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Recognition of mechanisms involved in bile resistance important to halting antimicrobial resistance in nontyphoidal *Salmonella*

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ABSTRACT

Increasing antimicrobial resistance in nontyphoidal *Salmonella* (NTS) is a global public health problem that complicates antimicrobial therapy. As an enteric pathogen, *Salmonella* must endure the presence of bile in the intestinal tract during the course of infection. In this study, we sought to identify *Salmonella* genes necessary for bile resistance and to investigate their association with antimicrobial resistance. Four genes related to bile resistance were identified, namely *rfaP*, *rfbK*, *dam* and *tolC*. The first three genes are involved in lipopolysaccharide synthesis, and *tolC* is associated with an efflux pump. Antimicrobial susceptibility testing showed increased susceptibility to polymyxin B and ciprofloxacin in *rfaP* and *tolC* mutants of *Salmonella*, respectively. Genetic analysis of 45 clinical isolates of NTS revealed that all isolates with reduced susceptibility to fluoroquinolones (minimum inhibitory concentration ≥0.125 mg/L) were associated with point mutations in the quinolone resistance-determining regions of the *gyrA* and *parC* genes. The efflux pump also played a role, as evidenced by the reduction in fluoroquinolone resistance when the TolC efflux pump was inhibited by Phe-Arg-β-naphthylamide, a competitive efflux pump inhibitor. Based on these results, we conclude that an intact membrane structure and the efflux pump system provide mechanisms enabling NTS to resist bile. Caution should be taken when using ciprofloxacin and polymyxin B to treat *Salmonella* enteric infection, as resistance to these agents involves the same mechanisms. Addition of an efflux pump inhibitor to fluoroquinolones may be an effective strategy to deal with the increasing resistance in NTS.

1. Introduction

Nontyphoidal *Salmonella* (NTS) infection is a leading cause of food poisoning and enteric infection [1]. NTS infection usually results in enteritis, although systemic infections, including bacteraemia and meningitis, may occur [2]. Antimicrobial therapy is recommended if NTS infection spreads beyond the intestine or if NTS enteritis occurs in immunocompromised individuals [3]. Ampicillin, chloramphenicol and trimethoprim/sulfamethoxazole were the drugs of choice in the treatment of invasive salmonellosis prior to the 1980s. Thereafter, extended-spectrum cephalosporins and fluoroquinolones were recommended instead because of their favourable pharmacokinetic properties and low prevalence of resistance [4,5]. However, increasing cases of ciprofloxacin- or ceftriaxone-resistant *Salmonella* have been reported in the 1990s and this issue has become a serious threat to public health worldwide [4].

To survive in the human intestinal tract and gallbladder, *Salmonella* must endure other environmental extremes, including the presence of bile [6]. Bile plays a major role in the emulsification and solubilisation of lipids. Bile also acts as a detergent with potent antimicrobial activity due to its ability to disrupt the phospholipids and proteins of cell membranes and to interfere with cellular homeostasis [6]. Enteric bacteria are intrinsically resistant to bile, owing to both the low permeability of the outer membrane bilayer to lipophilic solutes and active efflux mechanisms [7]. Mutations in genes for the synthesis of lipopolysaccharide (LPS) (the major component of outer membrane of Gram-negative bacteria), efflux pump genes (*acr* or *emr*) and regulatory genes (*marAB* and *phoPQ*) have been previously reported for enteric bacterial resistance to bile [7–9]. Although mechanisms of bacterial resistance to bile are partially known, little is known about their associations with antimicrobial resistance and their impact on therapeutic options. We hypothesise that the presence of bile may be a potential

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Table 1
Susceptibility of *Salmonella enterica* serovar Typhimurium SL1344 and five bile-sensitive mutants to antimicrobial agents.

Strain	Minimum inhibitory concentration (mg/L)[a]		
	Ciprofloxacin	Ceftriaxone	Polymyxin B
SL1344 (wild-type strain)	0.012/0.012/0.012	0.064/0.047/0.064	1.000/1.000/1.000
77 (*rfaP* mutant)	0.008/0.008/0.019	0.094/0.064/0.094	0.190/0.500/0.500
98 (*dam* mutant)	0.008/0.019/0.008	0.047/0.047/0.064	0.380/0.500/0.500
137 (*rfbK* mutant)	0.016/0.019/0.016	0.023/0.023/0.047	0.380/0.500/0.380
763 (*rfaP* mutant)	0.012/0.008/0.012	0.094/0.094/0.064	0.190/0.380/0.250
995 (*tolC* mutant)	0.002/0.002/0.003	0.047/0.047/0.047	0.500/1.000/1.000

[a] Antimicrobial susceptibilities of *Salmonella* isolates were determined by Etest. The experiment was repeated three times to confirm the reproducibility of the results.

selective pressure on antimicrobial-resistant strains. Thus, recognition of the mechanisms related to *Salmonella* resistance to bile could improve our understanding of NTS antimicrobial resistance and aid in the development of therapeutic strategies to combat the emerging resistance.

In this study, we sought to identify the genes necessary for bile resistance using molecular and microbiological methods. The antimicrobial susceptibility of mutants that lost their resistance to bile was evaluated. Clinical isolates of NTS were collected from the microbiology laboratory of Chang Gung Memorial Hospital (CGMH) in Taiwan. These isolates were tested for their antimicrobial susceptibility and resistance mechanisms, with a focus on ciprofloxacin resistance.

2. Materials and methods

2.1. Identification of genes necessary for resistance to bile

The bacterial strain used in the identification of genes necessary for bile resistance was *Salmonella enterica* serovar Typhimurium SL1344, which is a wild-type laboratory strain. Bile utilised in this study was a synthetic mixture containing salts of deoxycholic acid (50%) and cholic acid (50%) (Sigma-Aldrich, St Louis, MO).

Bile-susceptible *S.* Typhimurium strains were created by EZ-Tn*5*™ <R6Kγori/KAN-2> transposon mutagenesis as described previously [10]. Briefly, transposon Tn*5* was randomly introduced into the chromosome of *S.* Typhimurium and mutants were then grown on Luria–Bertani (LB) agar–kanamycin plates. Individual colonies were screened for a bile-susceptible phenotype by replicating them onto plates containing LB agar containing 32 mg/mL bile (physiological concentration in the intestine and gallbladder) [7]. Plates were incubated overnight at 37 °C and colonies that could not grow on bile-containing plates were visually selected. An enzyme-linked immunosorbent assay (ELISA) reader was used for further confirmation of the susceptibility of the isolates to bile by comparing the absorbance of suspensions in different concentrations of bile between the wild-type strain and mutants. Briefly, a dilution of bacterial cells from overnight culture, including the wild-type strain and visually selected mutated strains, were inoculated in 100 μL of LB broth containing 250 mg/mL bile and the media were serially two-fold diluted in LB broth. Plates were incubated overnight at 37 °C and the absorbance was read at 570 nm using an ELISA reader. Mutants with bile–absorbance curves significantly different from the wild-type strain at different bile concentrations were ultimately selected.

2.2. Characterisation of mutants that lost resistance to bile

Genomic DNA of individual mutants was first purified and then fragmented, self-ligated and transformed into *Escherichia coli* competent cells. Only cells containing Tn*5* were selected using LB agar–kanamycin plates. Plasmid DNA was then purified and subjected to automated DNA sequencing to identify the disrupted genes, as previously described [10]. The minimum inhibitory concentration (MIC) of *Salmonella* strains, including *S.* Typhimurium SL1344 and the selected bile-susceptible mutants, were determined by Etest (AB BIODISK, Solna, Sweden). Owing to their effectiveness in the treatment of NTS infection and different antimicrobial characteristics, antimicrobial agents including ciprofloxacin, ceftriaxone and polymyxin B were selected for examination. Susceptibility and resistance were defined according to the criteria suggested by the Clinical and Laboratory Standards Institute (CLSI) [11]. The experiment was repeated three times to confirm the reproducibility of the results.

2.3. Time–kill studies

The mutants and the wild-type strain were also used to examine the killing kinetics of the antimicrobial agents. The antimicrobial agents examined in the time–kill study included ciprofloxacin and polymyxin B. Briefly, an overnight bacterial suspension was diluted to 5×10^5 CFU/mL in 50 mL of fresh LB broth. Suspensions containing different antibiotic concentrations, including 0, 1× MIC and 10× MIC, were incubated at 37 °C. MIC values were determined for each of the study strains by Etest (Table 1). Bacterial counts were measured at 0, 2, 6, 12, 24 and 48 h post inoculation. All of the experiments were performed twice to confirm the reproducibility of the results.

2.4. Clinical isolates of Salmonella and antimicrobial susceptibility testing

Forty-five *Salmonella* clinical isolates from the microbiology laboratory of CGMH were collected for investigation. All isolates were derived from stool specimens and were identified by standard methods [12]. *Salmonella* serogroups were determined by the slide agglutination method using antisera specific to O antigen (Difco Laboratories, Detroit, MI). Serotypes of these isolates were determined by H antisera (Difco Laboratories) using the tube agglutination method. The MIC of various antibiotics to these isolates was determined by Etest (AB BIODISK) in accordance with the standard protocol of the CLSI [11]. *Salmonella enterica* serovar Choleraesuis SCB67 was used as positive control in antimicrobial susceptibility testing [13].

2.5. PCR amplification and DNA sequencing of gyrA and parC

To study the mechanisms of resistance, clinical isolates of *Salmonella* with a MIC ≥ 0.125 mg/L to ciprofloxacin were screened for all known genes for DNA gyrase A (*gyrA*) and topoisomerase IV (*parC*) by PCR [14]. Sequences of the primers for PCR amplification of *gyrA* and *parC* have been previously described [15]. Preparation of template DNA and the determination of gene sequences followed the procedures described previously [15,16]. The amplified fragment contained the quinolone resistance-determining region (QRDR) of *gyrA* and *parC* [17]. PCR products obtained after