Molecular mechanism of multi-drug resistance in *Shigella* Isolates from Rural China

S Mehata and GC Duan

Department of Epidemiology, College of Public Health, Zhengzou University, Henan, China

Corresponding author: Prof. Dr. Guangcai Duan, Department of Epidemiology, Zhengzhou University, Henan, China; e-mail: gcduan@public.zz.ha.cn

ABSTRACT

Diarrhoeal diseases and enteric infections are major causes of morbidity and mortality in the developing countries. Shigellosis was sixth in the rank of death caused by infectious disease in China. Present study assess the patterns of antimicrobial susceptibility and mutations in *marA* genes of *Shigella* isolates and its association. One hundred isolates of *Shigella* spp were tested to evaluate the antimicrobial susceptibility and mutation of *marA* through PCR-SSCP. The antimicrobial resistance profiles were: Amoxicilline-clavum (85%), Nalidixic acid (100%), Piperacilline (100%), Ciprofloxacin (25%) and Polymixin B (1.1%). *S. flexneri* isolates were more resistant than those of *S. sonnei* to Amoxicilline-clavum (96.6% versus 0.0%, P<0.001) and Ciprofloxacin (27.3% versus 8.3%, P=0.141). *S. felxneri* isolates were more resistant than those of *S. sonnei* to three or more antimicrobial agents (96.6% versus 8.3%, P<0.001). Overall mutation rate in *mar*A was 19%. Nucleotide sequencing showed mutations in three positionsÿcodon 6 (Delation of C), 319 (Ala Gly) and 374 (Addition of C). The mutation of *marA* may play a minor role and other mechanisms may contribute to the drug resistance of *Shigella* spp.

Keywords: antimicrobial resistance, *marA*, mutation, PCR-SSCP, *Shigella*.

INTRODUCTION

Shigellosis still remains a public health problem in developing countries because of poverty, poor sanitation, personal hygiene and poor water supply.[1] Antimicrobial therapy for shigellosis reduces the duration and severity of the disease and can also prevent potentially lethal complications. However, over the past few decades *Shigella* spp. have become resistant to most of the widely used antimicrobials.[2-4] At present, multi-drug resistance has complicated the selection of empirical agents for treatment of shigellosis, particularly in children.[5]

The chromosomal multiple antibiotic resistance (*mar*) locus of *Escherichia coli* and other members of the *Enterobacteriaceae* controls resistance to multiple, structurally unrelated compounds including antibiotics, household disinfectants, organic solvents and other toxic chemicals.[6,7] Present study assess the patterns of antimicrobial susceptibility and mutations in *marA* genes of *Shigella* isolates and its association.

MATERIALS AND METHODS

Bacterial Isolation and susceptibility testing: All 100 strains of *Shigella* spp. (88 *Shigella flexnari* and 12 *Shigella sonnei*) were isolated from the rural hospitals in Henan, China in 2006. Antibiotic susceptibility studies were performed by disc diffusion method (Kirby-Bauer) according to the Clinical and Laboratory Standard Institute (CLSI 2007)[8] for the following antibiotics: Amoxicilline-clavum, Nalidixic acid, Ciprofloxacin, Piperacilline and Polymixin B. *E. coli* ATCC 2922 was used as quality control strain for all tests of susceptibility.

DNA Extraction and PCR amplification: Chromosomal DNA was obtained as described by Kaufmann.[9] The extracted DNA was amplified in a 25ìL reaction mixture containing 17 ìL ddH_2O, 2.5 iL 10X Taq buffer, 2 iL dNTP mixture (2.5 mM)0.5 iL Taq Polymerase, 0.5 iL Primer 1, 0.5 iL Primer 2 (Tiangen Biotech Beijing co. Ltd) with 2 iL DNA template. All primers from the conserved regions of *marA* of *E. coli* were selected for PCR amplification. *MarA* primers were A1: 5'-GTC ACG TTA TCA ACT AGC-3' and A2: 5'- CTG CGT

Table-1: Drug resistant patterns between the S*higella* spp

Antimicrobials	S. Flexneri n=88(%)	S. Sonnei n=12(%)	P Value*
Amoxicilline-clavum	96.6	0	0.001
Nalidixic acid	100	100	na
Ciprofloxacin	27.3	8.3	0.141
Piperacilline	100	100	na
Polymixin B	1.1	0	0.880

*Fisher's exact probability; na= not applicable

Table -2: Multidrug-resistance patterns of 100 *Shigella* isolates

Shigella spp.	Resistance pattern*	Total	%
S. flexneri (n=88)	NAL, PI	3	3.4
	AMC, NAL, PI,	60	68.2
	AMC, CIP, NAL, PI	24	27.3
	AMC, PB, NAL, PI	1	1.1
S. sonnei (n=12)	NAL, PI	11	91.7
0	CIP, NAL, PI	1	8.3

*: AMC, Amoxicilline-clavum; NAL, Nalidixic acid; CIP, Ciprofloxacin; PI, Piperacilline; PB, Polymixin B.

AAA CAA AA-3'; amplification fragments contain 425 bp. The reaction mixture was subjected to initial denaturation at 95° C for 5min followed by 35 cycles at 94°C for 1min, 42.3 °C for 1min, 72°C for 1 min and a final cycle at 72°C for 10 min to complete the elongation of the PCR intermediate product.

Single-Strand Conformation Polymorphism (SSCP) Analysis: The SSCP of the digested PCR products with *TaqI* at 65^{0}C for 16 hours was analyzed by electrophoresis with 30 % acrylamide gels. In brief, 10ìL of the amplified PCR product was diluted with 100ìL of buffer (0.1% sodium dodecyl sulphate, 10mM EDTA). Ten microlitres of this dilution was mixed with 10ìL of loading buffer (95% formamide, 20mM EDTA, 0.05% each of bromophenol blue and xylene cyanol). The mixture was denatured by heating at 98° C for 5min, cooled on ice and loaded on to the non-denaturing gel at 200V for 4 hours at 4^{0}C. The gels were then silver stained and a photograph was taken. A DNA marker was run alongside the clinical isolates. A change in the banding pattern as compared with the DNA marker was taken as an indicator of mutation.

Nucleotide sequencing of the PCR products: PCR products of randomly selected mutated gene *marA* by SSCP analysis, were sequenced in **San Bo Yuan Zhi Biotechnology** Company (Beijing) using specific primer. The nucleotide sequence were edited with using Chromas (Version 1.62) and compared in BLAST of the NCBI database.

Statistics: Statistical analyses were conducted using SPSS13. Categorical variable were compared by the Chi-square and P values of less than 0.05 were considered statistically significant.

Ethics: Ethical approval was obtained from Ethical Review Committee of College of Public Health, Zhengzhou University, Henan (China).

RESULTS

Anitmicrobial susceptibility testing of 100 strains of S*higella* spp. showed a high degree of resistance to the commonly used antimicrobials, including Amoxicilline-clavum (85%), Nalidixic acid (100%), Piperacilline (100%), Ciprofloxacin (25%) and Polymixin B (1.1%). *Shigella flexneri* isolates were more resistant than those of *S. sonnei* to Amoxicilline-clavum (96.6% versus 0.0%, P<0.001) and Ciprofloxacin (27.3% versus 8.3%, P=0.141) (Table-1). Overall, *S. felxneri* isolates were more resistant than those of *S. sonnei* to three or more antimicrobial agents (96.6% versus 8.3%, P<0.001). The result of multi antimicrobials resistance pattern were displayed in Table-2.

The results of SSCP analysis of *mar*A are summarized in (Table-3). Study revealed that overall mutation rate in *mar*A was 19% (19.3% in *S. flexneri* and 16.7 % in *S. sonnei*) (Fig. 1) and the difference in mutation rate is not statistically significant. (P>0.05). DNA sequence analysis of mutated gene *marA* by SSCP analysis revealed mutations in three positionsÿcodon 6 (Delation of C), 319 (GCG GGG) (Ala Gly) and 374 (Addition of C).

DISCUSSION

When compared with those from other countries, our isolates were more resistant to the antibiotics previously recommended in the treatment of Shigellosis, such as Amoxicilline-clavum, Nalidixic acid, Piperacilline, Ciprofloxacin and highest ever reported, compared with 100% isolates were sensitive to Nalidixic acid from Mozambique and Ethiopia.[10,11] A study from Senegal showed resistance to Amoxicilline-clavum was 58%, which is also relatively lower than our findings.[12] A study from other part of China showed 74-80% of *Shigella* isolates remained susceptible to fluorinated quinolones[13] and a study from Banglore (India) revealed that *S.flexneri* strains were more resistant to Ciprofloxacin than those of *S. sonnei* (28.7 % versus 3.4%), which is also noted by our study (27.3% versus 8.3%).[14]

That overall 86 percent (96.6 % in *S. flexneri* versus 8.3 % in *S. sonnei*) of S*higella* isolates were found to be

Table-3: Association of gene *marA* with S*higella* spp

Mutation	*S. flexneri*		*S. sonnei*		Total		P Value*
	n	%	n	%	n	%	
Yes	17	19.3	2	16.7	19	19	
No	71	80.7	10	83.3	81	81	0.826
Total	88	100	12	100	103	100	

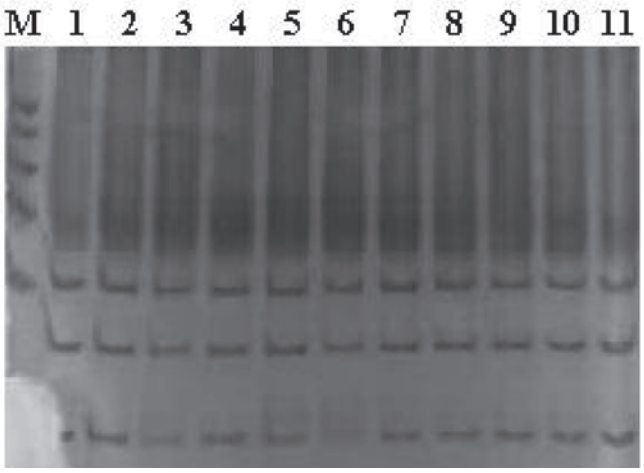

Fig. 1. PCR-SSCP patterns obtained by processing DNA template of *Shigella* spp. Where M extends for DNA Marker and lane 1-10 were digested *marA* (425 bp) with *Taq* I into 82, 139 and 204 bp. and the result showed altered profile (mobility shift) in Lane-6 of gene

resistant to three or more antimicrobial agents, which is a challenge for the effective assurance of effective treatment in China. Similar study from rural Mozambique showed multidrug resistance patterns were 65%,[11] whereas study from Ethiopia showed about 46% of the isolates were resistant to at least three of the most commonly used drugs.[15] Present study revealed that the resistance to antimicrobials varies in species necessitating for the identification of serogroups along with resistance patterns for the purpose of treatment of Shigellosis.

Study in *Escherichia coli* and other *Enterobacteriaceae* showed resistance to multiple antibiotics (nalidixic acid, ampicillin, chloramphenicol, and tetracycline) and overexpressed the *marA* gene, [16-18] while present study revealed that mutation in *marA* gene found *Shigella* spp. isolates from China and the rate of mutation was 19% (19.3% in *S. flexneri* and 16.7 % in *S. sonnei*), which may be associated with porin-mediated antibacterial resistance.

Our study suggested that mutation of *marA* may play a minor role in mechanism of multi drug resistance and other mechanisms may contribute to the drug resistance of *Shigella* spp.

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