

## Sodium Benzoate is Associated with *Salmonella typhi* Resistant to Chloramphenicol

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### Abstract

**Objective:** There are many factors that govern growth and resistant of *Salmonella typhi*. A study had reported that the use of sodium benzoate caused antibiotic resistant. However, no study has directly evaluated the effect of sodium benzoate exposure on *S. typhi* sensitivity to chloramphenicol. The aim of this study was to evaluate the resistance or sensitivity of *S. typhi* to chloramphenicol after sodium benzoate exposure. **Methods:** The study was conducted in seven groups: three treatment groups (sodium benzoate insensitive *S. typhi*+8 µg/mL, 16 µg/mL, and 32 µg/mL of chloramphenicol), three positive control groups (sodium benzoate sensitive *S. typhi*+8 µg/mL, 16 µg/mL, and 32 µg/mL of chloramphenicol), and one negative control groups (sodium benzoate sensitive *S. typhi*+0 µg/mL of chloramphenicol). The effect of sodium benzoate exposure to *S. typhi* sensitivity to chloramphenicol was measured after 24 hours. Spearman test was used to analyzed this association. **Results:** In this study, we found that the average *S. typhi* growth in the treatment groups (A, B, C) was 445 CFU/mL, 385 CFU/mL, and 171 CFU/mL, respectively. While in the positive control group (D, E, F) was not obtained any *S. typhi* growth. Average *S. typhi* growth in the negative control group was 430 CFU/mL. We found that sodium benzoate exposure inhibited *S. typhi* growth and affected *S. typhi* sensitivity to chloramphenicol ( $p < 0.05$ ). In addition, we found that 32 µg/mL chloramphenicol had the highest mean difference value, so this showed that the dose 32 µg/mL of chloramphenicol had the best effectiveness of various treatment groups ( $p < 0.05$ ). **Conclusions:** Sodium benzoate exposure can inhibit *S. typhi* growth and cause *S. typhi* resistant to chloramphenicol.

**Keywords:** *Salmonella typhi*, chloramphenicol, sodium benzoate, drug resistance

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### Introduction

*Salmonella enterica* subspecies *enterica* serotype Typhi (*Salmonella typhi*) belongs to the family of Enterobacteriaceae, Gram-negative, motile, non-lactose-fermenting bacilli. *S. typhi* causes typhoid fever, which is responsible for 16-17 million morbidities and 600.000 mortalities, annually.<sup>1,2</sup> Chloramphenicol is a drug of choice for typhoid fever. Chloramphenicol inhibits bacterial protein synthesis.<sup>3</sup> *S. typhi* has resisted to chloramphenicol in some countries. In Indonesia, chloramphenicol is still standard treatment for typhoid fever.<sup>3</sup> The chloramphenicol resistance can be caused by efflux pumps mechanism<sup>4</sup> and multidrug resistance mediated by plasmid.<sup>5</sup> The efflux pumps mechanism is divided into five families: ATP-binding cassette superfamily (ABC), major facilitator superfamily (MFS), multidrug and toxic compound extrusion family (MATE), small multidrug resistance family (SMR), and resistance-

nodulation division superfamily (RND). The mechanism of RND efflux pumps plays an important role in Gram-negative bacteria resistance.<sup>4</sup> The mechanism of plasmid-mediated multidrug resistance is associated with the encoding of chloramphenicol acetyltransferase.<sup>6</sup>

Typhoid fever is a water-and foodborne disease.<sup>1</sup> The most common way used to prevent food contamination from bacteria is the use of food preservative, such as sodium benzoate, which is used as bacteriostatic and fungistatic in acidic food and drink such as vinegar, carbonated drinks, jams, fruit juice, and condiments. As the result of long-term intake even though it is a small amount, the preservatives may cause harm to consumers within some sickness and in chromosomes level.<sup>7</sup> The study conducted by Dai *et al.*<sup>8</sup> has reported that the use of sodium benzoate caused antibiotic resistant. However, no study has directly evaluated the effect of sodium benzoate exposure on *S. typhi* sensitivity to chloram-

phenicol. Therefore, this study aimed to evaluate the resistance or sensitivity of *S. typhi* to chloramphenicol after sodium benzoate exposure.

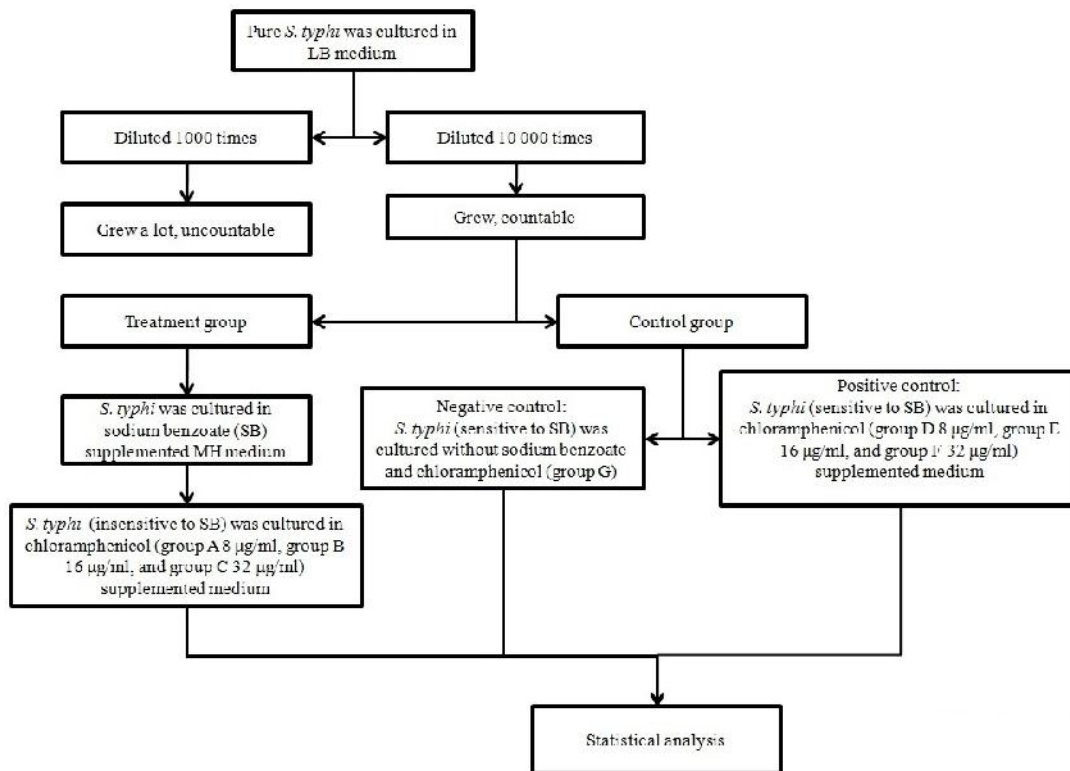
**Methods**

**Experimental Design.** This study was a true experimental. The design of this study was randomized post test-only control group design. This study consisted of seven groups: three treatment groups (sodium benzoate insensitive *S. typhi*+8 µg/mL, 16 µg/mL, and 32 µg/mL of chloramphenicol), three positive control groups (sodium benzoate sensitive *S. typhi*+8 µg/mL, 16 µg/mL, and 32 µg/mL of chloramphenicol), and one negative control groups (sodium benzoate sensitive *S. typhi*+0 µg/mL of chloramphenicol).

**Place and Time of the Research.** This research was conducted in the Microbiology Laboratory, School of Medicine, University of Islamic, Malang. This study was held in December 2010-January 2011.

**Study Variables: (1) Exposure of Sodium Benzoate.** Exposure of sodium benzoate in each group of study. Ordinal scale was used to measure this variable; **(2) Growth of *S. typhi*.** The amount of *S. typhi* growth in CFU/mL. Ratio scale was used to measure this variable.

**Research Procedures. (1) *S. typhi* culture and chemicals.** *S. typhi* culture was obtained from the Department of Microbiology, Brawijaya University, Indonesia. Lyso-genybroth (LB) medium and chloramphenicol were purchased from Difco Laboratories and Ranbaxy, respectively. **(2) Optimization of dilution and bacterial cultures.** *S. typhi* starter was diluted 1.000x and 10.000x. *S. typhi* was re-cultivated by growing on liquid LB medium. Then, *S. typhi* starter was grown on Mueller Hinton (MH) medium at pH 7-7.2, and was finally incubated at 37 °C for 24 hours. **(3) *S. typhi* exposure to sodium benzoate.** MH agar was supplemented with sodium benzoate (0.5% w/v, 1.0% w/v and 2% w/v) and was incubated for 24 hours. This MH agar was further inoculated with *S. typhi* and incubated for 24 hours at 37 °C. The *S. typhi* which was able to grow after repeated exposure of sodium benzoate was called preservative-induced mutant *S. typhi* or sodium benzoate-insensitive (SBI) *S. typhi*, and it was further propagated by growing in LB medium supplemented with 50% sodium benzoate which induces mutant of *S. typhi*. **(4) Chloramphenicol exposure to *S. typhi* mutant.** Two hundred and fifty mg chloramphenicol was dissolved in 100 mL sterile aquadest and was mixed in agar media containing *S. typhi* mutant. *S. typhi* concentration was measured by spectrophotometer (Lobomed). **(5) Anti-bacterial resistance test.** Bacteria



**Figure 1. Flow of Research Procedure.** This Procedure Consisted of Several Stages, i.e: (1) Preparation of *S. typhi* Culture and Chemicals, (2) Optimization of Dilution and Bacterial Cultures, (3) *S. typhi* Exposure to Sodium Benzoate, (4) Chloramphenicol Exposure to *S. typhi* Mutant, (5) Antibacterial Resistance Test

resistance test was performed with five Erlenmeyers (Pyrex) with 1.9 g of MH agar and 50 mL aqua. Chloramphenicol 8 µg/mL, 16 µg/mL, and 32 µg/mL and preservative-induced mutant *S. typhi* were added to Erlenmeyer A, B and C, respectively. Erlenmeyer D, E and F were supplemented with chloramphenicol– 8 µg/mL, 16 µg/mL, and 32 µg/mL, respectively- and starter bacteria (positive control). Erlenmeyer G was supplemented with starter bacteria only (negative control). All of Erlenmeyer were heated and stirred for 15 minutes for contents to dissolve. Agar media was sterilized for 30 minutes at 121 °C and put into petri dishes. Then, it was inoculated and incubated in for 24 hours at 37 °C. The flow of this research procedure was outlined in Figure 1.

**Statistical analysis.** The results of this study were analyzed using spearman test with a significant level  $p < 0.05$ .

## Results

**The effect of sodium benzoate exposure to *S. typhi* growth.** We found that sodium benzoate correlated with *S. typhi* growth and seem dose-dependent. There were 1.544 CFU/mL of *S. typhi* which exposure with 1% sodium benzoate. There was no *S. typhi* growth found in 2% sodium benzoate group. Contrary, there were overgrowths of *S. typhi* in 0.5% sodium benzoate group, thus could not be counted.

**The effect of sodium benzoate exposure to *S. typhi* sensitivity to chloramphenicol.** After exposure to sodium benzoate, all of group (A-G) performed serial of sensitivity tests. Table 1 provides the results of *S. typhi* sensitivity test to chloramphenicol after sodium benzoate exposure. Spearman test indicated that sodium benzoate exposure to *S. typhi* had significant differences to chloramphenicol sensitivity test. It showed that group C has the highest mean difference value, this indicated group C has the best effectiveness than other treatment groups ( $p < 0.05$ ).

Base on spearman test results which were significance  $p < 0.05$  and correlation coefficient 1.000, it indicated

that sodium benzoate exposure affected *S. typhi* growth and caused *S. typhi* resistant to chloramphenicol.

## Discussion

Sodium benzoate is a food preservative compound that contributes to the microbial resistance.<sup>8</sup> Mechanism of sodium benzoate to decrease *S. typhi* contamination had been reported previously by Ibrahim *et al.*<sup>9</sup> In this study, we found that the average *S. typhi* growth in the treatment groups (A, B, C) was 445 CFU/mL, 385 CFU/mL, and 171 CFU/mL, respectively. While in the positive control group (D, E, F) was not obtained any *S. typhi* growth. Average *S. typhi* growth in the negative control group was 430 CFU/mL. In this study, we found that sodium benzoate had a significant effect on the *S. typhi* growth ( $p < 0.05$ ). Based on previously theory, this effect is because sodium benzoate is accumulated in *S. typhi* membrane or surface then enters into *S. typhi* in neutral pH, dissociate and produce  $H^+$  ions and decrease *S. typhi* pH.<sup>9</sup>

The effects of sodium benzoate on bacteria growth depend on its dose. A sufficient dose of sodium benzoate will penetrate to the cell; react to the 50 S ribosomal subunit and inhibit the peptidyltransferase enzyme activity; disrupt bacterial metabolism, protein synthesis, and cell wall synthesis.<sup>10-12</sup> These mechanisms may lead to the absence of *S. typhi* growth at 2% sodium benzoate in this study. This result is consistent with the previous study.<sup>12</sup> Whereas an insufficient dose of sodium benzoate, it will accumulate limited in bacteria surface. In this setting, bacteria still survive, but with a lower membrane pH.<sup>12</sup> This condition explains *S. typhi* growth condition in 0.5% and 1% sodium benzoate groups in this study.

*S. typhi* resistance to chloramphenicol has been reported in Asia since 1980.<sup>13,14</sup> In this study, we found that *S. typhi* which resistance to sodium benzoate previously also resisted to chloramphenicol. See Table 1 to detail. No study has elucidated the association of sodium benzoate with *S. typhi* resistant to chloramphenicol. But, several studies had shown that sodium benzoate had an important role in antibiotic resistant mechanism.

**Table 1. Results of *S. typhi* Growth on Difference Dose of Chloramphenicol After Exposure to Sodium Benzoate**

Group	Mean	SD	Significancy ( $p$ )	Correlation coefficient
A (8 µg/mL chloramphenicol + SBI <i>S. typhi</i> )	445	± 42.079		
B (16 µg/mL chloramphenicol + SBI <i>S. typhi</i> )	385	± 30.971		
C (32 µg/mL chloramphenicol + SBI <i>S. typhi</i> )	171	± 46.121		
D (8 µg/mL chloramphenicol + SBS <i>S. typhi</i> )	0	± 0	0.001	1.000
E (16 µg/mL chloramphenicol + SBS <i>S. typhi</i> )	0	± 0		
F (32 µg/mL chloramphenicol + SBS <i>S. typhi</i> )	0	± 0		
G (0 µg/mL chloramphenicol + SBS <i>S. typhi</i> )	430	± 50.251		

SBI = sodium benzoate-insensitive; SBS = sodium benzoate-sensitive; = indicated in CFU/mL.

Shaista *et al.*<sup>15</sup> conducted a study about the association of five stabilizers including sodium benzoate on antibacterial activity of ginger garlic paste against five pathogens (*Escherichia coli*, *Staphylococcus aureus*, *S. typhi*, *Proteus mirabilis*, and *Enterobacter aerogenes*). They found that *E. coli* and *S. typhi* showed more resistance to ginger garlic paste after sodium benzoate exposure. A study by Rosner<sup>16</sup> showed that the use of chemotactic repellents including sodium benzoate caused *E. coli* resistance to chloramphenicol and ampicillin.

Other study conducted by Potenski *et al.*<sup>17</sup> analyzed about sensitivity of multiple antibiotics (tetracycline, chloramphenicol, nalidixic acid, and ciprofloxacin) to *S. enteritidis* after food preservative exposure including sodium benzoate. It showed multiple antibiotics resistant after food preservative exposure. The *S. typhi* survival in treatment groups (A, B and C) might be caused resistance mechanism that induced by sodium benzoate exposure. Based on theory, decreasing of membrane pH caused by sodium benzoate will decrease influx and increase efflux mechanism system in bacteria cell membrane. Therefore, it will disrupt cell membrane metabolism. In addition, low pH will also affect the plasmid stretching and change DNA structure which trigger bacterial resistance to antibiotics.<sup>18,19</sup>

Efflux pumps mechanism in *S. typhi* relate to RND family, which involves two transporters: AcrB and MexB (AcrB transporter is predominant). This process consists of three stages: access, binding, and extrusion.<sup>4</sup> The AcrB access will form a tripartite complex with periplasma adapter proteins or membrane fusion protein, so it causes the formation of a ligand for the extrusion process in the periplasma region.<sup>20</sup> Next AcrB involves several residues as proton or antiporter, such as Asp407, Asp408, Lys940, and Thr978 have a role in binding and extrusion of antimicrobial.<sup>21</sup>

Multidrug-resistance *S. typhi* can be caused by plasmid disruption. Plasmid DNA (IncHI1) can integrate into the chromosomes and play a role in the evolution of plasmid phenotype in multidrug-resistance *S. typhi*. Plasmids can be affected by low pH level such as caused by sodium benzoate.<sup>22</sup> Optimal pH for growth and plasmid stability is 6.39 and 6.41.<sup>23</sup> Decreasing pH caused by sodium benzoate will affect the growth and plasmid stability, and influence plasmid stretching that can cause the changes in DNA structure.<sup>19</sup> In addition, study by Hinrichs *et al.*<sup>24</sup> found that the modification of bacteria outer membrane that induced by low pH level alone increased antibiotic resistance.

Furthermore, changes in the plasmid code chloramphenicol acetyltransferase enzyme production may result in chloramphenicol resistance. Chloramphenicol acetyltransferase is a bacterial enzyme which detoxifies

chloramphenicol and causes chloramphenicol resistance through a catalytic reaction involving a histidine residue as base catalyst.<sup>25,26</sup> In catalytic reaction, this enzyme covalently is attached to an acetyl group from acetyl CoA to chloramphenicol; prevent ribosome bind to chloramphenicol.<sup>27</sup> Based on mechanisms that had been explained, in this study, they affected *S. typhi* resistance to chloramphenicol which previously exposure with sodium benzoate.

## Conclusions

This study confirmed that sodium benzoate could inhibit *S. typhi* growth and sodium benzoate insensitive *S. typhi* developed resistance mechanism to chloramphenicol. However, further studies considering gene-environment interactions should be conducted to investigate the associations of *S. typhi* resistance to chloramphenicol caused by sodium benzoate. It is necessary to study the correlation of *S. typhi* resistance to chloramphenicol caused by sodium benzoate clinically in human subjects.

## Conflicts of Interest Statement

The authors declare that there is no conflict of interest regarding the publication of this paper.

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