Plasmid-Mediated High-Level Ceftriaxone Resistance in a *Salmonella enterica* Serotype *typhimurium* Isolate

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Key Words

Gastroenteritis · *Salmonella typhimurium* · Extended spectrum beta-lactamase · Multidrug resistance

Abstract

Objective: To present the first documented case of acute infectious gastroenteritis caused by high-level ceftriaxone-resistant *Salmonella enterica* serotype *typhimurium* in Kuwait. **Subject and Methods:** Isolation from stool specimen and species identification of current enteric pathogen was carried out according to standard methods. Susceptibility to antibiotics was determined by the disc diffusion method on Mueller-Hinton agar. Minimal inhibitory concentrations (MICs) were measured with E-test strips. The production of extended spectrum beta-lactamase (ESBL) was studied by the double disc synergy method and E-test ESBL strips. Plasmid DNA isolation was performed by the rapid alkaline lysis method. Plasmid DNA was transferred by conjugation to a recipient strain of *Escherichia coli*. **Results:** The isolate of *S. enterica* serotype *typhimurium* was resistant to ceftriaxone (MIC >256 mg/l), cefotaxime and ceftazidime, and produced ESBL. Ceftriaxone and cefotaxime resistance were co-transferred on a 3.2-kb plasmid to the *E. coli* recipient strain. Loss of the 3.2-kb plasmid from the

transconjugant resulted in the co-loss of ceftriaxone and cefotaxime resistance confirming the carriage of ceftriaxone resistance on the 3.2-kb plasmid. **Conclusion:** Plasmid-mediated high-level resistance to ceftriaxone and ESBL production in *Salmonella* serotype *typhimurium* is an emerging problem among *Salmonella* that requires closer monitoring of antimicrobial resistance among these bacterial species.

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Introduction

Infections caused by nontyphoid *Salmonella* species are frequent and constitute a major health concern in many countries [1]. *Salmonella enterica* serotype *typhimurium* is an important pathogen with considerable versatility in its ability to acquire multidrug resistance determinants. Multiple drug resistance, including resistance to the oxyimino-beta-lactams (cefotaxime, ceftazidime) that have been used successfully as empirical therapy of severe forms of salmonellosis has emerged as an important problem in many countries of the world [2–6].

Ceftriaxone is the drug of choice for invasive *Salmonella* diseases, especially in children. The extensive usage

and inappropriate therapy of these antibiotics have produced selective pressure that has led to the appearance of ceftriaxone-resistant salmonellae in many countries [2–6]. The development of multiple drug resistance, including resistance to the oxyimino-beta-lactams in salmonellae threatens the efficacy of these antibiotics as a treatment of choice in severe forms of salmonellosis, especially in children.

Consequently, it is important to monitor *Salmonella* isolates for antimicrobial resistance, particularly resistance to clinically important antimicrobial agents such as ceftriaxone. In this report, we present the first documented case of acute infectious gastroenteritis caused by a high-level ceftriaxone-resistant *S. enterica* serotype *typhimurium* isolate in Kuwait.

Subject and Methods

Subject

A 10-month-old Bedouin girl presented with diarrhea and symptoms of upper respiratory tract infection on admission to the pediatric ward at the Al-Sabah Hospital, Kuwait. The patient was started on ampicillin injection (75 mg/kg div 8 h; in drip) and ventolin-atrovent mask. A stool sample was obtained for culture and susceptibility testing. The child became afebrile after 3 days. However, the stool culture grew *S. enterica* serotype *typhimurium*. The disease was self-limiting. No other family member was admitted to hospital with similar symptoms. There was no history of overseas travel in family members. The source and the means of transmission remain unknown.

Isolation and Identification of Bacterial Pathogen

The pediatric case of acute gastroenteritis was clinically and microbiologically investigated. The stool sample was inoculated into Selenite broth and onto Salmonella-Shigella and MacConkey agar, and the cultures were incubated aerobically at 37°C for 24 h. The species was identified using analytical profile index (API 20 E, Bio Merieux, France) and the speciation was done using serology with specific antisera (Bio Merieux).

Antibiotic Susceptibility Testing

Antibiotic susceptibility testing was performed by the disk diffusion method using Mueller-Hinton agar. The results were interpreted according to the current National Committee for Clinical Laboratory Standards guidelines [7]. The following antibiotics were tested: ampicillin, piperacillin, piperacillin-tazobactam, amoxicillin-clavulanate, cephalothin, cefuroxime, cefotaxime, ceftriaxone, cefoxitin, ceftazidime, gentamicin, amikacin, streptomycin, trimethoprim-sulphamethoxazole, ciprofloxacin, nalidixic acid, chloramphenicol and imipenem. *Escherichia coli* strain ATCC-25922 was used for quality control. Minimal inhibitory concentrations (MICs) of ampicillin, ceftazidime, ceftriaxone, cefotaxime, chloramphenicol, ciprofloxacin and imipenem were determined using E-test strips (AB Biodisk, Sweden) [8] according to the manufacturer's instructions.

Detection of Extended Spectrum Beta-Lactamase Production

Extended spectrum beta-lactamase (ESBL) production was detected using the double-disk synergy method, as described previously [7, 8], and with E-test ESBL strips. For the double-disk synergy test, a ceftazidime disk (30 µg) was placed 30 mm away from a disk containing amoxicillin/clavulanate (60/10 µg). ESBL production was considered positive when an enhanced zone of inhibition was visible, between the beta-lactam and beta-lactamase inhibitor-containing disk. With the ESBL Etest strip, ESBL production was positive if the ratio of the MIC of ceftazidime to the MIC of ceftazidime with clavulanate was greater than 8.

Plasmid Analysis and Transfer of Resistance Determinants

Plasmid DNA was isolated by the alkaline lysis method [9], separated by agarose gel electrophoresis on 0.6% (w/v) agarose gels in TAE buffer and stained with ethidium bromide. Plasmid sizes were estimated using *E. coli* V517 carrying plasmids of 35.8, 4.8, 3.7, 2.6, 2.0, 1.8 and 1.4 kb. Ceftriaxone resistance was transferred in conjugation experiments to *E. coli* JM109 strain mutated to nalidixic acid resistance as recipient. A donor-to-recipient ratio of 1:10 in brain heart infusion broth (BHIB) was incubated at 37°C for 18 h without shaking. Transconjugants were selected on brain heart infusion agar containing nalidixic acid (40 µg/ml) and ceftriaxone (10 µg/ml). Ten randomly selected transconjugants were screened for unselected resistance markers and plasmid content. The transfer frequency was calculated as the number of transconjugants per donor viable cell counts. Curing of antibiotic resistance determinants from the transconjugants was performed by growing the cells in BHIB at 44°C for 24 h. Following the initial 24-hour incubation, 1 ml of the culture was added to 19 ml BHIB and the incubation repeated. Cells were then grown on brain heart infusion agar at 37°C for 18 h to obtain single colonies, which were screened for the loss of antibiotic resistance by replica plating. Colonies that lost antibiotic resistance were screened for plasmid loss and tested for ESBL production.

Results

The stool culture yielded growth of nonlactose-fermenting, hydrogen sulphide-producing bacterium identified as *S. enterica* serotype *typhimurium* that was resistant to ampicillin, piperacillin, cephalothin, cefuroxime, ceftriaxone, ceftazidime and trimethoprim-sulphamethoxazole. It expressed intermediate susceptibility to the beta-lactam-beta-lactamase inhibitor combinations, piperacillin/tazobactam and amoxicillin/clavulanic acid. It was susceptible to cefoxitin, gentamicin, streptomycin, amikacin, nalidixic acid, ciprofloxacin, chloramphenicol, nitrofurantoin and imipenem. Results of MIC determination are presented in table 1. It shows high levels of resistance to ampicillin, ceftriaxone, cefotaxime and low-level resistance to ceftazidime. This resistance pattern was characteristic of isolates that produce ESBLs. Consequently, ESBL production was in-

vestigated by the double-disk (synergy) test and E-test ESBL strips. Results of both tests indicated that it produced ESBL.

Transfer of Plasmid-Mediated β-Lactam Resistance

The *S. enterica* isolate carried differently sized plasmids as shown in figure 1. Its ability to transfer resistance to extended spectrum cephalosporins was investigated in conjugation experiments. Transconjugants were obtained on ceftriaxone selection plates at a frequency of 1.2×10^{-6} /CFU. Ten transconjugant colonies were selected to screen for the co-transfer of unselected resistance markers of the parent strain. They were all resistant to ampicillin, cefotaxime and ceftriaxone but susceptible to ceftazidime. Except for ceftazidime, their MIC values were similar to those of the parental strain (table 1). They contained a 3.2-kb plasmid that was also present in the parent (fig. 1). The figure shows the plasmid contents of the donor, recipient, representatives of the transconjugants and a transconjugant cured of transferred plasmid. The DNA bands below the 3.2 kb band in the transconjugants are the open circular and linear forms of the 3.2-kb plasmid and were lost during curing. A large molecular size plasmid running together with the chromosomal DNA was detected in the recipient and transconjugants. To confirm whether or not the 3.2-kb plasmid in the transconjugants encoded resistance to ampicillin, ceftazidime and cefotaxime, and ESBL production, a transconjugant was selected and used in curing experiments. Five of 300 colonies screened after growth at 44°C lost resistance to ampicillin, cefotaxime and ceftriaxone together with the 3.2-kb plasmid (fig. 1). These colonies also failed to produce ESBL clearly associating the 3.2-kb plasmid with ESBL production in these colonies.

Fig. 1. Plasmid contents of *Salmonella* donor and *E. coli* transconjugants. Lane 1, *S. enterica* serotype *typhimurium* donor. Lane 2, *E. coli* recipient resistant to ciprofloxacin. Lanes 3, 4 and 6, *E. coli* transconjugants resistant to ceftriaxone and cefotaxime and containing the 3.2-kb plasmid. The lower bands are the open circular forms and the upper bands are the covalently closed circular forms of the same plasmid. Lane 5, *E. coli* transconjugant cured of ceftriaxone and cefotaxime resistance and the 3.2-kb plasmid.

Table 1. Antibiotic resistance patterns (MIC, mg/l) of *S. typhimurium* clinical isolate and *E. coli* transconjugant

Antimicrobial agents	<i>S. typhimurium</i> donor	<i>E. coli</i> recipient	<i>E. coli</i> transconjugants	Cured transconjugants
Ampicillin (≥ 32)	>256	4	>256	4
Ceftriaxone (≥ 64)	>256	0.05	>256	0.05
Ceftazidime (≥ 32)	64	0.2	6	0.2
Cefotaxime (≥ 64)	>256	0.2	>256	0.2
Imipenem (≥ 16)	0.38	0.2	0.2	0.3
Piperacillin/tazobacam (≥ 128/4)	3	1	3	1
Chloramphenicol (≥ 32)	3	2	2	2
Ciprofloxacin (≥ 4)	0.012	>32	>32	>32

Figures in parentheses indicate resistance breakpoint MICs.

Discussion

S. enterica serotype *typhimurium* is an important pathogen that has acquired novel beta-lactamases, and there has been a significant increase in multidrug-resistant nontyphoid *Salmonella* infections in the past few years [2].

Resistance to expanded-spectrum beta-lactam antibiotics, such as ceftriaxone is increasing in *Salmonella* isolates throughout the world [3–6, 10]. This report, the first documented in Kuwait, adds to the growing list of extended-spectrum cephalosporin-resistant *S. enterica* serotype *typhimurium* isolates.

Conjugation experiments and plasmid analysis revealed the presence of a 3.2-kb plasmid in the *S. enterica* serotype *typhimurium* donor and in the *E. coli* transconjugants. The transfer of ceftriaxone and cefotaxime resistance accompanied the transfer of the 3.2-kb plasmid to *E. coli*. Similarly, the loss of the 3.2-kb plasmid from the *E. coli* recipient during curing accompanied the loss of resistance to ceftriaxone and cefotaxime. These results confirmed the carriage of ceftriaxone and cefotaxime resistance and ESBL production on the 3.2-kb plasmid. The transfer of ceftriaxone and cefotaxime resistance to *E. coli* resulted in the reduction in ceftazidime MIC values from 64 to 6 mg/l, corresponding to the loss of ceftazidime resistance. This means that either ceftazidime resistance was not linked to ceftriaxone and cefotaxime resistance or that it was not expressed in the *E. coli* recipient following transfer.

Different types of plasmid-mediated cefotaxime-hydrolyzing enzymes have been described in *Salmonella* species [5–7]. The genes encoding these enzymes are located on a range of small 8- to 12-kb plasmids or on large >100-kb plasmids [5–7]. Although the type of ESBL enzyme encoded by the 3.2-kb plasmid isolated in this study has not yet been determined, it encoded only resistance to extended spectrum cephalosporins and appears to be the smallest plasmid yet reported for these enzymes.

Expanded spectrum cephalosporins, especially ceftriaxone, are frequently used empirically to treat *Salmonella* infections in children. The identification of locally acquired ESBL-mediated ceftriaxone-resistant *Salmonella* infection suggests that such infections could become more common in Kuwait, especially since the resistance to this drug is mediated by a plasmid.

Conclusion

This report indicates that the acquisition of plasmids carrying the genes in *Salmonella*, responsible for ESBL production and resistance to the expanded spectrum cephalosporins, is an emerging problem. Closer monitoring of antimicrobial resistance, particularly resistance to clinically important antimicrobial agents such as ceftriaxone is required because it is the recommended antibiotic for treatment of invasive *Salmonella* infections in children.

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