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Full Length Research Paper

Molecular Epidemiology of *Shigella sonnei* Isolated from Clinical cases in Tehran using RAPD-PCR Method

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To characterize *Shigella* clinical strains, especially *Shigella sonnei*, we studied 80*Shigella* strains recovered from 495 stool samples of patients with bloody diarrhea in three hospitals in Tehran, Iran, over the period of 2 years from May2010 to June2012. Serological assay classified the *Shigella* isolates as follows: 60(75%) *Shigella sonnei* isolates, 15 (18.75%) *Shigella flexneri* isolates,2 (2.5%) *Shigella boydii* isolates, and 3 (3.75%) *Shigella dysenteriae* isolates. In an antibiogram test, all *Shigella* strains were susceptible to ciprofloxacin, and ceftriaxone. They showed high degrees of sensitivity to nalidixic acid, gentamicin and amikacin. Approximately 98.66% of the *Shigella* isolates were resistant to co-trimoxazole. The RAPD-PCR fingerprinting was carried out using ARB11 primer. The PCR product was run and visualized in 1% agarose gels and stained with ethidium bromide. The RAPD-PCR profiles were grouped depending on the patterns of the amplified bands. In conclusion, these data mandate local monitoring of drug resistance and its consideration in the empirical therapy of *Shigella* infections. These results also demonstrated that RAPD-PCR analysis is more reliable than antibiotic susceptibility pattern analysis and could be used as a routine laboratory method for the identification of *Shigella* species, those causes epidemic strains in Iran.

Keywords: Shigella, Serotyping, RAPD-PCR.

INTRODUCTION

Infectious diseases kill about 11 million children each year and 99 percent of these deaths occur in the developing countries. Notably, of the 11 million deaths, 4 million die within the first year of their life (Dipika et al., 2004; http://www.who.int/infectious-disease-report accessed on 20th April 2004). Acute diarrheal diseases rank second amongst all deaths due to infectious diseases accounting for3.1 million deaths in under 5 years old children; 80 percent of these deaths occur in children below 2 years of age. Shigellosis is an important cause of diarrheal deaths. It has been reported that no less than 140 million cases of shigellosis occur worldwide with 600,000 deaths annually; 60 per cent of such deaths are seen in under 5 children (Dipika et al., 2004).Shigellosis is an acute gastroenteritis that is one of the most common causes of morbidity and mortality in children with diarrhea in developing countries. The disease is caused by microorganisms belonging to the genus Shigella spp. which can be subdivided into four serogroups - *S.sonnei*, *S.boydii*, *S.flexneri* and *S.dysenteriae*. Each of them has a number of serotypes, e.g., *S.dysenteriae* type 1-12,*S.sonnei* phase I and II,*S.boydii* type 1-18, *S.flexneri* type 1-6. However, three predominant strains are responsible for majority of shigellosis cases viz., *S.sonnei*, *S.flexneri* 2a and *S.dysenteriae* type 1, and among these species

S.sonnei is encountered mostly in industrialized countries, *S.flexneri*2a in developing countries and *S.dysenteriae* type 1 is the only epidemic as well as pandemic strain. *S.dysenteriae* type 1, which produces severe disease, may cause life-threatening complications, is usually multi drug resistant and can cause large epidemics and even pandemics with high morbidity and mortality (Kotloff et al., 1999). The disease is highly contagious due to its low infectious dose. The infective dose is as low as 10-100 organisms only (Dipika et al., 2004; Bradbury et al., 1984). Epidemics usually occur in areas with crowding and poor sanitary conditions (Craun et al., 2005). Essential events in the pathogenesis of Shigella infections include bacterial invasion of epithelialcells, escape from the phagosome, and induction of apoptosis in macrophages (Guichon et al., 2001). Factors affecting the emergence or decline of epidemic shigellosis are not clear, and shigella are generally believed to have only a human or a primate host. Recently, the World Health Organization has emphasized the need to understand the disease burden and epidemiology of Shigella infections in developing countries (World Health Organization, 1999). The identification of Shigella species is important because of both their clinical and their epidemiological implications. Serological testing is also needed for the identification of Shigella isolates (Talukder et al., 2002). Understanding of the antibiotic resistance patterns of shigella and molecular characterization of plasmids and other genetic elements are also epidemiologically useful. Comparison of plasmid profiles is a useful method for assessing the possible relatedness of individual clinical isolates of a particular bacterial species for epidemiological studies (Dutta et al., 2002). The present study was designed to isolate Shigella strains from clinical samples of patients with bloody diarrhea by culture methods and characterize them by appropriate biochemical, serological, and antibiogram tests. It was also designed to genetically characterize the isolates by using molecular techniques, such as random amplified polymorphic DNA- PCR (RAPD-PCR). Furthermore, this study was carried out to investigate the reliability of drug sensitivity patterns for the discrimination of epidemic strains of Shigella spp. isolated from epidemics of bacillary dysentery.

MATERIALS AND METHODS

Settings and study period

The study was conducted over a period of 2 years from May 2010 to June 2012 in three hospitals (Imam Khomayni, Shariati, and Sina) in Tehran, Iran. Stool specimens from 495 patients aged 2 months to 11 years were collected from three hospitals of the Tehran University of Medical Sciences and transferred to a clinical microbiology laboratory. The inclusion criteria were as follows: the age of the patients were in the range of 2 months to 11years, the patients had diarrhea that had lasted 7 days, and blood was detected by stool examination by an occult blood (OB) test.

Bacterial culture and isolation

All OB-positive samples were inoculated on xylose-lysin-desoxycholate agar and incubated at 37°C for 24 h. Non-lactose fermenting colonies were picked from the culture plates and were subjected to further analysis by biochemical tests (Motility, MR, Citrate, H2S, Indole, Lysine decarboxylase, Ornitin decarboxylase, ONPG) for the identification and isolation of possible Shigella colonies. The isolates, which were confidently identified as *Shigellasonnei*, were stored at 70°Cand transferred to a clinical microbiology laboratory in buffered glycerol-saline transport medium to Isfahan University for further studies. Two reference strains, *Shigella flexneri* ATCC 12022 and *Shigellasonnei* ATCC 9290 were used as control strains for comparison purposes.

Serological tests

The serotypes of all Shigella isolates were determined with commercially variable polyclonal antisera (Mast Co., Merseyside, United Kingdom) against all Shigella serotypes. The Shigella strains were subcultured on MacConkey agar plates, and serological tests were performed by the slide agglutination method, as described previously (Dutta et al., 2002).

Antibiogram analysis

Antibiotic resistance/sensitivity profile was analyzed following disk diffusion method (Hossain et al., 1998) using commercially available antibiotic disks (Mast Co.) The antibiotic disks used in this study were amikacin 30 µg, amoxicillin 30µg, ampicillin 10µg, ceftriaxone 30µg, co-trimoxazole 30µg, chloramphenicol 30 µg, ciprofloxacin 30µg, gentamycin10 µg, nalidixic acid 30µg, tetracycline 30 µg and trimethoprim 10µg.

Genotypic characterization

Random amplified polymorphic DNA (RAPD) analysis

For RAPD, genomic DNA was extracted as described by Sambrook (Alam et al., 2001). Briefly, genomic DNA was prepared from bacterial cultures grown overnight in Luria Broth at 37°C by pelleting 1.5 ml culture by centrifugation at 8,000 rpm for10 min, resuspended the pellet in 500µl of sterile distilled water and boiling at 100°C for 10 min. It was then snap cooled at –20°C for 5 min, centrifuged at 5,000 rpm for10 min at 4°C. The supernatant containing DNA template was amplified using ARB11primer (5'-CAT TCG ACC 3') (CLS, 2002). The reaction was performed

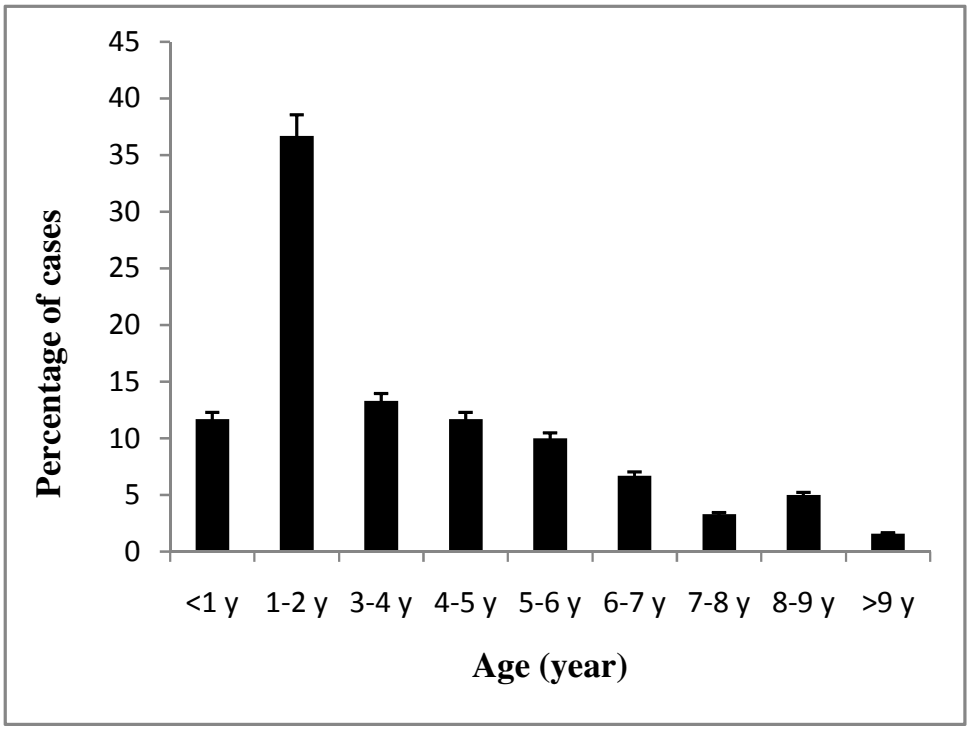

Figure 1. The rate of patient infected to shigellosis according of age. 73.3% patient were under 5 years and another (26.7%) had age ranging from 5 to 11 years.

in a total volume of 50µl per tube containing 5µl of 10X PCR buffer (500mM KCl,100 mMTris (pH 8.3), 25 mM $MgCl_2$), 3µl of template DNA, 5µl primer, 0.5µl of Taq DNA polymerase (Bangalore Genei, India), and 5µl of 2.5 mM each dNTP (dATP, dCTP, dGTP and dTTP). A negative control was assayed containing all components except template DNA. After initial denaturation at 94°C for 5 min the reaction mixture was run through 30 cycles of denaturation at 94°C for 30sec, annealing at 40°C for 1 min and extension at 72°C for 1 min, followed by a 10 min final extension period at 72°C.Theexpected size of the amplicons was ascertained by electrophoresis in 1.0% agarose gel with an appropriate molecular size marker (1 Kb DNA ladder) (CLSI, 2002; Sambrook et al., 1989).

RESULTS

Cases of shigellosis

Among 495 children with diarrhea, 148(30%) OB-positive patients from the ages of 2 months to 11 years old were enrolled in our study (Figure 1). Seventy percent of the 148 patients were under 5 years old. There were 89 males (60%) and 59 females (40%). Fever (91%), vomiting (69.7%), abdominal pain (69.8%), and convulsion (23%) were the most common presenting symptoms. Based on the biochemical and bacteriological properties, 80(54%) of these isolates were confirmed to be Shigella and finally 60 isolates confirmed to be *Shigellasonnei*.

Shigella serotypes

Based on our serological finding, 60 (75%) of the isolates were identified as *Shigella sonnei* and all patients were children under 11 years old (Figure 1), 15 (18.75%) were identified as *Shigella flexneri*, 2(2.5%)were identified as *Shigella boydii*, and 3 (3.75%) were identified as *Shigella dysenteriae*.

Antibiotic susceptibility analysis

The antimicrobial activities of eleven commonly used antibiotics against all the *Shigella* isolates were determined by the Kirby-Bauer method and according to the recommendations of the CLSI (20). The results of the antibiotic susceptibility tests for four species of Shigella isolates are shown in Table 1. It was shown that out of 60 isolate of *shigella sonnei*, 62% were resistant to ampicillin, 96% were resistant to co-trimoxazole, 15% were resistant to amoxicillin, 4% were resistant to nalidixic acid, and 15% were resistant to tetracycline, 2% were resistant to trimethoprim and 100% were susceptible to amikacin, ceftriaxone, chloramphenicol,

Table 1. Antimicrobial resistance patterns of shigella isolates from Iran

Antibiotic resistance pattern	No. of Shigella isolates (n=80)		
	S. sonnei (*n*=60)	*S. flexneri* (*n*=15)	*S. dysenteriae* (*n*=03)
Amx	9	6	2
Amk	0	0	1
Amp	23	8	1
Cro	0	0	0
Stx	58	60	60
Chl	0	2	0
Cip	0	0	0
Gen	0	2	1
Nal	58	4	1
T	3	4	1
Tr	1	4	0

Amx, amoxicillin; Amk, amikacin; Amp, ampicilin; Cro, Ceftriaxone; Stx, c0-trimoxazole; Chl,chloramphenicol; Cip, ciprofloxacin; Gen, gentamycin; Nal. nalidixic acid; T,tetracycline; Tr, trimethoprim.

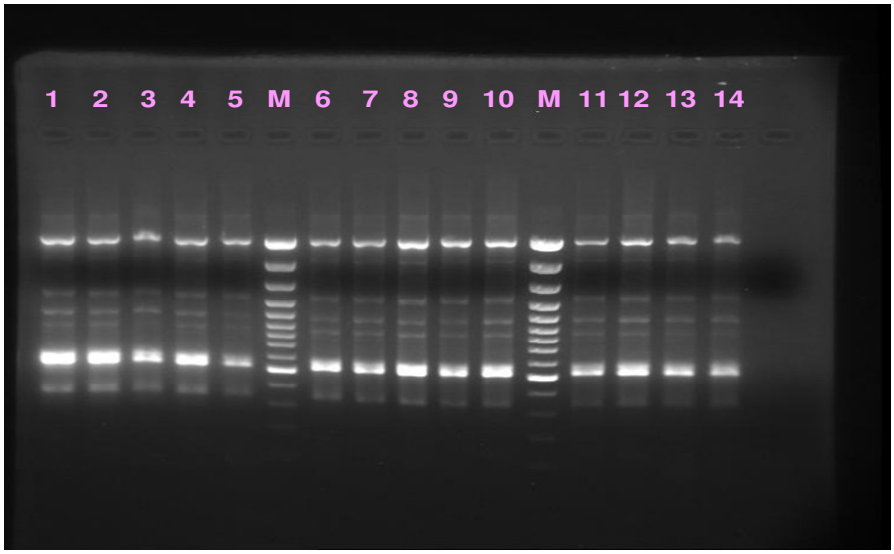

Figure 2. RAPD-PCR profiles of 14 representative *shigella sonnei* species in 1% agarose gel. Lane 6, 12 are super coiled DNA ladder (the marker). 39 patients (65%) had common pattern band. The data related to another species not shown.

ciprofloxacin, gentamicin. Likewise, out of 15 isolate of Shigella flexneri 43% were resistance to amoxicillin, 47.5% were resistant to ampicilin, 17.4% were resistant to gentamycin and chloramphenicol,100% were resistant to co-trimoxazole all isolates were susceptible to amikacin, ceftriaxone and ciprofloxacin. Out of *Shigella dysenteriae*, 14.3% were resistant to tetracyclineand ciprofloxacin, 42.8% were resistant to amikacin and nalidixicacid, 57% were resistant to gentamycin, 71.4% were resistant toamoxicillin, 28.5% were resistant to ampicillin and all isolates were susceptible to co-trimoxazole, ceftriaxone and chloramphenicol. Theremaining isolates were resistant to one or two antibiotics.

Genotypic characterization

Analysis of all 60 isolates revealed that the *Shigellasonnei* species produced five different RAPD pattern with bands ranging from 5–11. One common band of 500bp was observed in all the isolates, while a band of 325bp was evident in 65%of the isolates. This finding indicated that, there are close relationship between these isolates, as well as 15 isolates of *S. flexneri* showed 3 RAPD profiles with bands ranging from 7–11 and 2 isolates of *S.boydii* showed 2 RAPD profiles with bands ranging from 7 to 9 and 3 isolates of *S. dysenteriae* showed single RAPD profile with 7 bands (Figure 2).

DISCUSSION

Epidemiological studies of shigellosis are generally based on standard culture methods of diagnosis. Shigellosis is an important public health problem with high morbidity and also mortality mainly among children in developing countries situations where overcrowding and poor personal hygiene are rampant. Bacillary dysentery caused by members of the genus Shigella is prevalent in many countries with temperate climates. It is a disease of children from 6 months to 10 years of age, although it can affect susceptible individuals of any age who are subject to poor sanitation (Bhattacharya et al., 1987. In our study most of the patients (73.33%) were under 5 years of age. Similar results were reported from a survey of Shigella infections in the United States from 1974 to1980, in which the age group with the highest rate of infection comprised children less than 5 years of age, and also is in agreement to results come from study that, has down by Sh. Farshad et all in Shiraz. Iran. (Blaser et al., 2003; Shohreh et al., 2006). This reflects the fact that in general young children are less likely than older children to practice good hygienic habits. Serotyping of the isolates showed that *S.sonnei* was the most frequent species (75%) isolated in the period of our study. Most cases of dysentery, especially those due to *S.sonnei* infection, are mild and do not require antibiotic therapy (1). More than half of our patients (50.9%) showed mild clinical symptoms. However, the severity of the clinical symptoms is related to the bacterial species, the age of the patients, the immunity of the patient, and the inoculums dose. While Iran is a developing country, the prevalence of *S. sonnei* in Iran is similar to that shown by the results of some of studies that have been done in Israel, the United States, Canada, and other developed countries. It has been found that S. sonnei is the predominant species in those countries and is more common in children than in adults (Ashkenazi et al., 2003; Faruque et al., 2002). However, in Taiwan and Bangladesh the infections are mostly caused by *S. flexneri* (Faruque et al., 2002; Chiou et al., 2001; Tacket et al., 1984). It has been suggested that factors involved in natural selection may have been the main reason for these discrepancies (Pan, 1997). In case management of shigellosis antibiotics play a central role. Use of appropriate antibiotic hastens recovery, shortens the duration of excretion of pathogen in stool and possibly prevents complications. However, these should be chosen carefully considering the sensitivity pattern of the circulating strains (Dipika et al., 2004).

Antimicrobial resistance patterns are valuable as a guide to empirical therapy, as a typing method, and as an indicator of the dissemination of antimicrobial resistance determinants (SilviaY et al., 1998). By analyzing trends in the resistance patterns of the various Shigella species, we found that S. sonnei is currently significantly more resistant than the other Shigella species (Table 1).

This finding is of special importance, because at present *S. sonnei*is the predominant species in Iran. According to our findings and other reports, the rate of resistance to the antimicrobial agents used to treat shigellosis in young children, namely co-trimoxazole, has reached 96 to 100% (Ashkenazi et al., 2003; DeLappe et al., 2003; Mermel et al., 1997; Oh et al., 2003). However, the overall susceptibility patterns of the test strains focus on the fact that the strains were not frequently exposed to expanded- or broad-spectrum antibiotics. Therefore, due to the lack of variability in susceptibility patterns, the antimicrobial resistance pattern was not a useful epidemiological marker in our study. Antimicrobial resistance in enteric pathogens is of great significance in the developing world, where the rate of diarrheal diseases is highest. The progressive increase in antimicrobial resistance among enteric pathogens in developing countries is becoming a critical area of concern. During the present investigation it was observed that most of the isolates were resistant to amoxicillin while variable resistance patterns were observed with different species of *Shigella* with respect to different antibiotics (Anjana et al., 2009). Furthermore the RAPD-PCR is a DNA fingerprinting technique, which is one of the most sensitive and efficient method for distinguishing different strains of a species and it can be used as a strong and useful method for diagnosis in ecological and epidemiological studies. And this is in agree with previous studies have done by Warner et al (Warner and Oliver, 1999). it has been reported to be an effective subtyping method for several other species, namely *Listeria, Yersinia* (Rasmussen et al., 1994), *Staphylococcus* (Young et al., 1994), *Streptococcus* (Sepala et al., 1994) and *Aeromonas* (Sharma et al., 2005). Finally in this study it was shown that RAPD- PCR method has a relative good discrimination power, and according to its high discrimination power, the ability of typing and reproducibility, low cost, rapidity and facility, is a good method for typing of Shigella isolates in molecular epidemiology studies.

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