In vitro Susceptibility Study of *Salmonella typhimurium* Mutant to Stress Factors

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ABSTRACT

Background and Objective: In this study, we compared the susceptibility of *Salmonella typhimurium phoP* mutant and its parent to stress conditions that the pathogen may encounter in a host.

Methods: For this purpose, we used the *phoP* deletion mutant constructed in our previous study. In order to test the in vitro susceptibility of the mutant to stress factors, the effect of acidic pH, heat, bile salts and polymyxin on growth of the mutant was examined. Then, minimum inhibitory concentration and minimum bactericidal concentration of bile salts and polymyxin were determined. *Salmonella typhimurium* 14028 was used as the parent strain.

Results: The mutant was highly susceptible to bile salts and polymyxin in comparison with the parent strain, but no difference was observed in their susceptibility to acid and heat.

Conclusion: This study confirms the role of the *phoP* in resistance of *Salmonella* to polymyxin and bile salts. Mutation in the *phoP* leads to susceptibility of the mutant to bile and cationic antimicrobial peptides.

Keywords: In vitro, Mutant, *Salmonella typhimurium*, Disease Susceptibility.

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INTRODUCTION

Salmonella typhimurium is an enteric pathogen belonging to the family Enterobacteriaceae, with ability to grow in a broad range of adverse environmental conditions (1, 2). It causes infections in humans and animals ranging from self-limited gastroenteritis to bacteremia and systemic infections (3). Although *S. typhimurium* encounters several adverse environments such as low pH of stomach, bile salts, low oxygen in small intestine and cationic antimicrobial peptides on epithelial cells during the infections, there are different regulatory systems in the organism to overcome these stresses (4). The PhoP/PhoQ two-component regulatory system is one of the major regulators of virulence in *S. typhimurium*. The regulator phoP modifies expression of 3% of the *Salmonella* genes in response to the periplasmic Mg^{+2} concentration detected by the phoQ protein (5). This regulatory system also controls the expression of genes required for the virulence and resistance of *S. typhimurium* to antimicrobial peptides, bile salts and acidic pH (6-8).

In our previous study, we constructed a *phoP* deletion mutant of *S. typhimurium* by SOEing PCR method. In this method, a kanamycin cassette was replaced with the *phoP* gene of *S. typhimurium*, and later confirmed by PCR technique. This study aimed to examine the in vitro susceptibility of the constructed mutant to the stresses that the pathogen may encounter in a host, and compare the results with its parent.

MATERIAL AND METHODS

Bacterial strains used in this study included *S. typhimurium* 14028 (American Type Culture Collection**)** and a *phoP* mutant of *S. typhimurium* 14028 that was generated by SOEing PCR method in our previous study (9).

The media used in this study were Luria-Bertani (LB) agar and broth purchased from Difco laboratories, Detroit, MI. Chemicals including HCL, OXbile and polymyxin were purchased from Merck, NEOGEN and Sigma-Aldrich, respectively.

The tests included growth at pH 4.5, growth in presence of bile salts, resistance to polymyxin as a representative of cationic antimicrobial peptides and growth at 42 ˚C. In order to assess the growth at pH 4.5, the pH of LB broth was adjusted to 4.5 using HCL. Then, the inoculated media were incubated at 37 ˚C and growth was evaluated after 1 and 3 days by spectrophotometer. To test growth at 42 ˚C, terminal OD at 600nm was determined 24 h after the inoculation.

For the bile salts and polymyxin susceptibility testing, minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) assays were performed. The strains were subjected to various concentrations of bile salts ranging from 0.07-10%. For this purpose, LB broth with 10% bile salts was prepared and then two-fold serial dilutions were made in a 96-well microplate. The whole microplate was inoculated with 100 µl of LB broth except for column 1. Next, 200 µl of LB broth with 10% bile salts was added to the wells of column 1 using a multichannel pipette. Finally, 100 µl from column 1 was transferred into wells in column 2. After proper mixing by pipetting up and down, 100 µl of the mixture was transferred to column 3, reaching gradual two-fold decreasing concentration of bile salts in LB down to column 9. Column 9 contained plain LB broth as a positive control. Finally, each well was inoculated with 10 µl of 100-time diluted overnight *Salmonella* culture. The plate was incubated overnight at 37 ˚C. Next morning, turbidity of wells was assessed visually to determine at what concentration *Salmonella* was capable of growth. MIC was determined as the lowest concentration that prevented bacterial growth. Same procedures were used for polymyxin except for that 20 µg of polymyxin per ml of LB broth was inoculated into column 1 for making serial dilutions.

In order to determine MBCs for the bile salts and polymyxin, samples were taken from wells that showed no bacterial growth in the MIC assay. The samples were plated out for single colonies onto plain LB agar. The plates were incubated overnight at 37 ˚C to give viable bacteria time to produce colonies. The plates were then examined for growth and MBC was determined as the lowest dilution at which no growth was observed following subculture onto plain LB agar (10).

RESULTS

Growth in LB at pH 4.5 was not decreased in the mutant strain on post-inoculation day 1 and day 3 compared to the parent. The Growth rate

of the mutant strain at 42 ˚C showed no difference with the parent strain (Table 1).
The mutant was highly sensitive to bile salts and the MIC for bile salts was determined as 1.25%. However, the parent strain grew in all concentrations of bile salts (Table 2). To determine MBC for the mutant strain, the samples were taken from all concentrations that inhibited growth of the mutant, and transferred onto LB agar. After 24 h incubation at 37 ˚C, MBC for bile salts was determined as 2.5%.
The results showed a decrease in resistance of the mutant strain to polymyxin. The MIC of polymyxin was 2.5 µg/ml and 0.3 µg/ml for the parent and mutant strains, respectively. MBC of polymyxin was 2.5µg/ml and 1.25µg/ml for the parent and mutant strains, respectively. According to the results, the MIC and MBC of polymyxin for the parent strain were equal (Table 3).

Table 1- The growth results of the parent and mutant strains in LB at pH 4.5 and 42 ˚C.

Strains	First day	Third day	37 ˚C	42 ˚C
Parent	0.7962	0.8254	0.9008	0.8439
Mutant	0.6971	0.7154	0.8894	0.8308
Control	0.0591	0.0596	0.023	0.035

Table 2- MIC of bile salts for the parent and mutant strains

Strain	MICs (% bile salts)							
	10%	5%	2.5%	1.25%	0.62%	0.3%	0.15%	0.07%
Parent	+	+	+	+	+	+	+	+
Mutant	-	-	-	-	+	+	+	+

Table 3- MIC of polymyxin for the parent and mutant strains

Strain	MICs (µg/ml polymyxin)							
	20	10	5	2.5	1.25	0.62	0.3	0.15
Parent	-	-	-	-	+	+	+	+
Mutant	-	-	-	-	-	-	-	+

DISCUSSION

In our previous study, we constructed a *phoP* deletion mutant of *S. typhimurium* by SOEing PCR method. In this method, the *phoP* gene of *S. typhimurium* was deleted by replacement of a kanamycin cassette. In the present study, we compared the susceptibility of *S. typhimurium phoP* mutant and its parent to the stress factors that the pathogen may encounter in a host. The results of the present study indicate no difference between the growth rates of the mutant and parent strains at pH 4.5 and 42 ˚C. Meanwhile, a decrease in resistance to bile salts and polymyxin was observed in the mutant when compared with the parent strain. Karasova et al. compared the resistance of different mutants of *Salmonella enteritidis* to stress factors (10). Similar to the results of the present study, they confirmed the role of the *phoP* gene in resistance to polymyxin and bile salts. Previous studies reported that phoP regulates the expression of some genes by activation of other transcriptional regulatory systems such as PmrA/PmrB, which controls the expression of a group of genes mediating the modification of lipopolysaccharides and resistance to antibacterial peptides and polymyxin (7,11). Based on Yixin et al. different PhoP-mediated modifications in lipid A are necessary for resistance to different antimicrobial peptides (12). Some researchers reported that the PhoP/PhoQ is also required for high-level resistance to bile in *Salmonella,* and the *phoP* plays a major role in this resistance (8, 13, 14). A study has shown that a phoP-null strain is more than five-fold less resistant to bile (15). Few studies have been conducted on in vitro susceptibility of different mutant strains of *Salmonella* to stress factors. Similar to some of these studies, the experiments in the present study were not done in replicates due to some

limitations. Therefore, it is recommended to perform the experiments in triplicate to increase the accuracy of findings (13). In agreement with other studies on the role of *phoP* in resistance of *Salmonella* to polymyxin and bile salts, the present study demonstrates that the mutation in the *phoP* leads to susceptibility of the mutant to bile and cationic antimicrobial peptides.

CONCLUSION

According to the result of the present study, mutation in the *phoP* is a promising approach for attenuation of the *Salmonella* species.

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CONFLICT OF INTEREST

There is no conflict of interest.

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