[5]Tetracycline (Tet): zone of inhibition; <14: Resistant(R); 15-18: Intermediate (I); >19: Susceptible(S).
[6]Chloramphenicol (CHL): zone of inhibition; <12: Resistant(R); 13-17: Intermediate (I); >18: Susceptible(S)

spp including Gram negative stain reaction, lactose negative colonies on MCK and SS agar plates and typical *Shigella* like biochemical properties, in particular, a K/A reaction on TSI , (+/-) reaction to MR/VP, (-) reaction to Indole, (-) citrate utilization. Isolate S122 showed a rather different reaction to key biochemical tests including an indole positive reaction. Further analysis by slide agglutination assay using polyvalent antisera revealed that of 13 outbreak isolates analyzed 6 (46.1%), i.e., S33, S143, S144, S201, S601, S603 were *Shigella sonnei*, 2 (15.4%), i.e., S121 and S123 were *Shigella dysenteriae*, 2 (15.4%), i.e., S146 and S203 were *Shigella flexneri* and 3 (23.1%), i.e. S41, S91 and S122 were non-agglutinable (NAG) *Shigella*.

Based on antibiotic susceptibility testing the rate of resistance for the isolates to amoxicillin-clavulanic acid, chloramphenicol, tetracycline and trimethoprim-sulfamethoxazole was 69.23% (9/13), 53.84% (7/13), 61.53% (8/13) and 76.92 % (10/13), respectively (Table 1).

DNA from all 13 outbreak *Shigella* isolates could be digested with endonuclease *Mlu*I and were typable. The reference strain of *Citrobacter koseri* CIP 105177 cleaved by *Mlu*I restriction endonuclease used as a molecular marker in the ribotyping (Fig. 1). Reproducibility was perfect and typeability was 100%. A total of 4 distinct ribotypes were obtained. The number of bands composing each ribotype varied from 9 to 13 and fragment sizes ranged from 1.2 to 16.8 Kbp (Fig. 1).

Examination of the dendrogram demonstrated that ribotyping analysis divided *Shigella* isolates into 4 distinct but related subgroups (Fig. 2). The major cluster included 6 outbreak *S. sonnei* isolates (S33, S143, S144, S201, S601 and S603) which gave the same single profile corresponding to international ribotype Da/e2/f/g of *S. sonnei* (8). The second major cluster included 4 outbreak isolates (S41, S91, S121 and S123) which also generated similar ribotype profiles. The isolates S121 and S123 were identified as *S. dysenteriae* by serotyping testing while the other two isolates, i.e., S41 and S91 were non-agglutinable (NAG). The ribotype profiles of these two major clusters showed 57% homology. The ribotyping profiles of the outbreak isolates, S146 and S203 which were identified as *S. flexneri* by serologic testing, were similar to corresponding international ribotype B1/2/2a/2b' of *S. flexneri* (8). The isolate S122 showed a unique ribotype pattern. The isolate S122 was non-agglutinable with serotyping testing.

DISCUSSION

Shigellosis, or 'bacillary dysentery', is an intestinal infection that is found throughout the world, despite the fact that it is a major public health problem in many developing countries, where it causes about

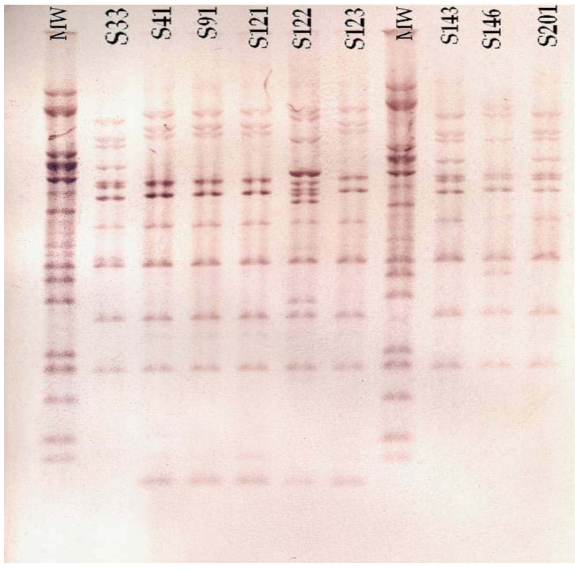

Fig. 1. Ribotype profiles of *Shigella* isolates; Lanes MW: Molecular Weight Marker **(*Citrobacter koseri*** CIP 105177 DNA was cleaved by *Mlu*I restriction endonuclease and the fragments were used as molecular size standards (the sizes are from top to bottom, respectively in base pairs): 16752, 12482, 7330, 6552, 5752, 5098, 4405, 3023, 2778, 1696, 1444 and1171).

5 to 10 percent of childhood diarrhea (9). Humans are the only known reservoirs for *Shigella* spp, and transmission can occur through the fecal-oral route (10). As few as 10-100 organisms can cause infection, enabling person-to-person transmission where hygienic conditions are poor, in developed and developing countries alike (11, 12).

Outbreaks of shigellosis can occur throughout the year, but in most communities the incidence is highest when the weather is hot and dry. In fact the scarcity of water might limit handwashing and other hygiene measures that facilitate transfer of very small number of bacteria needed to cause infection (13, 14).

The current study deals with an outbreak that took place in late summer and early autumn 2006 in a vast area of Nain & Aredestan county**,** located at 130 km distance to the east of Isfahan city and 320 km to the south east of Tehran on the road to Yazd, Iran. A substantial number of people were affected by the disease and the outbreak spread to the neighboring areas. The health officials took appropriate and necessary measures to contain the spread of the disease. The outbreak eventually abated with climate change and starting of cold season, but not as quickly as it struck initially. At the late stage of outbreak our research laboratory was assigned the task of carrying out an investigation on the causative agents and pattern of outbreak.

The rate of isolation of *Shigella* spp. in the present study estimated to be 8.9% which was lower than those reported by previous Iranian investigations (15, 16). The low rate of isolation as observed in the present study may be attributed to poor sample collection, storage and transport of specimens and an incomplete repertoire of skills.

In our study *Shigella sonnei* was found to be the dominant species, while quite interestingly other *Shigella* species, i.e*., S. dysenteriae* and *S. flexneri* were also isolated from the patients. Previous studies from different parts of the country have also supported these observations and confirmed the ranking of *S. sonnei* as the major causative agent of shigellosis in Iran (15, 16). However, isolation of various *Shigella* species from patients in our study makes the interpretation of the results a difficult task and

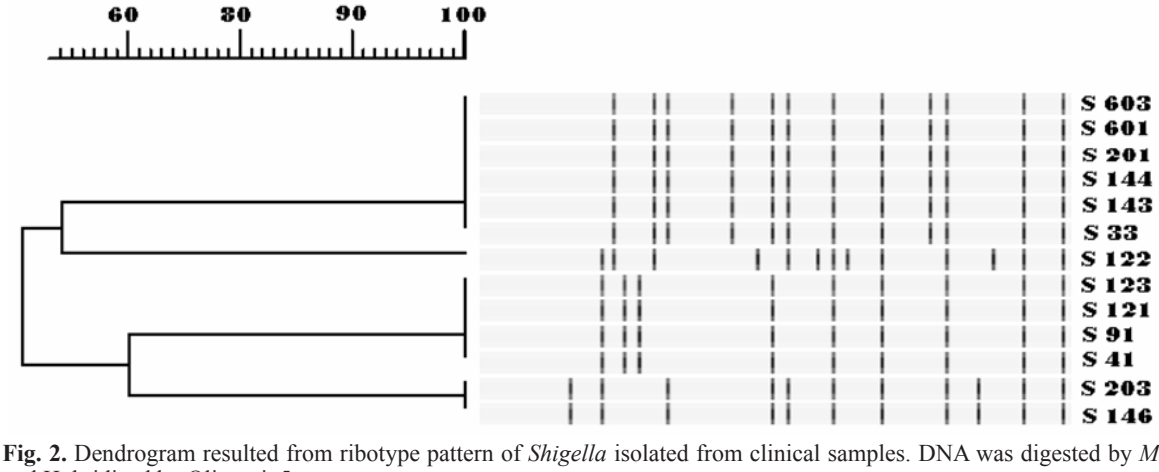

Fig. 2. Dendrogram resulted from ribotype pattern of *Shigella* isolated from clinical samples. DNA was digested by *Mlu*1 and Hybridized by Oligomix5**.**

rather intuitive. We speculate that this observation might be due to the timing of investigation, that is, the late stage of outbreak when an assorted types species, be it endemic, sporadic or epidemic, in a set of location could be widespread and recoverable. In other words, this phenomenon might be interpreted as non-outbreak-associated isolation for each species. Molecular microbiological analyses of pathogenic bacteria are applied to differentiate species into types in order to associate different clinical cases with outbreaks. The differentiation can be made using molecular typing methods, including ribotyping (8, 17) which have been previously used by others in *Shigella* outbreaks (18, 19).

In the present study usefulness of established molecular typing method such as ribotyping as an accurate tool in outbreak diagnosis and short scale studies with a limited number of strains was determined since ribotyping differentiated the clinical isolates into three distinct patterns corresponding to three *Shigella* species. However, for countries with limited resources such as our country ribotyping can be expensive or impractical in large scale studies.

The current study reaffirmed the significance of timing as a paramount necessity and the quality of proper specimen collection, storage and transport as a prerequisite in gaining robust and conclusive microbiological results in a shigellosis outbreak investigation. Having considered, the relatively fastidious nature of the organism and the fact that *Shigella* are particularly sensitive to elevated temperatures and are difficult to cultivate at later stages of the illness; proper specimen collection at the site of an outbreak, correct packaging and rapid transport to the laboratory are of utmost importance in ensuring the isolation of organisms. Delays in transporting specimens to the laboratory are not advisable as the yield of bacteria may fall significantly (20).

In conclusion, this diarrheal outbreak appeared to be the result of shigellosis. Despite the fact that *Shigella sonnei* was the predominant organism isolated from patients, the causative agent of outbreak diarrhea remains obscure, since other *Shigella* species were also involved. The serologic testing supports this conclusion, as do the molecular patterns of the *Shigella* isolates.

Although the isolates produced four separate ribotyping patterns, the four patterns were very closely related and were species specific, however since the specimens were collected at the late stage of outbreak, it was very likely that a collection of endemic and epidemic cases was screened resulting in isolation of various *Shigella* species.

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