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RESEARCH ARTICLE

Mechanism of antimicrobial resistance in *Shigella* isolates

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ABSTRACT

INTRODUCTION: Shigellosis still remains a public health problem in developing countries because of poverty, poor sanitation, personal hygiene and poor water supply. 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. has become resistant to most of the widely used antimicrobials. This study assessed the patterns of antimicrobial susceptibility and mutations in *marA* and *marR* genes of *Shigella* isolates and its association.

MATERIALS AND METHODS: Fifty three isolates of *Shigella* spp. were tested to evaluate the antimicrobial susceptibility by disc diffusion method (Kirby-Bauer) according to the Clinical Laboratory Standard Institute (CLSI) for the following antimicrobials: ciprofloxacin, norfloxacin, ampicillin, tetracycline, chloramphenicol, trimethoprim, gentamicin and streptomycin and mutation on *marAR* genes by using polymerase chain reaction–Single strand conformation polymorphism analysis.

RESULTS: Study revealed that there was significant association in between resistant to ciprofloxacin, norfloxacin and gentamicin with mutation in *marA* gene (87.5% vs 51.1%, P<0.05; 87.5%Vs 51.1%, P<0.05 and 90% vs 48.8%, P<0.05, respectively). However, there was no significant association in between resistant to tetracycline, streptocycin and ampicillin. Similarly, it was noted that the association in between antimicrobial resistance with mutation in *marR* like ciprofloxacin (O% vs 57.8%, P<0.05); norfloxacin (O% vs 57.8%, P<0.05), chloramphenicol (O% vs 70.3%, P<0.05); gentamicin (O% vs 60.5%, P<0.05) and trimethoprim (42.6% vs 100%, P<0.05), suggest that mutation in *marR* is protective factor for antimicrobial resistance.

CONCLUSIONS: The study revealed that mutation in *marR* is preventive factors for antimicrobial resistance like ciprofloxacin, norfloxacin, chloramphenicol, gentamicin and trimethoprim.

KEY WORDS: Antimicrobial resistance, *marA, marR*, *Shigella*

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INTRODUCTION

Diarrhoeal diseases and enteric infections are major causes of morbidity and mortality in the developing countries. In the United States, Shigellosis is an important cause of gastroenteritis, resulting in an estimated case of 450,000 each year.[1] Prompt treatment with effective antimicrobial agents may shorten the duration of clinical symptoms and carriage, and reduce the spread of infection.[2,3] Tetracycline, Chloramphenicol, Ampicillin, and Trimethoprim-sulfamethoxazole have all in succession been used as first-line antimicrobial drugs in China along with oral rehydration solution and antiprotozoal drugs. However, over the past few decades *Shigella* spp. have become progressively resistant to most of the first-line drugs used.[4,5] At present, multi-drug resistance has complicated the selection of empirical agents for the treatment of Shigellosis, particularly in children.[6]

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.[7] Present study assess the patterns of antimicrobial susceptibility and mutations in *marA* and *marR* genes of *Shigella* isolates and its association.

MATERIALS AND METHODS

Bacterial isolation and antimicrobial sensitivity: The *Shigella spp.* were isolated from stool of acute gastroenteritis patients attending out-patient department at Sunsari district hospitals and private clinics at Inaruwa Municipality of Nepal from May 2008 to October 2009, after fallowing standard bacteriological processing. Antimicrobial sensitivity study was performed by disc diffusion method (Kirby-Bauer) according to the Clinical Laboratory Standard Institute (CLSI) for the following antimicrobials: ciprofloxacin,norfloxacin, ampicillin, tetracycline, chloramphenicol, trimethoprim, gentamicin and streptomycin. *Escherichia coli* ATCC 25922 was used as quality control strain for all sensitivity tests.

DNA extraction and PCR amplification: Fifty-three strain of *Shigella* spp. (28 *S. flexneri* and 25 *S. dysenteriae*) were isolated in 2009 from stool of acute gastroenteritis patients attending out-patient department at district hospitals in Eastern Nepal after following standard bacteriological processing. For genomic DNA extraction, strains were grown overnight in LB medium at 37°C and a loopful of the colony was suspended in 1000µL of ddH_2O in microcentrifuge tube. The sample was boiled for 10 min at 100°C and then centrifuged at 12,000 rpm for 5min. The supernatant was stored as a source of DNA at −20°C and sent to molecular epidemiology laboratory of Zhengzhou University, China for further molecular analysis. The extracted DNA was amplified in a 25 µL reaction mixture containing 17 µL ddH_2O, 2.5µL 10X Taq buffer, 2 µL dNTP mixture (2.5 mM each), 0.5µL Taq Polymerase, 0.5µL Primer 1, 0.5µL Primer 2 (Tiangen Biotech Beijing co. Ltd) with 2µL DNA template. All primers from the conserved regions of *marAR* of *Escherichia coli* were selected. *marA* primers were A1: 5'- CTG CGT AAA CAA AA – 3' and A2: 5'- GTC ACG TTA TCA ACT ACG-3'; amplification fragments contain 425 bp. *marR* primers were R1: 5'- AAA CAA GGA TAA AGT GTC A-3' and R2: 5'- AAT GGT AAT AGC GTC AGT A-3'; amplification fragments contain 647 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 (*marA*)/ 51° C (*marR*) for 1 min, 72° C for 1 min and a final cycle at 72° C for 10 min.

Restriction enzyme digestion: In a 25 µl reaction mixture, 15 µl of PCR product was digested with 2.5 units of each restriction enzyme in separate 0.2 ml tubes for 16 hrs at 68°C. The restriction enzyme was *TaqI*. Ten µl of the restriction enzyme digested PCR product was separated by electrophoresis through a 2 % agarose gel containing ethidium bromide in 1 × Tris-acetate-EDTA (TAE) buffer at 80 V for 30 min and was then visualized under UV light. The enzyme digested product of *marA* into 82, 139 and 204 bp and *marR* into 289 and 358 bp.

Single-strand conformation polymorphism (SSCP) analysis: The SSCP of the PCR products were analysed by electrophoresis with 30% acrylamide gel. In brief, 10µL of the amplified PCR product was mixed with 10µL 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 then loaded on to the non-denaturing gel at 200V for 4 hours at 4°C. The gel was then silver stained and a photograph was taken. A DNA marker was run alongside the clinical isolates. A change in the banding patterns as compared with the DNA marker was taken as an indicator for mutation.

Statistics: Statistical analyses were conducted using SPSS 11.5. Categorical variables were compared by the Pearson and continuity correction Chi-square, and $P<0.05$ was considered statistically significant.

RESULTS

Study also revealed that the rate of mutation with *marA* gene was 67.9% in *S. flexneri* and 44% in *S. dysenteriae* strains, similarly, with *marR* gene was 46.4% in *S. flexneri* and 52% in *S. dysenteriae* strains (Table 1).

It is noted that there is no significant association with number of antimicrobial resistance by a *Shigella* isolates with mutation in *marAR* gene (Table 2).

Table 3 describes the association among antimicrobial resistant phenotype and mutation in *marA* gene. Study revealed that 87.5% resistant phenotypes with ciprofloxacin showed mutation in *marA* gene, however, the mutation was noted in 51.1% non-resistant phenotype. Similarly, 87.5% resistant and 51.1% non resistant phenotype of norfloxacin showed mutation, 57.1%resistant and 54.5% non resistant phenotype of tetracycline and streptomycin showed mutation. The rate of mutation among phenotype resistant with gentamicin was 90% whereas non resistant phenotype showed 48.8%.

Table 4 describes the association among antimicrobial resistant phenotype and mutation in *marR* gene. Study revealed that none of the resistant strains of ciprofloxacin and norfloxacin showed mutation in *marR* gene whereas 57.8% non resistant strains had mutation. Similarly, the mutation was observed in 47.6% resistant and 54.5% non resistant phenotype with tetracycline and streptomycin. Similarly, mutation was observed in none of the resistant phenotype and 70.3% non resistant phenotype of chloramphenicol. And, also mutation was observed in 42.6% resistant and 100% non resistant phenotype of trimethoprim.

Table 1. Association of *Shigella* spps. strain with mutation in gene *marAR*

Gene	Species	Mutation		Total	p-value
		Yes	No		
marA		n (%)	n (%)		
	S. flexneri	19(67.9)	9(32.1)	28	
	S.dysenteriae	11(44)	14(56)	25	0.08
	Total	30(56.6)	23(43.4)	53	
marR					
	S. flexneri	13(46.4)	15(53.6)	28	
	S. dysenteriae	13(52)	12(48)	25	0.68
	Total	26(49.1)	27(50.9)	53	

Table 2. Association of resistance to antimicrobials with mutation in gene *marAR*

Gene	Resistant to antimicrobials	Mutation		Total	p-value
		Yes	No		
marA		n (%)	n (%)		
	Two or less	6 (54.5)	5(45.5)	11	
	Three or more	24(57.1)	18(42.9)	42	0.87
	Total	30(56.6)	23(43.4)	53	
marR					
	Two or less	6(54.5)	5(45.5)	11	
	Three or more	20(47.6)	22(52.4)	42	0.68
	Total	26(49.1)	27(50.9)	53	

DISCUSSION

In present study, we found *S. flexneri* was predominant strains and similar study also noted the same result[8] whereas, some author reported *S. dysenteriae* were common in Nepal[9]and other developing countries like China[10]. However, *S. sonnei* was predominant in developed countries.[11,12] In present study, there is high rate of mutation in gene *marA* and *marR* as 56.6% and 46.6% respectively among the *Shigella* isolates. Study from other Gram negative bacteria revealed that antibiotics such as B-lactams[13]chloramphenicol and fluoroquinolones permeate the Gram-negative outer membrane via porins. As such, changes in porin copy number, size or selectivity will alter the rate of diffusion of these antibiotics.[14] One of the first examples of antibiotic resistance due to porin loss was a clinical isolate of *S. marcescens* that exhibited resistance to both aminoglycosides and B-lactams. Additional examples have since been reported with various bacterial isolates, including *E.*

Table 3. Association of selected antimicrobials with gene *marA*

Antimicrobials	Resistance	Mutation		Total	p-value
		Yes n(%)	No n(%)		
Ciprfloxacin	Yes	7(87.5)	1(12.5)	8	
	No	23(51.1)	22(48.9)	45	0.05*
	Total	30(56.6)	23(43.4)	53	
Norfloxacin	Yes	7(87.5)	1(12.5)	8	
	No	23(51.1)	22(48.9)	45	0.05*
	Total	30(56.6)	23(43.4)	53	
Tetracycline	Yes	24(57.1)	18(42.9)	42	
	No	6(54.5)	5(45.5)	11	0.87*
	Total	30(56.6)	23(43.4)	53	
Chloramphenicol	Yes	10(62.5)	6(37.5)	16	
	No	20(54.1)	17(45.9)	37	0.56
	Total	30(56.6)	23(43.4)	53	
Streptomycin	Yes	24(57.1)	18(42.9)	42	
	No	6(54.5)	5(45.5)	11	0.87*
	Total	30(56.6)	23(43.4)	53	
Ampicillin	Yes	30(56.6)	23(43.4)	53	--
	Total	30(56.6)	23(43.4)	53	
Gentamicin	Yes	9(90)	1(10)	10	
	No	21(48.8)	22(51.2)	43	0.01*
	Total	30(56.6)	23(43.4)	53	
Trimethoprim	Yes	24(51.1)	23(48.9)	47	
	No	6(100)	0(0)	6	0.02*
	Total	30(56.6)	23(43.4)	53	

* Continuity corrected chi square

coli[15], *Enterobacter cloacae*[16] and *S. enterica.*[17] In *E. aerogenes*, B-lactam resistant isolates often exhibit loss of a porin along with the expression of B-lactamase,[18] but resistance can also result from mutations that lead to a narrowing of the porin channel.[19] In *E. cloacae*, meropenem resistance resulted from loss of porins.[20]B-Lactam resistance of *K. pneumoniae* often results from loss of porins as well, though usually in conjunction with B-lactamase production.[21] A mutation in a porin gene in *N. gonorrhoeae* was shown to be responsible for resistance of this organism to B-lactams and tetracycline.[22]

CONCLUSIONS

Study revealed that there is significant association in between ciprofloxacin, norfloxacin and gentamicn with mutation in *marA* gene. However, there is no significant association in between tetracycline, streptocycin and ampicillin. Similarly, it is observed that negative association in between antimicrobial resistance with mutation in *marR* like ciprofloxacin, norfloxacin, chloramphenicol, gentamicin and trimethoprim.

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CONFLICT OF INTEREST: None to declare.

FINANCIAL INTEREST: None to declare.

Table 4. Association of selected antimicrobials with gene *marR*

Antimicrobials	Resistance	Mutation		Total	p-value
		Yes n(%)	No n(%)		
Ciprfloxacin	Yes	0(0)	8(100)	8	
	No	26(57.8)	19(42.2)	45	0.01*
	Total	26(49.1)	27(50.9)	53	
Norfloxacin	Yes	0(0)	8(100)	8	
	No	26(57.8)	19(42.2)	45	0.01*
	Total	26(49.1)	27(50.9)	53	
Tetracycline	Yes	20(47.6)	22(52.4)	42	
	No	6(54.5)	5(45.5)	11	0.68
	Total	26(49.1)	27(50.9)	53	
Chloramphenicol	Yes	0(0)	16(100)	16	
	No	26(70.3)	11(29.7)	37	0.001
	Total	26(49.1)	27(50.9)	53	
Streptomycin	Yes	20(47.6)	22(52.4)	42	
	No	6(54.5)	5(45.5)	11	0.68*
	Total	26(49.1)	27(50.9)	53	
Ampicillin	Yes	26(49.1)	27(50.9)	53	--
	Total	26(49.1)	27(50.9)	53	
Gentamicin	Yes	0(0)	10(100)	10	
	No	26(60.5)	17(39.5)	43	0.001*
	Total	26(49.1)	27(50.9)	53	
Trimethoprim	Yes	20(42.6)	27(57.4)	47	
	No	6(100)	0(0)	6	0.008*
	Total	26(49.1)	27(50.9)	53	

* Continuity corrected chi square

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