

# Plasmid Profile, Antibiotic Resistance, and Phenotypic Virulent Strains of *S. flexneri*

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## Abstract

Shigellosis is an acute gastroenteritis caused by *Shigella* species. The purpose of this study was to determine plasmid profile, antibiotic resistance and phenotypic virulent by Congo red between *S. flexneri* strains. The isolated bacteria were identified by standard bacterial and biochemical methods. Plasmids were isolated by alkaline lysis method. Antibiotic susceptibility test was performed according to "Kirby-Bauer" method. Serological reactions were carried by slide agglutination tests with both polyclonal and monoclonal antiserum kits. Virulent strains were isolated on a TSA plate contained Congo red dye concentration. From 350 isolated *Shigella* species, 142 (40.57%) were *S. flexneri*. Eleven distinct plasmid profile patterns were identified. Of *S. flexneri* isolates, 95% were resistant to tetracycline, 85.6% to SXT and 75.3% to ampicillin. All the isolates were sensitive to ciprofloxacin. Our results showed that 39% were serotype II. 45.56% of *S. flexneri* were Congo red positive. Antibiotic resistant determination in each case may prevent drug resistance increase. Since Congo red binding test is cheap and simple it can be used to determine virulence properties of *S. flexneri*.

**Keywords:** *S. flexneri*, Plasmid profiles, Congo red, Antibiotic resistant

## Introduction

Shigellosis is an acute gastroenteritis caused by *Shigella* species, including *S. dysenteriae*, *S. flexneri*, *S. boydi* & *S. sonnei* (1-5). Volunteer challenge studies showed that shigellosis can be evoked by an extremely small inoculum (10-100 organisms) and the time of onset of symptoms is influenced by the size of the challenge (1, 2). In healthy adults, dysentery is a self-limited disease but it can be fatal to infants & young children (6). There are approximately 164.7 million cases of which 163.2 million in developing countries and 1.5 million in industrial countries (7). Each year 1.1 million people are estimated to die from *Shigella* infection and 61% of all deaths attributable to shigellosis involve children less than 5 yr old (7). Epidemics usually occur in areas with crowding & poor sanitary condition, where transmission from person to person is common, or when food or water is contaminated by the organism (1, 6-10). *S. sonnei* is classically

predominant in Thailand (71%) (7). *S. flexneri* type 2a is predominant in developing countries (60%) and in Iran (1, 7, 11). *S. dysenteriae* type 1 (Sd1) is the only cause of epidemic dysentery (7). It is known that *S. dysenteriae* and *S. flexneri* are the predominant species in the tropical countries (1, 6, 7).

Many of the bacterial virulence determinants that mediate the complex interactions are encoded by large plasmids (6). Determination of plasmid profile has been shown to be a powerful tool in epidemiological studies when used as a fingerprint for a strain (6, 12). The plasmid profile may aid in differentiation of strains, identifying a source of infection or evaluating the efficiency control measures (12, 13). Resistance in *Shigella* to multiple antibiotics such as sulfonamides, streptomycin, ampicillin, chloramphenicol and tetracycline was first reported in Japan shortly after their introduction as therapeutic agents for the treatment of shigellosis

(5). Resistance to tetracycline has increased dramatically since the first appearance of resistance in 1953 in *S. dysenteriae* (4, 14). Agar medium containing Congo red differentiates virulent and virulent colonies of *Shigella* strains.

The aim of this study was to determine plasmid profile, antibiotic resistance, and phenotypic virulent strains by Congo red binding and production of haemolysin of *S. flexneri* strains in Tehran.

## Materials and Methods

**Bacterial strains** *Shigella flexneri* strains were isolated from fecal specimens of children attending to the 3 children hospitals (Markaz-e tebbi Kudakan, Ali Asgar Hospital and Mofid Hospital) from January 2001 to December 2003. After identification of strains by standard microbiological and biochemical tests, such as growth on SS agar, MacConkey agar or XLD agar, TSI agar for detection of K/A, gas(-) and H<sub>2</sub>S(-) production were done. Lysin decarboxylase test and ONPG were checked. The *S. flexneri* strains were stored at -70 °C in peptone and glycerol. *S. flexneri* strains were routinely grown at 37 °C in Mac Conkey agar.

References strains of *S. flexneri* 2a obtained from Bahar Afshan Company and *S. flexneri* 2a, 1, 6 Newcastle, from Iran Reference Laboratory, Mast assure™ *Shigella* antisera (3).

**Plasmid isolation** Plasmids were isolated by alkaline lysis method (2, 3, 15, 16). Briefly, in this method, a single colony was picked up and grown in 5 ml BHI broth for overnight. Then cells were lysed by the addition of SDS and NaOH. Then, proteins were precipitated by phenol/ chloroform solution (1: 1). The precipitated DNA was washed with 70% ethanol and suspended in TE buffer. Plasmid bands were separated by electrophoresis through agarose gel (0.8%) in TAE buffer. DNA fragments visualized by ethidium bromide (0.5% µg/ ml) staining and photographed under UV light illumination.

**Antibiotic susceptibility** Antibiotic susceptibility (antibiogram) testing was performed accord-

ing to “Kirby- Bauer” method on Muller- Hinton agar and used 0.5 MacFarland standard based on NCCLS (17). Following antibiotics, disks of nalidixic acid, ampicillin, gentamicin, trimethoprim/ sulfamethoxazole, cephalotin, tetracycline, kanamycin, amikacin, chephalexin, ciprofloxacin were used.

**Serological typing** Serotyping of *S. flexneri* isolates type 2 was confirmed by a slide agglutination test using 2 following commercial serotype kits:

- 1- A commercially available antiserum kit (Bahar afshan) specific for all types
- 2- Monoclonal antibody reagent (Mast co Ltd) specific for *S. flexneri* 2(a,b)

In this method, isolated strains were subcultured on MacConkey agar plates. After 18 h, serological reactions were carried out by slide agglutination tests (3, 6, 18). Two drops of sterile 0.85% saline solution were placed on to a cleaned slide. A loop of organisms from a fresh culture was emulsified to each drop of saline to produce a distinct turbidity. A drop of a monovalent or polyvalent antiserum was added on it. Saline solution was added on control drop. After mixing the suspension by tilting the slide back and forth for 60", the agglutination reaction was detected under indirect light.

**Determination of Congo red binding ability and haemolysin production** Phenotyping analysis for screening virulent of strains of *S. flexneri* was done by Congo red dye. A colony of fresh culture of isolated bacteria were cultured on a plate containing TSA (Tryptic Soy agar) and Congo red solution at final concentration of 0.003% to detect red pigmentation colony ( 19, 20).

## Results

From 350 isolates of *Shigella* species, 142 (40.57%) were *S. flexneri*, of which, 100 randomly were enrolled in this study. 40.7% of patients were female, with the age distribution from 6 months to 7 yr old.

**Plasmid profile analysis** Analysis of plasmid DNA, revealed that all of the isolated strains

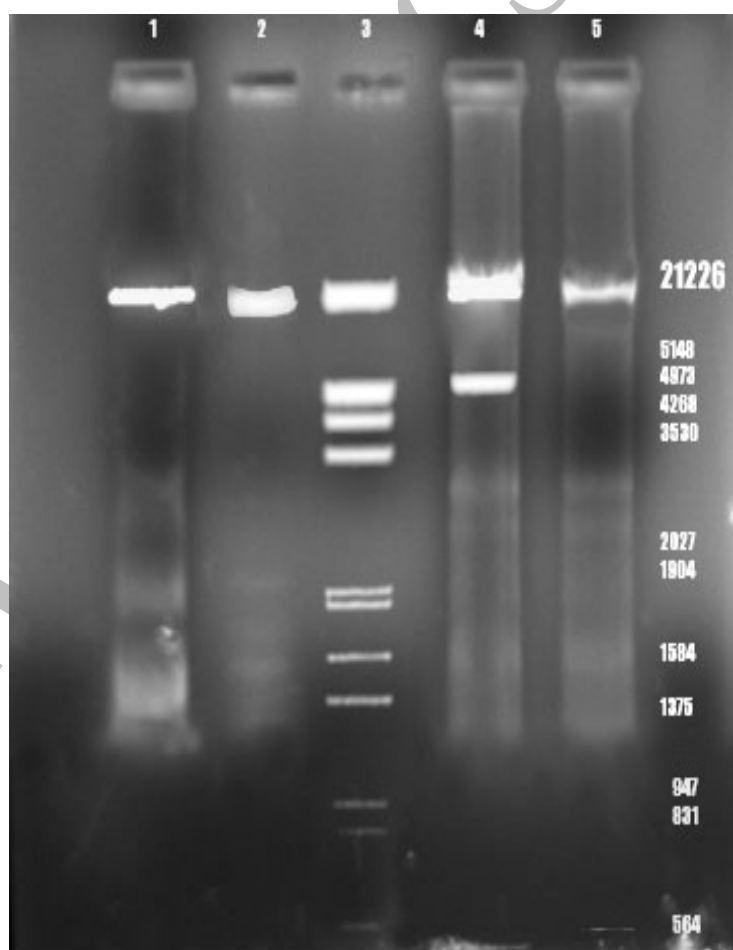
contained multiple plasmids (1-5 plasmid bands) which ranged from 0.564 kb to 21.226 kb, and forming a number of unique banding patterns. Plasmids of the same size were present in multiple strains, for example, most of strains (78.8%) harbored the 21.226 kb plasmid. Eleven distinct plasmid profiles were identified. Only bright and sharp bands were concerned, faint bands were interpreted as relaxed forms of the brighter bands. The plasmid profiles of some strains are shown in Table 1, Fig.1. From 10 isolates (17.3%), no plasmid bands were detected.

**Antibiotic resistance** Antibiotic resistance and sensitivity of *S. flexneri* strains is shown in Table 2. Multiple resistance of strains to am-

picillin, tetracycline, trimethoprim- sulfomethoxazole and cephalixin were 56.7%.

**Serological typing** Our biochemical results confirmed with serological slide agglutination. There were different serotypes of *S. flexneri*, but strain 2 was the most prevalent serotype, because 38.9% of them were agglutinated with monoclonal antibody 2 (a, b).

**Determination of Congo red binding ability and haemolysin production** In this study, 45.56% of *S. flexneri* were Congo red positive colonies (red colony) on TSA contained 0.003% Congo red dye. All the Congo red positive colonies (100%) produced  $\beta$  haemolysin on blood agar.



**Fig. 1:** Plasmid profiles detected in some *S. flexneri* strains. 1, 2, 4, 5: samples, 3: Lambda DNA/*EcoRI*+*HindIII* Marker, Fermentas.

**Table 1:** Plasmid profiles detected between isolates of *S.flexneri* strains.

Plasmid groups	Plasmid bands( kb)								%
	0.56%	0.83%	0.94%	1/375	2/027	3/530	5/148	21/226	
I	-	-	+	+	+	+	-	+	3.5
II	-	-	-	+	-	-	+	+	1.75
III	-	-	-	-	-	-	+	+	1.75
IV	-	-	-	+	+	+	+	+	3.5
V	-	-	-	+	+	-	+	+	1.75
VI	-	-	+	-	+	-	-	+	3.5
VII	+	-	-	-	+	+	-	+	1.75
VIII	-	-	-	+	+	+	-	+	1.75
IX	+	-	-	-	-	-	-	+	1.75
X	-	-	-	-	-	-	-	+	61.40
XI	-	-	-	-	-	-	-	-	17.6

**Table 2:** The results (%) of antibiogram between isolates of *S. flexneri* strains.

Antibiotics	CN <sub>30</sub>	AN <sub>30</sub>	AM <sub>10</sub>	Te <sub>30</sub>	CF <sub>30</sub>	SXT	GM <sub>10</sub>	NA <sub>30</sub>	CP <sub>5</sub>
Resistance (R)	70.3%	29.6%	91.3%	95%	27.3%	85.6%	16.6%	1.2%	-
Intermediate (I)	28.7%	25.3%	-	1.2%	5%	2.1%	5%	1.2%	-
Sensitive (S)	1%	45.1%	8.7%	3.8%	67.7%	12.3%	78.4%	97.6%	100%

## Discussion

Of 350 isolated *Shigella*, 142 (40.57%) were *S. flexneri*. In a study, of 734 stool samples collected from patients with acute diarrhea, 123 (16.8%) yielded *Shigella* species, 7.5% *S. flexneri*, 5.2% *S. sonnei*, 2.6% *S. dysenteriae*, and 1.5% *S. boydii* (20). This result besides other studies showed that *S. flexneri* was the most prevalent species in developing countries including Iran (1, 21, 22). Our results confirmed these findings. Stool samples of 810 patients with diarrhea dysentery showed that *S. flexneri* (48%) was the most common sero-group (23), which agreed with our results.

In this study, the male to female ratio was (1.4/1) which was similar to another study (24).

Of 33 *S. flexneri* isolated with the exception of one strain isolated in 1998 which were sensitive to all of antimicrobials, all the other isolates

were resistant to at least 6 drugs and supposed to be multiply drug resistance (6). Our results similarly showed that 56.7% of isolated *S. flexneri* strains had multi drug resistance to ampicillin, tetracycline, and SXT. Besides, in both studies, 100% of strains were sensitive to ciprofloxacin. In the study of Turner et al., 100% of isolated *S. flexneri* strains were resistant to carbenicillin, streptomycin, chloramphenicol, tetracycline, ampicillin, ticarcillin. However, 13 isolates were resistant to SXT (39.3%) and nalidixic acid (6%). All the strains were susceptible to cephalotin, colistin, kanamycin, amikacin, ciprofloxacin, cefoxitin & cefotaxime (5). Results of this study showed more resistance to tetracycline, ampicillin, and SXT, which might be due to geographic differences or indiscriminate use of these drugs. Kaisar et al. study, showed that 42% of isolates were resis-

tant to three commonly used antibiotics, ampicillin, tetracycline and thrimethoprim-sulfamethoxazole (3) which was close to our result. Eleven distinct plasmid patterns were identical and plasmid size ranged from 0.564 kb to 21.226 kb. Plasmids of the same size were present in multiple strains, for example, most of strains (78.8%) harbored the 21.226 kb plasmid. Besides, the type strain of *S. flexneri* 2a from Reference Lab. of Iran, harbored 2 plasmid bands, 21.226 kb and 5.148 kb.

Of 33 *S. flexneri* isolates, 11 distinct profiles were detected (6). The type strain of *S. flexneri* ATCC 9403 harbored four plasmids with 2 same size of bands (18kb, 1.7kb) (5). Similarly, in both studies not only 11 plasmid profiles were detected but also, from type strains of *S. flexneri* 2a, 2 distinct plasmid bands were isolated. However, due to different type strains between two studies, different plasmid sizes were detected. The ability of *Shigella* spp. to bind to Congo red of agar medium is generally correlated with their virulence properties. In this study, 45.56% of *S. flexneri* isolates were Congo red positive colonies on TSA contained 0.003% Congo red dye. Maurelli showed the relationship between the virulence of *S. flexneri* 2a and their ability to absorb Congo red (19). The Congo red positive (CR<sup>+</sup>) isolates of the various *Shigella* strains all produced distinctly red colonies by 24 h of incubation; whereas the *E. coli*, *V. cholera*, *N. meningitidis* strains required at least 48 h of incubation to absorb sufficient dye to easily distinguish CR<sup>+</sup> and CR<sup>-</sup> colonies (20). This property is correlated to invasiveness property of *S. flexneri* in cell culture (25). Our unpublished results confirm these data too. In another study, all Congo red positive strains of *Shigella dysenteriae* type 1, *Shigella flexneri*, *Shigella boydii* and *Shigella sonnei* were haemolysine positive (26). Similarly, in our study all 45.56% Congo red positive *S. flexneri* strains were haemolysin positive on Blood agar.

It is concluded that *S. flexneri* isolation is important, because it is the dominant strain in developing countries and may cause death in acute

cases. To determine virulence properties of *S. flexneri*, using Congo red binding test is very cheap, rapid, and simple.

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### References

1. Azizi F, Hatami H, Janghorbanai M (1373). *Epidemiology and control of common acute diseases in Iran*. Shaid Beheshti Medical University. (In Persian).
2. Chiou CH, Hsu WB, Wei HL (2001). Molecular epidemiology of a *Shigella flexneri* outbreak in a Mountainous township in Taiwan, Republic of China. *JCM*, 39 (3): 1046-58.
3. Kaisar A, Zhahirul Islam T, Aminul Islam M (2003). Phenotypic and genotypic characterization of provisional serotype *S. flexneri* 1c and clonal relationship with 1b strains isolated in Bangladesh. *JCM*, 41 (1): 110-17.
4. Hartman AB, Essiet II, Isenbarger DW (2003). Epidemiology of tetracycline resistance determinants in *Shigella* spp and enteroinvasive *E. coli*: characterization and dissemination of tet (A)-1. *JCM*, 41 (3):1023-32.
5. Turner SA, Luck SH, Sakellaris H (2003). Molecular epidemiology of SRL pathogenicity Island. *Antimicrobial Agents Chemotherapy*, 47(2): 727-34.
6. Min SK, Park EH, Kim MH (2002). Molecular epidemiological characteristics of *S. flexneri* strains isolated in Busan during the period 1998 to 2002: Antibiotogram, plasmid profile and serotype correlation. *Health Environ*, 12: 38-47.
7. WHO (2002). Initiative for vaccine research (IVR). Available from: [www.google.com](http://www.google.com).
8. Hale TL, Keusch GT (2000). *Shigella*. *Med Micro*. Available: [www.google.com](http://www.google.com)

9. Kotloff KL, Winickoff JP, Ivanoff B, Clemens JD, Swerdlow DL, Sansonetti PJ (1999). Global burden of *Shigella* infections: implications for vaccine development and supplementation of control strategies. *Bull WHO*, 77: 651-66.
10. Martin DI, Gustafson TL, Pierce GV (1986). Contaminated produce, a common source for outbreaks of *Shigella* gastroenteritis. *AM J Epidemiol*, 124: 299-305.
11. Willer EM, Lima RL, Gimenes L (2004). In vitro adhesion and invasion inhibition of *S. dysenteriae*, *S. flexneri* & *S. Sonnei* clinical strains by human milk proteins. *BMC Microbiol*, 4(1): 180-90.
12. Litwin CM, Storm AL, Ryam KJ (1991). Molecular epidemiology of *Shigella* infections: plasmid profiles, serotype correlation and restriction endonuclease analysis. *J Microbiol*, Jan: 104-108.
13. Shahed N, Huq MI, Cohen ML (1984). Usefulness of plasmid profiles for differentiation of *Shigella* isolates in Bangladesh. *JCM*, Aug: 300-301.
14. Haltalin KC, Nelson JD, Ring RD, Hintoni L (1967). Double-blind treatment study of shigellosis comparing ampicillin, sulfadiazine placebo. *J Pediatr*, 70: 970-81.
15. Sambrook R (2001). *Molecular Cloning*. CSHL press (Vol 1), pp.:32-7.
16. Kado CI, Liu ST (1981). Rapid procedure for detection and isolation of large and small plasmids. *J Bacteriol*, 145:1365-73.
17. Kaiser AT (etal) (2002). Phenotypic and genotypic characterization of serology atypical strains of *S. flexneri* type isolated in Dhaka, Bangladesh. *JCM*, 40(7): 2490-97.
18. Maurelli AT, Blackmon B, Roy Curtiss III (1984). Loss of pigmentation in *S. flexneri 2a* is correlated with loss of virulence and virulence-associated plasmid. *Infect Immun*, Jan: 397-401.
19. Payne SM, Richard A, Elstein F (1977). Detection and differentiation of Iron-responsive virulent mutants on Congo red agar. *Infect Immun*, Oct: 94-98.
20. Tacket CO, Shahid N, Huq MI (1984). Usefulness of plasmid profiles for differentiation of *Shigella* isolates in Bangladesh. *JCM*, 20: 300- 301.
21. Lee WS, Puthucheary SD (2003). Species distribution and antibiotic resistance of *Shigella* isolates in an urban community in Malaysia. *Med J Malaysia*, 58(2): 262-67.
22. Egah DZ, Banwat EB, Audu ES, Allanana JA, Danung ML, Damen JG, Badung BP (2003). Multiple drug resistant strains of *Shigella* isolates in Jos central Nigeria. *Niger Postgrad Med*, 10(3): 154-56.
23. Vasilev V, Andron N, Japeth R Agmon (2004). Variability of *S. flexneri* serotypes in Israel during a period of two years: 2000 & 2001. *Epidemiol Infect*, 132(1): 51-6.
24. Daskaleros PA, Payne SM (1987). Congo red binding phenotype is associated with hemin binding and increased infectivity of *Shigella flexneri* in the HeLa cell model. *Infect Immun*, 55(6): 1393-98.
25. Sharma K, Rishi P, Grewal JS, Ram S, Tiwari RP (2001). Correlation between Congo red binding and contact haemolysin production in *Shigella* species. *Microbs*, 106(413): 31-38.
26. Sankaran K, Ramachandaran V, Subrahmanyam YV, Rajarathnam S, Elango S, Roy RK (1989). Congo red mediated regulation of levels of *Shigella flexneri 2a* membrane proteins. *Infect Immun*, 57(8): 2364-71.