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Characterization of *Shigella* Strains by Plasmid Profile Analysis and Antibiotic Susceptibility Patterns in a Pediatric Hospital in Ahvaz

Amin Sakhaei,[1] Mohammad Savari,[2] Leili Shokoohizadeh,[3,4] Maryam Hadian,[5] and Alireza Ekrami[3,4,*]

[1]Department of Microbiology, Karaj Islamic Azad University, Karaj, IR Iran
[2]Department of Microbiology, School of Medicine, Tehran University of Medical Sciences, Tehran, IR Iran
[3]Infectious and Tropical Diseases Research Center, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, IR Iran
[4]Department of Medical Laboratory Sciences, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, IR Iran
[5]Laboratory Department, Abuzar Hospital, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, IR Iran

*Corresponding author: Alireza Ekrami, Infectious and Tropical Diseases Research Center, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, IR Iran. Tel: +98-6133738285, Fax: +98-6133738285, E-mail: aekrami@yahoo.com

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Background: High incidences of dysentery and diarrhea were reported in a pediatric hospital in Ahvaz, Iran during March to April, 2013.
Objectives: A cross-sectional study was therefore undertaken to identify the causative agents.
Patients and Methods: A total of 230 diarrhea samples were collected from the patients and analyzed by routine bacteriological methods. Bacterial identification, serological assay, antimicrobial susceptibility testing, extended spectrum β-lactamases (ESBLs) screening and plasmid profile analysis were performed according to the standard guidelines.
Results: A total of 70 *Shigella* strains including %70 (n = 49) S. *sonnei* and 30% (n = 21) S. *flexneri* were isolated from diarrhea samples. Most of the *Shigella* isolates showed high degrees of resistance to ampicillin, ulafamethoxazole- trimethoprime and cefexim. Concurrent resistance to sulafametoxazole- trimethoprime and ampicillin was the most common resistance pattern. Overall, 11.4% of *Shigella* isolates showed the ESBL producer criteria. The plasmid profile patterns of all the strains were determined by a modified alkaline lysis method. By plasmid profile analysis 23 genotypes were identified among all the isolates, 14 and 9 genotypes among the S. *sonnei* and S. *flexneri* respectively. S. *sonnei* and S. *flexneri* isolates demonstrated unique plasmid profiles.
Conclusions: These data demonstrated that S. *sonnei* strains are the main cause of shigellosis as the prevalent *Shigella* serotype in Iran. We also found that the antibiotic resistance rates are increasing among *Shigella* strains. Plasmid profile analysis is more reliable than antibiotic susceptibility patterns in epidemiologic studies.

Keywords: Dysentery, Shigellosis, Extended Spectrum β-Lactamase (ESBL)

1. Background

Microbial Infections, particularly gastrointestinal infections, impose monumental suffering on the afflicted individuals, and this puts a great burden on the medical system. Gastrointestinal pathogens have evolved a remarkable array of virulent traits that enable them to colonize the intestinal tract, adhere to the epithelium, and produce toxins. These toxins directly damage the epithelial cells or intestinal barrier function (1). *Shigella* is a gram negative bacterium and one of the major causes of bacterial diarrheal disease which induces death in both developed and developing countries. About 14,000 cases of the disease are reported in the United States yearly, and about 72% of those cases are caused by the subgroup S. *sonnei* (2). In endemic regions of the developing world, shigellosis is principally a pediatric disease. In developed countries, however, it occurs more frequently in overcrowded or poor areas (2, 3)

Most of the morbidity due to *Shigella* infection (shigellosis) in developing countries is reported in children of less than five years. *Shigella* is transmitted through faecaloral route (and may be via food or water) and through person-to person spread (3, 4).

Shigella spp are divided into four serogroups: S. *flexneri*, S .*sonnei*, S. *boydii*, and S. *dysenteriae*. S. *flexneri* is the most common agent of shigellosis in developing countries and tropical areas (e.g., Iran). However, epidemiological shift in shigellosis and the change in the pattern of antibiotic resistance among *Shigella* strains have been reported in recent years in Iran and the world (5, 6). As with other infectious diarrheal diseases, treatment of shigellosis includes both rehydration and antibiotics. Treatment for shigellosis depends upon the accessibility of effective antimicrobial agents. Active antibiotics reduce the length of symptoms and eliminate *Shigella* from the stool more rapidly (6).

Shigellosis occurs very often (especially in summer) in

Khuzestan province which is located in southwestern Iran. Many Shigelloses reported from different area in Khuzestan. Few studies have, however, investigated this infection in that area (7). The ability of *Shigella* to invade epithelial cells in colon or rectum is associated with the presence of large plasmids (120 - 140 Mdal) that encode the invasion plasmid antigens (Ipa). Strains lacking this plasmid are avirulent (8). Plasmid profile analysis has been used as an epidemiological tool for investigating the outbreak of infectious diseases. Plasmid profile analysis may be useful in identifying the source of infection, discriminating strains or assessing the ability of control procedure (5, 8). So far, plasmid profile analysis for typing *Shigella* strains in Ahvaz has not been evaluated. From March to April, 2014, a high incidence of dysentery was reported in Abuzar pediatric hospital in Ahvaz, the capital of Khuzestan.

2. Objectives

This cross-sectional study aimed at (a) determining the frequency distribution of *Shigella* spp in diarrhea samples (b) detecting antimicrobial susceptibility patterns and (C) analyzing plasmid to discriminate between *Shigella* strains.

3. Patients and Methods

3.1. Sampling

A total of 230 diarrhea samples were collected from children with dysentery who were less than five years of age in Abuzar pediatric hospital in Ahvaz.

3.2. Isolates Identification

Identification of *Shigella* is difficult and tricky using biochemical or even molecular diagnosis. A single specimen was obtained from each patient and identification was done according to standard procedure (4, 9). Samples were immediately inoculated on plates of Mac Conkey and XLD agar. The media cultures were incubated at 37°C for 24 hours. The samples were sent to the microbiology laboratory for further diagnostic processing. *Shigella* isolates were confirmed by general biochemical tests commonly used in microbiology labs. After the identification of *Shigella* by conventional methods, the isolates were serotyped by slide agglutination test using specific antisera (Baharafshan Co, Iran).

3.3. Antimicrobial Susceptibility Testing

Susceptibility to antimicrobial agents was determined based on disk diffusion methods according to CLSI criteria using commercially available disks (Mast, UK) including ampicillin (AMP 10 μg), ciprofloxacin (CIP 5 μg), norfloxacin (NOR 30 μg), ceftazidime (CAZ 30μg), imipenem (IMP 10 μg), nalidixic acid (NA 30μg), trimethoprim-sulfamethoxazole (SXT 25μg), gentamicin (GM 30 μg), kanamycin (K 30 μg), and cefexime (CFM 30 μg). According to CLSI standards for the detection of ESBL producers, each isolate with inhibition zone diameter ≤ 22 mm for ceftazidime or ≤ 27 mm for cefotaxime was considered as a probable ESBL-producer by using disc diffusion screening test. Phenotypic verification of ESBL production was done by double disk synergy and combination disk tests (10, 11).

3.4. Plasmid Extraction

Plasmid DNA was extracted from the *Shigella* spp using GF-1 plasmid DNA extraction kit (Vivantis Co, Malaysia). Using a combination of alkaline lysis-SDS and mini-column spin technologies, we can isolate up to 20μg of plasmid DNA from bacterial cultures. The extracted plasmid DNA was separated by horizontal electrophoresis in a 0.8% agarose slab gel in trisacetate-EDTA buffer at room temperature and 60 V for 4 hour. After electrophoresis, the gel was stained with ethidium bromide and photographed by a gel documentation system. The molecular masses of the unknown plasmid DNA were assessed by comparison of their motilities with two DNA ladders with 10 kb and 23 kb molecular masses.

3.5. Analysis of Similarity Among Strains

The similarities among the isolates on the basis of their plasmid profiles were analyzed with an online program (insilico.ehu.es). Plasmid profiles were compared with Dice method and the similarity of coefficients was analyzed by unweighted pair group method (UPGMA) to cluster and generate dendrograms through the average linkage procedure.

4. Results

A high incidence of shigellosis occurred from March to April 2014. In the 230 diarrhea samples collected from the patients, the most commonly affected group was 1 - 5 year old children. About 30% (n = 70) of diarrhea samples were positive for *Shigella* spp. The most common *Shigella* spp was S. *sonnei* (70%; n = 49) followed by *S.flexeneri* (30%; n = 21%). The results of the antibiotic susceptibility testing of S. *sonnei* and S. *flexneri* are shown in Table 1. The highest rate of resistance against ampicillin (89%: 100%), cefexime (86%: 75%), sulfamethoxazole- trimethoprim (63%: 50%), and the lowest rate of resistance against nalidixic acid (18: 2.5%), cholermphenichol (14%: 18%) were detected in S. *sonnei* and S.flexnri respectively. Both speices were fully susceptibile to gentamicin, norfloxacin, imipenem, norfloxacin, kanamycine and ciprofloxacin. The most common multiple resistance patterns were sulfamethoxazole- trimethoprim, ampicillin (28.5%), and sulfamethoxazole- trimethoprim, cefexime (27%). Based on CLSI criteria, 23.8% (n = 5) of S. *flexneri* and 6.1% (n = 3) S. *sonnei* were identified as the ESBL producer.

Table 1. Antibiotic Resistance Patterns of *Shigella* Isolates From the Pediatric Hospital in Ahvaz [a,b]

Antibiotics	Resistance Rate		All *Shigella* spp.
	S. *sonnei*	S. *flexneri*	
AMP	89 (n = 43)	100 (n = 21)	91.4 (n = 64)
CAZ	63 (n = 31)	0	63.2 (n = 31)
CFM	86 (n = 42)	75 (n = 16)	82.5 (n = 58)
SXT	63 (n = 31)	47.6 (n = 10)	58.5 (n = 41)
NAL	18 (n = 9)	14.2 (n = 3)	17 (n = 12)
CIP	0	0	0
GM	0	0	0
IPM	0	0	0
AMK	6 (n = 3)	0	4.5 (n = 3)
SXT-CFM	34.6 (n = 17)	9.5 (n = 2)	27 (n = 19)
SXT-NAL	8 (n = 4)	4.7 (n = 1)	7 (n = 5)
SXT-AMP	32.6 (n = 16)	19 (n = 4)	28.5 (n = 20)
SXT-AMP-NAL	8.1 (n = 4)	4.7 (n = 1)	7 (n = 5)
AMP-NAL	10.2 (n = 5)	4.7 (n = 1)	8.5 (n = 6)

[a] All values are presented as %.
[b] Abbreviations: AMP, ampicillin; CAZ, ceftzidime; CFM, cefexime; SXT, trimetoprim sulfametomethoxazol; NAL, nalidixic acid; CIP, ciprofloxacin; GM, gentamicin; IPM, imipenem; AMK, amikacin.

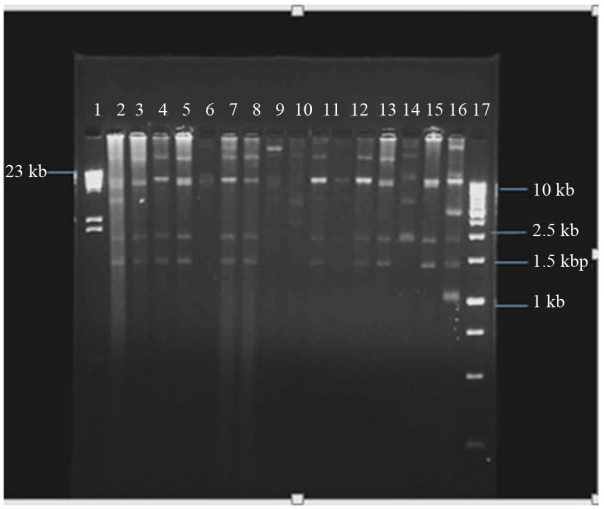
4.1. Plasmid Profiles

Analysis of plasmid DNA revealed that all 70 isolates harbored multiple plasmids, with an average of 5.5 plasmids (range, 5 to 6 plasmids) in each isolate of all strains and a mean of 7and 8 plasmids in each isolate of S. *sonnei* and S. *flexneri*, respectively. Figure 1 shows the plasmid patterns of all *Shigella* strains. In total, 23 different plasmid profile patterns were found among all isolates. Plasmid analysis identified 14 and 9 genotypes among the S. *sonnei* and S. *flexneri* strains, respectively. The sizes of the plasmids from among all isolates ranged from 1 to > 23 kb. Plasmids of 1.5 to 2.5 kb were the most frequently detected and were seen in about 95% of the isolates.

4.2. Genetic Similarity Among the Isolates

The genetic similarities among the 70 *Shigella* strains based on their plasmid patterns are represented by the dendrogram shown in Figure 2. Similarities ranged from about 55% to 100%. The organisms were clustered into 18 groups (78%), with more than 80% similarity between the groups. S. *sonnei* and S. *flexneri* strains clustered in different groups.

Figure 1. Plasmid Profiles of 15 Representative *Shigella Sonnei* And *Shigella Flexneri* Isolates in 0.8% Agarose Gel



Lane 1 DNA ladder 23 kb as the marker, lanes 2, 6, 9, 11, and 14: *Shigella flexneri*; lanes 3 - 5, 7 - 16 *Shigella sonnei*; lane 7, DNA ladder 10 kb as the Marker.

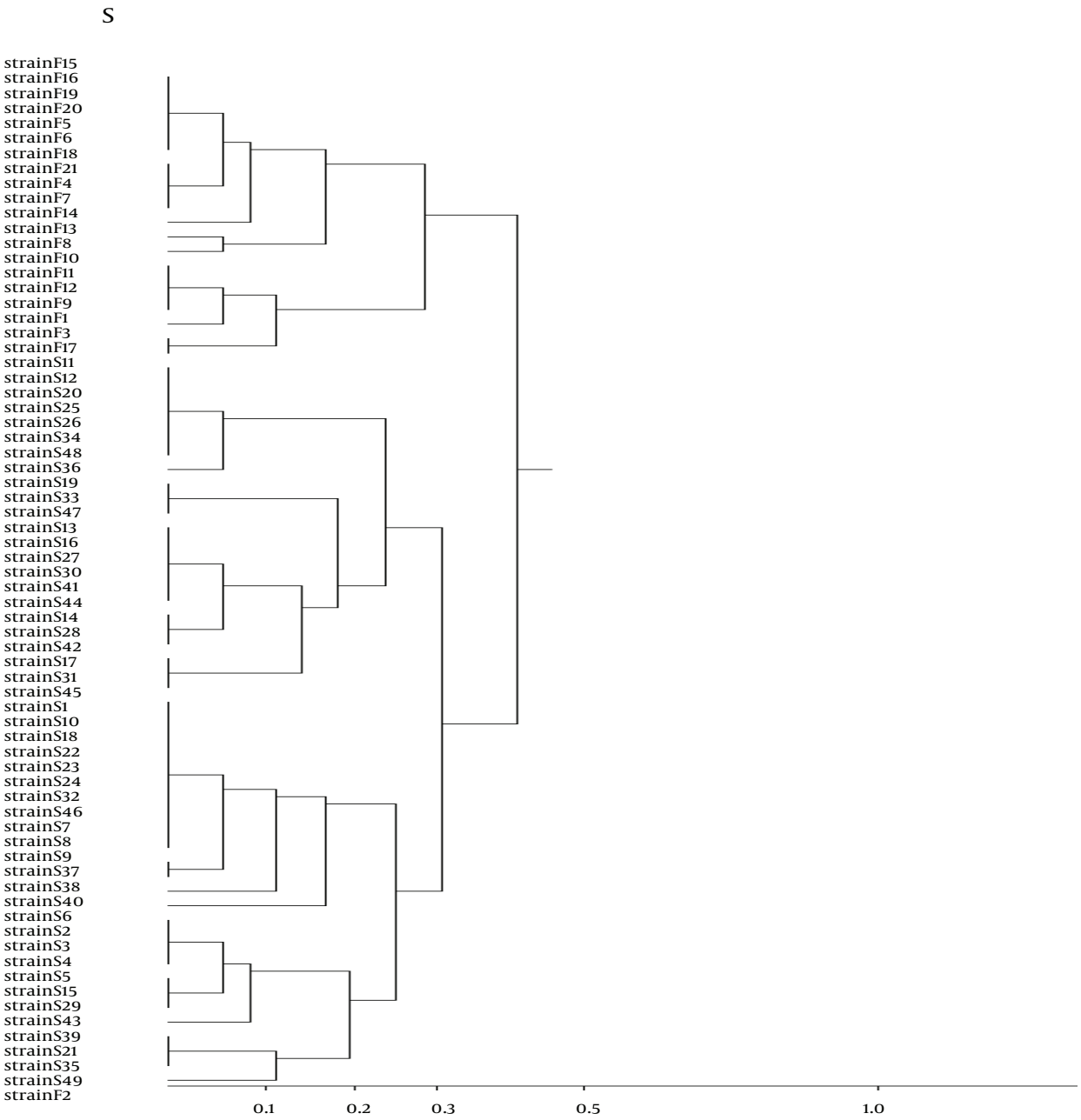


Figure 2. Clustering and Generating Dendrogram of Similarity Among the *Shigella* Isolates on the Basis of Their Plasmid Profiles

5. Discussion

Gastrointestinal diseases are the focus of attention at many hospitals, a common cause of admission to many of the clinics all over the world, and a significant and often preventable cause of death. Food-borne bacterial diarrhea, particularly shigellosis, is an emerging health threat that is attributable to the increased consumption of unsafe foods (12). Many cases of Shigellosis occurs annually in Ahvaz. The typical seasonal increase in shigellosis occurs during the spring and summer because of the hot weather (13). Our results showed that S. *sonnei* was the most common (70%) cause of dysentery and the children below 5 were the highest risk group for shigellosis in Abuzar pediatric hospital in Ahvaz. S. *sonnei* is the major cause of shigellosis in industrialized countries (4, 14). It has been recently reported as the dominant *Shigella* serotype in Iran. Unlike our results,

Khaghani et al. reported that S. *flexneri* was the most frequently isolated *Shigella* species in Ahvaz from 2008 to 2010 (13). So, we may face the microbiologic shift from S. *flexneri* to S. *sonnei* in Ahvaz. It is now found that many strains of Shigellea spp are resistant to commonly prescribed antibiotics, like tetracycline, ampicillin and sulfonamides, and multi-resistant strains are increasing (15). Antibiotic susceptibility testing showed high resistance against ampicillin, sulfamethoxazole- trimethoprim and cefexime in both S. *sonnei* and S. *flexneri* strains in children. Resistance to ceftazidime in S. *sonnei* strains is higher than S. *flexneri* strains. Ampicillin and sulfamethoxazole-trimethoprim are commonly used as the first-line drugs for the therapy of shigellosis while second-line drugs including the third generation of cephlosporines and fluoroquinolones are used for the treatment of children and adults respectively (11, 16). Unluckily, it has been recently reported that the prevalence of resistance to some commonly used antibiotics is increasing in *Shigella* spp in Iran (17, 18). Similar to our results Ranjbar et al. in 2013 also reported susceptibility to gentamicin, kanamycin, and Amikacin (11).

Resistance to ciprofloxacin and gentamicin was found to be low among *Shigella* isolates under study. Similarly, Ranjbar et al. and Farshad et al. from Iran and Vrints et al. from Belguim reported low or no resistance to Ciprofloxacin (5, 11, 19). In our study, the prevalence of ESBL producing *Shigella* isolates accounted for 11.4 % of all *Shigella* isolates which is larger than the reports from Iran and other countries (11, 20, 21). Plasmid profiling is useful for short episodes such as the occurrence of outbreaks in a hospital. By plasmid analysis similarities ranges were detected from 60 to 100% (Figure 2). *Shigella* strains clustered in 18 groups with over 85% similarity. Our findings indicated that although there is no common group or cluster between S. *sonnei* and S. *flexneri* strains, *Shigella* strains were closely related in this study. Indeed, each *Shigella* species have unique clusters. So, we may use plasmid profile analysis to discriminate between *Shigella* isolates in epidemiological investigations in hospitals. Our results also showed that within the species S. *sonnei* and S. *flexneri*, the plasmid profiles illustrate more strains than the antimicrobial susceptibility pattern. However, since plasmid profile analysis have a low discriminatory power and reproducibility; further studies using powerful typing techniques are needed to draw any conclusion regarding the genes which encode ESBLs and the putative sources of infection of *Shigella* (8, 22, 23). In fact, plasmid analysis has some shortcomings such as the lack of stability of plasmids in particular strains and cannot be used if the strains do not contain plasmid to begin with.

In conclusion, our data provided a local monitoring of the causative agents of diarrhea based on which a pragmatic program for the therapy of *Shigella* infections should be made. Our results also showed that the plasmid profiles distinguished more strains than the antimicrobial susceptibility pattern did. However, more robust methods are necessary to provide further information in such epidemiological studies.

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Footnotes

Authors' Contribution: Amin Sakhaei, Alireza Ekrami, and Leli Shokohizadeh developed the original idea and the protocol and wrote the manuscript, Mohammad Savari and Maryam Hadian contributed to the development of the protocol abstracted data, and prepared the manuscript.

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