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Characterization of Multiple-Antibiotic-Resistant *Salmonella typhimurium* Strains: Molecular Epidemiology of PER-1-Producing Isolates and Evidence for Nosocomial Plasmid Exchange by a Clone

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We characterized epidemiologic and genetic features of nosocomially originated multiple-antibiotic-resistant *Salmonella typhimurium* isolates from two hospitals. A total of 32 multiply resistant strains, isolated during a 28-month period, were studied. Four resistance phenotypes were distinguished on the basis of the results of disc diffusion tests. Group 1 was resistant to chloramphenicol, gentamicin, tobramycin, amikacin, and the newer cephalosporins because of the production of an extended-spectrum β-lactamase (PER-1). Group 2 exhibited the same pattern plus resistance to sulfamethoxazole-trimethoprim (Sxt). Except for Sxt resistance, dominant phenotypes of both groups were transferred on an identical plasmid, pSTI1 (81 MDa). Group 3 was resistant to ampicillin, chloramphenicol, gentamicin, tobramycin, and Sxt. This pattern was also transferred on an 81-MDa plasmid (pSTI2) which differed from pSTI1 on the basis of *Eco*RI and *Hin*dIII restriction fragments. Group 4 was resistant to ampicillin, chloramphenicol, and tetracycline, and a 74-MDa nonconjugative plasmid was detected. Restriction fragment length polymorphism of RNA-encoding DNA and arbitrarily primed PCR tests revealed that bacteria from groups 1, 2, and 3 were clonally related. Epidemiologic data also supported the clonal-dissemination hypothesis. We conclude that *S. typhimurium* isolates acquire and exchange multiple-resistance plasmids in hospital microflora.

Extended-spectrum β-lactamases are encoded on conjugative plasmids, transposons, or integrons. These mobile genetic elements readily spread under selective antibiotic pressure (7). Antibiotics are consumed regularly and extensively in hospitals. This constant antibiotic pressure selects multidrug-resistant and extended-spectrum β-lactamase-producing bacteria and enables their transmission among hospitalized patients. Consequently, a multiresistant microflora that is mostly composed of common human colonizers appears in hospitals. As a human pathogen, the genus *Salmonella* is not a typical member of this hospital microflora. Therefore, extended-spectrum β-lactamases with multiple antibiotic resistance are rarely associated with this genus (1, 6, 13). Moreover, plasmid profiles of salmonellae are believed to be relatively stable (16–18). However, because of the prophylactic and therapeutic use of antibiotics and their use as growth promoters in poultry and food animals, antibiotic-resistant strains are becoming more common (10).

Extended-spectrum β-lactamases are mutants of classical enzymes such as TEM-1, TEM-2, and SHV-1. Recently, a novel extended-spectrum β-lactamase, PER-1, has been discovered, and sequence analysis revealed that it does not belong to the TEM or SHV family (12). PER-1 was recognized in a *Pseudomonas aeruginosa* isolate from a Turkish patient (11a) and so far has not been reported from any other country. We isolated PER-1-producing *Salmonella typhimurium* strains from fatal nosocomial cases in 1992 at two university hospitals in Istanbul (19). During another study 17 months later, we again detected PER-1-producing *S. typhimurium* strains at one of these hospitals, indicating that the clone may have been present in the hospital microflora for more than 1 year. Although multiple-drug-resistant and extended-spectrum β-lactamase-producing salmonellae have been reported before, all of them were from short-term nosocomial outbreaks and produced relatively common enzymes (1, 6, 13).

We characterized resistance genes from nosocomial multiple-drug-resistant *S. typhimurium* strains and analyzed the molecular epidemiology of PER-1-producing isolates.

MATERIALS AND METHODS

Bacterial strains. Bacterial isolates were taken from the culture collections of the microbiology departments of two universities. The university hospitals are 20 km from each other on opposite sides of the Bosporus (the sea channel connecting the Black Sea to the Sea of Marmara). *S. typhimurium* strains resistant to more than two antibiotic groups were included in the study. Thirteen isolates from the Anatolian-side hospital (hospital A) and 19 from the European-side hospital (hospital E) fulfilled the criterion. The patients from whom these strains were isolated were initially hospitalized because of other diseases and became infected with the strains in the hospitals; thus, all isolates were nosocomially acquired. The most recent isolate was obtained in April 1994, while the first one was isolated 28 months before. Serotypes of the isolates were initially identified using O and H antiserum obtained from Difco Laboratories (Detroit, Mich.) and later confirmed at a reference laboratory.

Resistance tests, transconjugation, and plasmid analysis. Disc diffusion tests were done on Mueller-Hinton agar with commercial antibiotic discs (Oxoid Ltd., Basingstoke, United Kingdom) according to the recommendations of the National Committee for Clinical Laboratory Standards (11).

Escherichia coli K12 J53-1 (*pro* Nal^r) was used as the recipient in transconjugation experiments. Donor and recipient bacteria were mated on Mueller-Hinton agar plates at 37°C. Transconjugants were selected on Mueller-Hinton agar containing nalidixic acid (100 mg/liter) plus either ampicillin (64 mg/liter), cefotaxime (16 mg/liter), or sulfamethoxazole-trimethoprim (Sxt) (25 to 5 mg/liter).

The rapid procedure of Kado and Liu (8) was used to detect plasmids, and their sizes were estimated by comparing their migration to that of known plasmids on 0.9% agarose. Extracts were run on agarose gels at 12 V/cm for 2 h and stained with ethidium bromide. Alkaline lysis was the preferred plasmid isolation method for restriction enzyme digestion (14). Plasmid DNA was digested to completion in 25-μl volumes with 10 U of either *Eco*RI or *Hin*dIII (Stratagene,

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TABLE 1. Oligonucleotides for hybridization and PCR analysis

Primer or probe	Nucleotide sequence (5′ to 3′)	Accession no. (Ac),[a] position (p), and/or reference (r)
PER	ATG AAT GTC ATT ATA AAA GC	p, 308; r, 12
PER	AAT TTG GGC TTA GGG CAA GAA A	p, 1234
TEM	TTA CCA ATG CTT AAT CAG TGA G	Ac, X64523; p, 477
TEM	ATG AGT ATT CAA CAT TTC CGT G	p, 1359
SHV	CCA GCA TGG CCG CGA CCC T	Ac, M59181; p, 663
ERIC1R	GGA TTC AC[b]	r, 21
ERIC2	AAG TAA GTG ACT GGG GTG AGC G	r, 21

[a] Accession numbers are from the EMBL and GenBank databases.
[b] Last eight nucleotides of the 3′ end of the original ERIC1R sequence.

Cambridge, United Kingdom) and separated on a 0.9% agarose gel at 40 V for 4 to 6 h.

CAT assay and isoelectric focusing. The chloramphenicol acetyltransferase (CAT) tube assay was performed with positive and negative controls (4).

β-Lactamases were released by freezing and thawing a dense suspension of bacteria in 0.1 M phosphate buffer (pH 7.0) five times. After centrifugation for 15 min at 12,000 × *g*, crude extracts were applied to an ampholine gel with a pH range of 3.5 to 10, which was prepared according to the formulation of Matthew et al. (9) but supplemented with 10% sucrose. After focusing at 10 W for 90 to 120 min, the enzymes were located by 1 mM nitrocefin in 0.1 M phosphate buffer, pH 7.0. Estimations of pI values were made by comparison to the standards TEM-1 (5.4), TEM-2 (5.6), and SHV-1 (7.6).

Selection of oligonucleotides. TEM and PER probes were obtained by labelling PCR products of $bla_{\text{TEM-1}}$ and $bla_{\text{PER-1}}$. An SHV probe was designed with the OLIGO primer analysis software (MedProbe, Oslo, Norway) and compared for sequence homology with sequences in the EMBL and GenBank databases (Table 1). The TEM and PER probes were labelled with a random labelling kit (Boehringer Mannheim), and the SHV probe was labelled with an end tailing kit (Boehringer Mannheim). All labelling was performed according to the instructions of the manufacturer.

ERIC1R and ERIC2 are previously described primers for arbitrarily primed PCR (AP-PCR) tests (21). However, because it has been suggested that short primers provide better discrimination, we ordered the last eight nucleotides of the 3′ end of ERIC1R for AP-PCR (20).

Genomic DNA preparation, hybridization, ribotyping, and AP-PCR. Genomic DNA was extracted from overnight plate cultures by the guanidium thiocyanate (GuSCN)-diatom method (2). Bacteria were collected from the plates with 2 ml of 0.1 M Tris HCl (pH 6.4), and 200 μl of these dense suspensions was lysed with 1 ml of GuSCN plus 13 mg of Triton X-100 and 0.1 ml of diatom suspension for 30 min at 60°C (DNA binds to diatom in high concentrations of GuSCN at pH 6.4). After centrifugation at 12,000 × *g* for 15 s, supernatants were discarded. The diatom pellet was washed twice with 1 ml of GuSCN, twice with 1 ml of 70% ethanol, and once with 1 ml of acetone and left for 10 min at room temperature to dry. DNA was eluted at 50°C with 100 μl of a low-ionic-concentration Tris-EDTA buffer (10 mM Tris HCl, 1 mM EDTA [pH 8.0]). Genomic DNA was digested to completion with the enzyme *Eco*RI for 6 h. Fragments were separated on 0.7% agarose at 20 V for 14 h.

The colony hybridization method is described elsewhere (14).

Digested genomic DNA and plasmids were transferred to a positively charged nylon membrane (Boehringer Mannheim) by the method of Southern (15). All hybridization and washing steps were carried out under high-stringency conditions. The probe for ribotyping was prepared from a commercial solution of 16S-23S RNA (Boehringer Mannheim). Avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim) was used for synthesis and labelling of the probe instead of the Klenow enzyme of the random labelling kit (Boehringer Mannheim); otherwise, the hexanucleotide and digoxigenin labelling mixtures were from this same kit.

Amplification reactions were carried out in a volume of 50 μl for AP-PCR. A master mixture containing 200 μM deoxynucleoside triphosphate, 1 μM primer, 1.5 mM $MgCl_2$, and *Taq* polymerase (in increments of 2.5 U) (Stratagene) was prepared each time. PCR was run for 40 cycles at a denaturation temperature of 95°C for 1 min, an annealing temperature of 36°C for 2 min, and an extension temperature of 72°C for 3 min. Products were separated on a VisiGel separation matrix (Stratagene) at 80 V for 1 h, stained with ethidium bromide, and visualized with a UV lamp.

RESULTS

Characterization of resistance phenotypes. A total of 32 *S. typhimurium* isolates, 13 from hospital A and 19 from hospital E, fulfilled the criterion of being resistant to more than two groups of antibiotics. All of them belong to the same serotype (4,5,12:i:1,2) and the same biotype as API 20E (identification number, 6704150) (BioMerieux, Lyon, France).

We identified four resistance phenotypes. Bacteria belonging to group 1 (R 1) were found to be resistant to chloramphenicol, gentamicin, tobramycin, amikacin, and extended-spectrum cephalosporins (Table 2). They were sensitive to cefoxitin, and double-disc synergy tests were positive, suggesting that they were producing an extended-spectrum β-lactamase. Bacteria from group 2 (R 2) were resistant to the same antibiotics as were R 1 and were also resistant to Sxt. Group 3 isolates (R 3) were resistant to ampicillin, chloramphenicol, gentamicin, tobramycin and Sxt. The single isolate of group 4 (R 4) was resistant to ampicillin, chloramphenicol and tetracycline.

Resistance transfer and characterization of the plasmids. Although donor strains had four resistance phenotypes, the recipients were expressing three phenotypes and harboring three plasmids (Fig. 1). Resistance to chloramphenicol, gentamicin, tobramycin, amikacin, and cephalosporins was spontaneously transferred from R 1 and R 2 with the transfer of 81-MDa plasmids at a frequency of 10^{-8}. The *Eco*RI restriction patterns of these plasmids were similar, so both were designated pSTI1 (data not shown). The transconjugant of R 3 harbored an 81-MDa plasmid (pSTI2) and was resistant to ampicillin, chloramphenicol, gentamicin, tobramycin and Sxt. The *Eco*RI and *Hin*dIII restriction fragment length patterns of plasmids pSTI1 and pSTI2 were completely different (Fig. 2). We could not achieve any successful transconjugation with the R 3 isolate, which carried a single plasmid, pSTI3 (74 MDa). Sxt resistance from R 2 was not transferred as well.

These experiments revealed that plasmids pSTI1 and pSTI2

TABLE 2. Resistance phenotypes of *S. typhimurium* isolates, responsible plasmids, and isolation places

Group	Plasmid	Phenotype[a]	No. of isolates from hospital: A	No. of isolates from hospital: E
R 1	pSTI1[b]	Cm^r $Ctax^r$ Gm Tm Ak^r	9	13
R 2	pSTI1	Cm^r $Ctax^r$ Gm Tm Ak^r Sxt^r	0	2
R 3	pSTI2[c]	Cm^r Ap^r Gm Tm^r Sxt^r	3	4
R 4	pSTI3[d]	Cm^r Ap^r Tc^r	1	0

[a] Ak, amikacin; Ap, ampicillin; Cm, chloramphenicol; Ctax, cefotaxime; Gm, gentamicin; Sxt, sulfamethoxazole-trimethoprim; Tc, tetracycline; Tm, tobramycin.
[b] CAT and PER-1 are encoded.
[c] CAT and pI 7.0 (non-TEM, non-SHV) β-lactamase are encoded.
[d] CAT and pI 5.8 β-lactamase are encoded.

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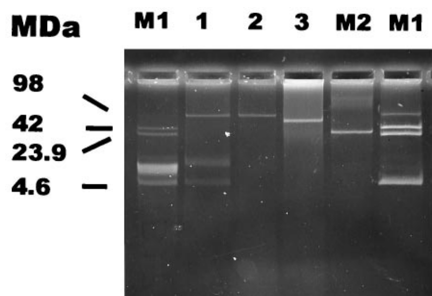

FIG. 1. Plasmids encoding the resistance phenotypes and known plasmids as molecular markers. Lanes: M1, NCTC 50192 (98, 42, 23.9, and 4.6 MDa); M2, pUZ8 (40 MDa); 1, pSTI1 (R 1; 81 MDa); 2, pSTI2 (R 3; 81 MDa); 3, pSTI3 (R 4; 74 MDa).

(each 81 MDa) were encoding the dominant resistance phenotypes of R 1 and R 3, respectively.

CAT assay and characterization of β-lactamases (isoelectric focusing and hybridization). The CAT assay was positive for all isolates; thus, resistance to chloramphenicol was due to chloramphenicol acetyltransferase.

Isoelectric focusing showed that pSTI1 and pSTI2 were encoding single β-lactamases that focused at pIs of 5.3 and 7.0, respectively. With the colony hybridization method, all bacteria belonging to phenotypes R 1 and R 2, their transconjugants, and plasmid pSTI1 gave a positive signal with the PER probe. The PER-1 gene was found to be located on an approximately 3.4 kbp *Eco*RI fragment of PSTI1. Thus, the enzyme with a pI value of 5.3 that was hybridizing with the PER probe was designated PER-1. Plasmid pSTI2 did not hybridize with the PER, TEM, or SHV probes. Therefore, we concluded that the enzyme with a pI value of 7.0 did not belong to either the TEM or SHV family. The single isolate of R 4 produced a β-lactamase focusing at pI 5.8, which was not further characterized.

Ribotyping and AP-PCR. Two RNA-encoding DNA restriction fragment length polymorphism (RFLP) (rDNA) patterns

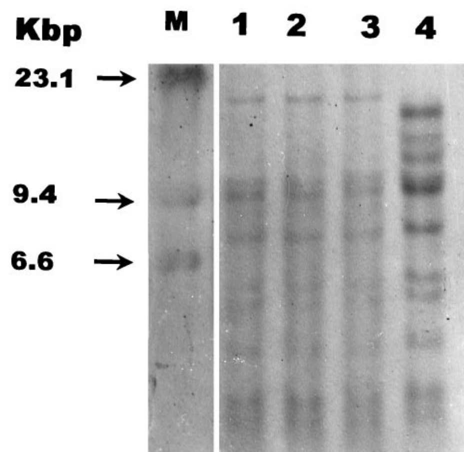

FIG. 3. rDNA RFLP of *Eco*RI-digested chromosomal DNA hybridized by digoxigenin-labelled 16S-23S rDNA probe. Lanes: M, digoxigenin-labelled *Hin*dIII-digested lambda DNA; 1, ribotype of R 1; 2, ribotype of R 2; 3, ribotype of R 3; 4, ribotype of R 4.

(ribotypes) were identified among the selected representatives. Ribotypes of R 1, R 2, and R 3 were similar and distinguishable from that of R 4 (Fig. 3). AP-PCR results were in agreement with the results of ribotyping. Except for the single isolate of group 4, isolates selected as representative were similar with regard to their AP-PCR product patterns (Fig. 4). The DNA fingerprints obtained with the above methods suggested that the isolates of group 1, 2, and 3 were clonally related.

Retrospective analysis of patient data. Totals of 22 and 2 isolates demonstrated R 1 and R 2 phenotypes, respectively. Of these strains, 9 were isolated at hospital A and 15 were isolated at hospital E (Table 2). The first patient was one who had been transferred from another hospital to hospital A with a case of nosocomial neonatal meningitis caused by the first *S. typhimurium* isolate of this study. Another patient, who was

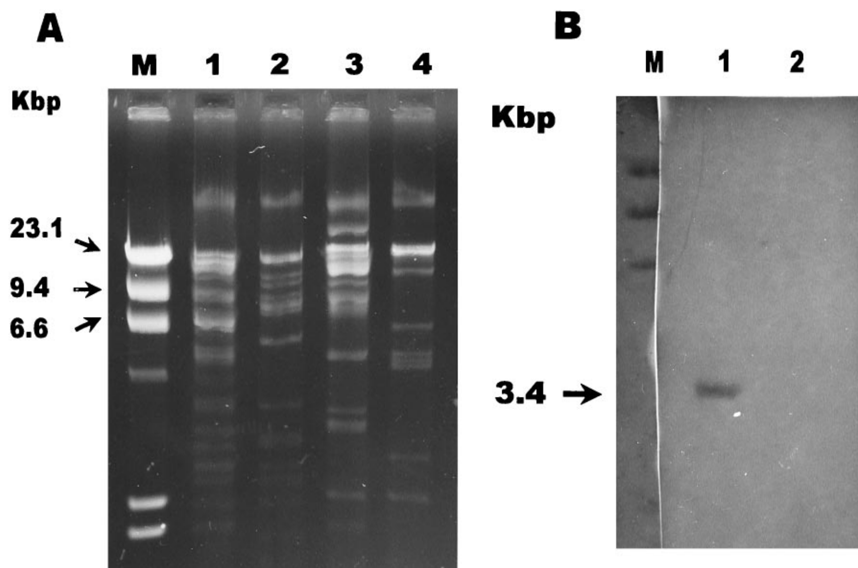

FIG. 2. (A) Agarose gel electrophoresis of *Eco*RI- and *Hin*dIII-digested plasmids. Lanes: M, *Hin*dIII-digested lambda DNA; 1, *Eco*RI-digested pSTI1; 2, *Eco*RI-digested pSTI2; 3, *Hin*dIII-digested pSTI1; 4, *Hin*dIII-digested pSTI2. (B) Hybridization of the digested fragments with digoxigenin-labelled PER probe. Lane M, digoxigenin-labelled *Hin*dIII-digested lambda DNA. Lanes 1 and 2 correspond to lanes 1 and 2 in panel A. The PER-1 gene has a *Hin*dIII restriction site in the middle, so there is no positive hybridization signal in the *Hin*dIII-digested plasmid.

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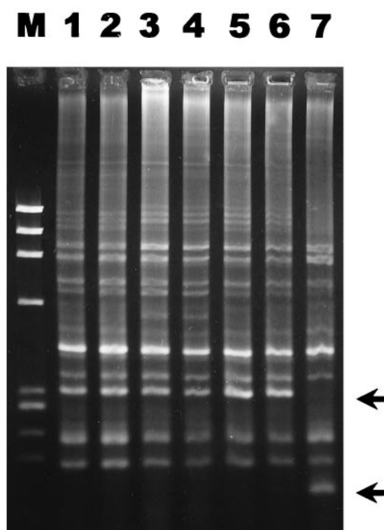

FIG. 4. AP-PCR patterns of randomly selected representatives of resistance phenotypes from both hospitals. Lanes: M, *Hae*III-digested φX174 DNA (Stratagene); 1 and 2, R 1; 3 and 4, R 2; 5 and 6, R 3; 7, R 4. The arrows indicate the main differences between the seventh lane and the others.

admitted to hospital E, was transferred from hospital A and was already infected with the R 1 isolate of *S. typhimurium*. Nearly all the patients were from pediatric surgery or neonatal intensive-care services.

A patient was found who had been excreting the isolate exhibiting the R 1 phenotype for more than a year. A total of seven strains, three from hospital A and four from hospital E, showed the R 3 phenotype. We could not find enough data to show a link between the hospitals for this phenotype, but the first of the hospital E isolates was also detected on the Anatolian side. The four isolates from the European side were very recent.

DISCUSSION

We identified four groups of isolates with different resistance patterns, namely, R 1, R 2, R 3, and R 4. Three plasmids, pSTI1, pSTI2, and pSTI3, were responsible for the dominant phenotypes. DNA fingerprinting with rDNA RFLP and AP-PCR suggested that R 1, R 2, and R 3 were clonally related. Although the relative mobilities of pSTI1 and pSTI2 were similar on agarose gels, their restriction patterns were different. This difference indicated that resistance patterns were changed because of the exchange of plasmids rather than by transposon or integron exchange. We conclude that if *S. typhimurium* isolates can persist long enough in hospital microflora, they will acquire and exchange multiple-resistance plasmids.

Phage typing, plasmid profiles, and antibiotic resistance patterns are the classical methods of an epidemiological investigation. Recently, molecular biology methods, such as rDNA RFLP, pulsed-field gel electrophoresis, and AP-PCR were applied to the field. These methods depend mainly on the comparison of fingerprints of either total DNA or a specific part of it. The discriminatory power of rDNA RFLP and AP-PCR depends on the selected restriction enzyme and primer sequences, respectively. *Eco*RI was previously suggested to be discriminative when coupled with a 16S-23S RNA probe for rDNA RFLP of *S. typhimurium* (5). The primers that we selected for AP-PCR were also known sequences and had been shown to differentiate clones of *Enterobactericeae*. The data based on the ribotyping and AP-PCR similarity of groups 1, 2, and 3 of this study revealed the clonal relatedness of *S. typhimurium*. A retrospective analysis of patient data further supported the hypothesis of the spread of a single clone between hospitals.

Several studies have indicated the stability of plasmid profiles of salmonella species, and thus plasmid analysis has been suggested as a useful method for epidemiological investigations (16–18). However, in this study, despite having similar rDNA RFLP and AP-PCR patterns, groups 1 and 3 harbored different plasmids.

Extended-spectrum β-lactamases are believed to be encoded on mobile genetic elements such as transposons and integrons. Integrons are recently described genetic elements, and with the aid of integron integrase they are able to exchange resistance gene cassettes (3). In other words, a single plasmid could be found with different multiple-resistance compositions. Because salmonellae are believed to have very stable plasmid profiles, the probability of exchanging transposons or gene cassettes on a common plasmid seemed to be high. If this were the case, we would find differences at very few restriction fragments of digested plasmids. Thus, to answer the question of whether clones exchanged two plasmids, pSTI1 and pSTI2, or the same plasmid with only the gene cassettes or transposons changed, we compared the RFLP patterns of pSTI1 and pSTI2. If only several restriction fragments were different, it would be essential to look for sequence homology between the fragments other than the one that carried $bla_{\mathrm{PER\text{-}1}}$. However, RFLP patterns obtained with two enzymes were quite different for these plasmids; thus, restriction enzyme analysis strongly suggested that pSTI1 and pSTI2 were different plasmids. The sequence analysis of $bla_{\mathrm{PER\text{-}1}}$ showed that it was only distantly related to the TEM or SHV enzymes. Although extended-spectrum β-lactamases are almost always the mutants of classical enzymes, the ancestor of PER-1 has not yet been identified (12). Our experiments with colony hybridization revealed that $bla_{\mathrm{PER\text{-}1}}$ was not uncommon among nosocomial *P. aeruginosa* isolates in Istanbul, Turkey (unpublished data). However, we did not detect PER-1 in any nosocomial species other than *P. aeruginosa* and *S. typhimurium* at hospitals A and E and could not find an explanation of why $bla_{\mathrm{PER\text{-}1}}$ was not disseminated among other species.

Probably, an *S. typhimurium* strain acquired plasmid pSTI1 in 1992 and the strain was disseminated between hospitals by the transfer of infected patients. The actual number of patients infected with multiply resistant strains must be much greater than that which we detected. Asymptomatic carriers or mild cases, which were unnoticed, could be an explanation for the persistence and dissemination of this clone in the hospital microflora.

S. typhimurium is one of the most common serotypes isolated from humans with salmonellosis. Although it is not recommended to treat gastroenteritis due to this organism with antibiotics, invasive complications such as bacteremia, sepsis, and meningitis do require antibiotics. Therefore, increasing multiple-resistance and extended-spectrum β-lactamase incidence among *S. typhimurium* deserves special attention, particularly in neonatal infections.

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