In Vivo Acquisition of Ceftriaxone Resistance in Salmonella enterica Serotype Anatum
Lin-Hui Su,¹ Cheng-Hsun Chiu,²* Chishih Chu,³ Mei-Hui Wang,² Ju-Hsin Chia,¹ and Tsu-Lan Wu¹

Department of Clinical Pathology, Chang Gung Memorial Hospital,¹ Department of Pediatrics, Chang Gung Children’s Hospital,² and Department of Microbiology and Immunology, Chang Gung University College of Medicine,³ Taoyuan, Taiwan

Received 6 August 2002/Returned for modification 17 September 2002/Accepted 12 November 2002

Salmonellosis is an important public health problem throughout the world (21, 31). Although most salmonella infections are self-limiting, serious sequelae, including systemic infection and death, can occur (9, 21, 31). Antibiotics are essential for the treatment of invasive infections caused by salmonella. However, rates of resistance to drugs like ampicillin, trimethoprim-sulfamethoxazole, and chloramphenicol have been escalating in many areas of the world (9, 18, 31). Expanded-spectrum cephalosporins and fluoroquinolones are recommended in such a setting.

Since 1991, Salmonella species that are resistant to expanded-spectrum cephalosporins have increasingly been reported in several countries (1–4, 11, 13, 14, 27, 33). Many epidemiological investigations further demonstrated that the use of antimicrobial agents in livestock is the principal cause of the emergence and dissemination of resistance to antimicrobial agents, including expanded-spectrum cephalosporins, in strains of Salmonella (7, 12, 32, 34). It is generally believed that the resistant salmonellas spread from food animals to humans through the food chain (7, 12, 32, 34).

However, a number of investigations found that some of the Salmonella isolates from food animals and humans expressed different molecular typing patterns, suggesting that the distribution of these isolates was not entirely due to clonal spread (11, 33, 34). Many members of the family Enterobacteriaceae contain plasmid-mediated β-lactamases (5). By coping with antimicrobial selective pressure in the environment, it is reasoned that salmonella could acquire resistance from other members of the family Enterobacteriaceae through the exchange of genetic elements.

Cephalosporins are not common drugs used either as therapeutic agents or as growth promoters in livestock in Taiwan (20). A clinical isolate of Salmonella enterica serotype Anatum that was resistant to ceftriaxone was identified in a patient in our hospital. The emergence of resistance to ceftriaxone during therapy for infections caused by Salmonella and E. coli appears to have been the cause of treatment failure in this patient. We used molecular methods in the study described here to investigate the in vivo acquisition of resistance determinants in this strain of S. enterica serotype Anatum.

MATERIALS AND METHODS

Case report. In August 2001, a 70-year-old female patient was admitted to the Chang Gung Memorial Hospital because of a fever and a large pressure sore in the sacral area that was unresponsive to topical antimicrobial treatment. She had had a history of diabetes, which required insulin therapy, for more than 10 years.

Results of laboratory examinations showed leukocytosis and pyuria. She began an empirical regimen of cefazolin plus gentamicin and underwent wound debridement. Culture of her urine yielded S. enterica serotype Anatum, and culture of tissue specimens from the wound obtained during the debridement operation grew the same Salmonella strain isolated from her urine (strain SA812S), along with E. coli (strain EC812S). The antimicrobial susceptibility patterns of the isolates are shown in Table 1.

On day 5 of treatment, she remained febrile, and ceftriaxone was administered for the treatment of possible bacteremia. The fever ultimately abated, while the wound infection did not improve significantly. On the 20th hospital day, she again received surgical debridement. Culture of the necrotic tissue grew S. enterica serotype Anatum (strain SA831R) and expanded-spectrum β-lactamase (ESBL)-producing E. coli (strain EC831R). By susceptibility testing, however, S. enterica serotype Anatum SA831R was found to be resistant to ceftriaxone (Table 1). Her condition deteriorated 2 days after the operation, and hypotension and fever appeared. A culture of her blood grew S. enterica serotype Anatum, which was resistant to ceftriaxone. The treatment was shifted to ciprofloxacin, but multorgan failure developed, and the patient died 5 days later.
Bacterial strains and antimicrobial susceptibility. The Salmonella isolates were identified and serotyped by standard methods (16). The susceptibilities of these isolates to antimicrobial agents were determined by the E-test in accordance with the recommendations of the manufacturer (AB Biodisk, Solna, Sweden). The MICs of cefotaxime and ceftazidime in the presence of clavulanate were determined by a standard broth microdilution method. The double-disk diffusion assay was used to screen for the presence of ESBLs by the methods of the National Committee for Clinical Laboratory Standards (23). Table 1 lists the bacterial strains used in this study, including four clinical isolates derived from the patient. S. enterica serotype Anatum SA812S and E. coli EC812S were isolated from wound pus and were susceptible to ceftriaxone. S. enterica serotype Anatum SA831R and E. coli EC831R were derived from the necrotic necrotic tissue and were resistant to ceftriaxone.

Genotyping. Infrequent-restriction-site PCR has been shown to be effective in discriminating Salmonella isolates (29). In the present study, the genetic relatedness of the clinical isolates was investigated by using this method (30). The only modification was that 15 μl of adapter-ligated DNA template was used in the final amplification. Each isolate was examined at least twice to ensure the reproducibility of the results.

PCR amplification and DNA sequencing. To determine the ESBL types, three primer sets previously described for the detection of the blaTEM, blaSHV, and blaCTX-M genes were used in the amplification procedure (15, 19, 25). The PCR products were purified by using a Wizard PCR Prep kit (Promega) and were sequenced with an ABI 377 automatic sequencer (Perkin-Elmer Applied Biosystems). The nucleotide sequences obtained were compiled and analyzed by using Pegen software (Intelligenetics). The search for homologous sequences in the GenBank database was done with FASTA software through the Internet.

Plasmid analysis, plasmid DNA purification, and DNA-DNA hybridization. The organisms were checked for the presence of plasmids by a method used earlier (17). The plasmid DNA was extracted and purified by a CsCl gradient method described earlier (24). Restriction fragment profiles were generated with restriction endonuclease BglII by the procedure recommended by the manufacturer (New England BioLabs Inc., Beverly, Mass.), and the fragments were electrophoresed in a 0.8% agarose slab gel. DNA-DNA hybridization was performed by the method of Southern (26), except that the probe for the target β-lactamase gene was prepared and labeled with digoxigenin-11-DUTP (Roche). After hybridization, the sample was exposed to X-ray film with an intensifying screen.

Electroporation and conjugation. To examine the transferability of the plasmid harboring the β-lactamase gene, 10 μg of pSA831R DNA extracted from S. enterica serotype Anatum SA831R was transferred into E. coli HB101 by electroporation. In the present study, the genetic relatedness of the clinical isolates was investigated by using this method (30). The only modification was that 15 μl of adapter-ligated DNA template was used in the final amplification. Each isolate was examined at least twice to ensure the reproducibility of the results.

Antimicrobial susceptibility. The susceptibilities of the isolates to the antimicrobial agents tested are shown in Table 1. The MICs differed for ceftriaxone-sensitive and -resistant isolates. Much higher MICs were obtained for two ceftriaxone-resistant isolates which were resistant to ampicillin, cefixime, cefotaxime, ceftiraxone, cefuroxime, cephalothin, chloramphenicol, and sulfamethoxazole-trimethoprim but susceptible to ceftazidime, ciprofloxacin, gentamicin, and imipenem. The double-disk diffusion assay showed that S. enterica serotype Typhimurium LBNP4417 (Nalr Strr) was used as the recipient. Transconjugants were selected by using ceftriaxone resistance as a selection marker. E. coli competent cells were prepared as described earlier (8). The transformant was used as the donor in the subsequent conjugation experiment. Bacterial conjugation was performed as described earlier (6), except that S. enterica serotype Typhimurium LBH101 was used as the recipient. Transconjugants were selected on MacConkey agar medium containing appropriate drugs (ceftriaxone, streptomycin, and nalidixic acid).

RESULTS

Antimicrobial susceptibility. The susceptibilities of the isolates to the antimicrobial agents tested are shown in Table 1. The MICs differed for ceftriaxone-sensitive and -resistant isolates. Much higher MICs were obtained for two ceftriaxone-resistant isolates which were resistant to ampicillin, cefixime, cefotaxime, ceftiraxone, cefuroxime, cephalothin, chloramphenicol, and sulfamethoxazole-trimethoprim but susceptible to ceftazidime, ciprofloxacin, gentamicin, and imipenem. The double-disk diffusion assay showed that S. enterica serotype Anatum SA831R and E. coli EC831R were ESBL producers.

Genotyping and DNA sequencing. Molecular typing demonstrated indistinguishable patterns between S. enterica serotype Anatum SA812S and SA831R as well as between E. coli EC812S and EC831R, suggesting that they each had the same origins. However, only the two ceftriaxone-resistant isolates possessed the CTX-M-type β-lactamase gene. Nucleotide sequence analysis of the PCR products revealed that both iso-
plasmid found in S. enterica EC831R; lane 5, E. coli EC812S; lane 3, S. enterica serotype Anatum SA831R; lane 4, E. coli EC831R; lane 5, E. coli HB101; lane 6, E. coli HB101/pSA831R; lane 7, S. enterica serotype Typhimurium LBNP4417; lane 8, S. enterica serotype Typhimurium LBNP4417/pSA831R.

Plasmid analysis. Figure 1 shows the plasmid profiles of all the isolates studied. S. enterica serotype Anatum SA831R did not carry any plasmid, while E. coli EC812S harbored three large plasmids. Similar plasmid profiles were found for S. enterica serotype Anatum SA831R and E. coli EC831R, except that an extra, smaller plasmid was found in these two isolates. This plasmid, arbitrarily named pSA831R, was extracted from S. enterica serotype Anatum SA831R and was successfully transferred into E. coli HB101 by electroporation. The transformant, E. coli HB101/pSA831R, was used as the donor in the subsequent conjugation experiment with S. enterica serotype Typhimurium LBNP4417 as the recipient. The conjugation efficacy (the number of transconjugants per the number of recipients) was estimated to be as low as 10^−9. The antimicrobial susceptibility patterns of the transconjugant, E. coli HB101/pSA831R, and transconjugant, S. enterica serotype Typhimurium LBNP4417/pSA831R, were similar to the pattern of the parent strain, S. enterica serotype Anatum SA831R (Table 1). Both strains also produced ESBLs. By using the PCR product of bla_{CTX-M-3} as the probe, DNA-DNA hybridization showed that bla_{CTX-M-3} was carried on pSA831R, the common plasmid found in S. enterica serotype Anatum SA831R, E. coli EC831R, E. coli HB101/pSA831R, and S. enterica serotype Typhimurium LBNP4417/pSA831R (Fig. 1).

The plasmid DNA was extracted from all plasmid-carrying isolates, including E. coli HB101/pSA831R and S. enterica serotype Typhimurium LBNP4417/pSA831R, and was subjected to digestion with BglII. The restriction fragment patterns of the plasmids are shown in Fig. 2. The same pattern was observed in S. enterica serotype Anatum SA831R, E. coli HB101/pSA831R, and S. enterica serotype Typhimurium LBNP4417/pSA831R. The size of the bla_{CTX-M-3}-carrying plasmid, pSA831R, was estimated to be 95.1 kb. While E. coli EC831R harbored the 95.1-kb plasmid pSA831R, it showed a restriction fragment pattern different from those of the other strains due to the presence of the other three plasmids (Fig. 1 and 2).

DISCUSSION

In the present study, isolates from a patient hospitalized for the treatment of wound and urinary tract infections caused by Salmonella and E. coli were evaluated. Even though the patient was given appropriate drugs, along with surgical debridement, the unexpected emergence of ceftriaxone resistance during treatment still led to a fatal outcome in the patient. Such emergence of resistance during treatment for infections is more frequently reported in patients receiving macrolides (22) or quinolones (26). Previous reports on the expanded-spectrum cephalosporins generally focused on characterization of the ESBLs or reporting of epidemiological data to link isolates from different sources (2–4, 11, 13, 14, 27, 33). Compelling evidence, as demonstrated in this study, for the in vivo acqui-
sition of extrachromosomal DNA which carried a resistance gene by the originally susceptible pathogen in one patient is lacking.

The emergence of antimicrobial resistance can develop in several ways, including the acquisition of new genes by the preexisting susceptible bacteria via horizontal gene transfer. In this study, indistinguishable molecular fingerprints indicated the identities of the resistant and susceptible strains. In vivo transfer of an extrachromosomal DNA between Enterobacteriaceae (C. H. Chiu, unpublished data). The CTX-M-3 producing enteric bacilli may act as the reservoir of the resistance-conferring plasmid for the two ceftriaxone-susceptible isolates in this study. It remains unclear whether one of the two ceftriaxone-susceptible isolates acquired the resistance plasmid before the other one did or simultaneously. We have tried to transfer plasmid pSA831R from SA831R to EC812S and from EC831R to SA812S by conjugation. The experiments failed due to the lack of proper selection markers. No matter what the actual direction of transfer is, bacterial conjugation appears to be the primary mechanism of resistance gene transmission between the two species of bacteria. The ubiquity of plasmid-borne resistance may be explained in part by the stamina of conjugation itself. Conjugation can occur even in environments that otherwise kill a bacterium, allowing plasmids to replicate by a horizontal gene transfer in the presence of antibiotics (10).

There has been growing evidence suggesting that the antibiotics fed to food animals may contribute to the resistance of human pathogens, in particular, Salmonella (7, 11, 12, 32). It has also been shown previously that clonal spread could not entirely explain the distribution of antimicrobial resistance between humans and animals (11, 33, 34). The results of the present study indicate that Salmonella can acquire resistance from other bacteria through the exchange of genetic materials in vivo. Furthermore, resistant enteric bacilli can act as a reservoir that keeps the resistance gene ready to be transferred to other bacteria. Resistance to antimicrobial agents is increasing in all Salmonella serotypes (1, 4, 11, 14, 18, 27, 33). To control the escalating resistance in salmonellae, in addition to banning inappropriate antibiotic use in food animals, parallel efforts should also be made to reduce the prevalence of resistant gram-negative enteric bacilli in hospital settings.

ACKNOWLEDGMENTS

This work was supported in part by grant CMRP1313 (to C.-H.C.) from Chang Gung Memorial Hospital, Taoyuan, Taiwan. We thank He Chao-Chen for expert laboratory work.

REFERENCES


