JOURNAL OF CLINICAL MICROBIOLOGY, July 2003, p. 2940–2945 Vol. 41, No. 7
0095-1137/03/$08.00+0 DOI: 10.1128/JCM.41.7.2940–2945.2003

Molecular Epidemiology and Characterization of Plasmid-Encoded β-Lactamases Produced by Tunisian Clinical Isolates of *Salmonella enterica* Serotype Mbandaka Resistant to Broad-Spectrum Cephalosporins

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Received 9 October 2002/Returned for modification 21 January 2003/Accepted 13 March 2003

We studied 31 clinical isolates of *Salmonella enterica* serotype Mbandaka resistant to broad-spectrum cephalosporins and recovered in Tunisia over a 5-year period. The transferability of this resistance was demonstrated by conjugation experiments. Thirty of the 31 isolates were positive in the double-disk synergy test. By isoelectric focusing analysis, all of the isolates were found to produce a band of β-lactamase activity with a pI of 5.9. Three of these isolates produced an additional band with a pI of 7.6. PCR and DNA sequencing identified these β-lactamases as TEM-4 and SHV-2a, respectively. The remaining isolate, highly resistant to ceftazidime but susceptible to cefepime, produced a β-lactamase that focused at pI 7.8. No synergy was detected by the double-disk synergy test. Sequence analysis of the *bla* gene amplified by PCR showed that the plasmid-mediated AmpC-type enzyme was ACC-1a. Fingerprinting analysis by repetitive-element PCR and enterobacterial repeat intergenic consensus-PCR suggested that 29 of the 31 *Salmonella* serotype Mbandaka isolates belonged to the same clonal population.

Salmonella enterica is a major endemic and epidemic pathogen in animals and humans worldwide. Animals and their products, particularly meat, chicken eggs, and milk, are major sources of human infection. The incidence of nontyphoid *Salmonella* infections has increased considerably in many countries, but with marked differences among countries (http:/www.who.int/emc/diseases/zoo/SALM-SURV). In Tunisia, studies done in the 1980s suggested that the most common *S. enterica* serotype isolated from human and animal sources was serotype Wien (25). In contrast, recent reports show that *S. enterica* serotype Enteritidis is becoming the predominant serotype in both humans and other animals in Tunisia. Other serotypes, such as Mbandaka, Braendrup, Typhimurium, Anatum, and Infantis, are also frequently encountered in this country (12–14). Antibiotics are necessary to treat most children and neonates with salmonellosis, broad-spectrum cephalosporins (e.g., ceftriaxone) being the primary drugs of choice. Over the past 2 decades and particularly in developing countries, *Salmonella* strains resistant to broad-spectrum cephalosporins have been reported (30). These strains produce extended-spectrum β-lactamases (ESBLs), such as TEM and SHV (TEM-3, TEM-4, TEM-25, TEM-27, SHV-2, and SHV-5) (1, 4, 7, 8, 16, 18, 22, 25, 33, 41, 42), CTX-M-2 (10, 23), CTX-M-3 (7), PER-1 (46), and PER-2 (11) and, more recently, plasmid-mediated AmpC-type enzymes, such as DHA-1 (21), CMY-2 (19, 20, 31, 35, 47–49), and ACC-1 (43). In North Africa, SHV-2 has been reported in *Salmonella* serotypes Wien (25), Typhimurium (22), and Mbandaka (27), TEM-3 has been reported in *Salmonella* serotype Typhimurium (1), CMY-2 has been reported in *Salmonella* serotype Senftenberg (31), and ACC-1 has been reported in *Salmonella* serotype Livingstone (43). Only TEM-25 and SHV-2 have been reported in *Salmonella* serotype Mbandaka (27, 41).

We studied 31 *Salmonella* serotype Mbandaka strains resistant to broad-spectrum cephalosporins and isolated from 1995 to 1999 at La Rabta University Hospital in Tunis, Tunisia. The β-lactamases were characterized by isoelectric focusing and molecular methods. Mechanisms involved in the transmission of *bla* genes among these *Salmonella* strains were investigated by conjugation experiments. We also used antimicrobial susceptibility patterns, plasmid profiles, and enterobacterial repeat intergenic consensus (ERIC)-PCR and repetitive-element PCR (rep-PCR) fingerprinting (36) for epidemiological characterization.

MATERIALS AND METHODS

Bacterial strains and media. Thirty-one isolates of *Salmonella* serotype Mbandaka obtained from 1995 to 1999 at La Rabta University Hospital in Tunis, Tunisia, were studied (Table1). The strains were isolated from stools, blood cultures, the urinary tract, and pus (Table 1). All isolates were identified with the API 20E system (Biomerieux SA, Marcy l'Etoile, France). Isolates were serotyped on the basis of somatic O, phase 1 flagellar, and phase 2 flagellar antigen expression by agglutination tests with antisera (Bio-Rad SA, Marnes-la-Coquette, France). The following media, from Bio-Rad SA, were used for bacterial culturing: Mueller-Hinton (MH) agar, salmonella-shigella agar, Trypticase soy (TS) broth and agar, and Drigalski agar.

Antimicrobial susceptibility testing. We used the disk diffusion assay with MH agar in accordance with French National Antibiogram Committee guidelines (Communiqué 2002; www.sfm.asso.fr/Sect4/atbfr.html). The isolates were tested

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TABLE 1. Date and site of isolation and β-lactamase and epidemiological characterizations of Tunisian clinical isolates of *Salmonella* serotype Mbandaka resistant to broad-spectrum cephalosporins

Isolate	Yr of isolation	Site of isolation	pI(s)	β-Lactamase(s)	Resistance cotransferred[a]	Plasmid profile	rep-PCR profile	ERIC-PCR profile
$MMAS_1$	1998	Stools	5.9	TEM-4	G, TM, N	P1	A	A1
$MMAS_2$	1997	Stools	5.9	TEM-4	G, TM, N	P2	A	A2
$MMAS_3$	1997	Pus	5.9	TEM-4	G, TM, N	P3	A	A2
$MMAS_4$	1997	Stools	5.9	TEM-4	G, TM, N	P3	A	A2
$MMAS_5$	1997	Stools	5.9	TEM-4	G, TM, N	P1	A	A1
$MMAS_6$	1997	Stools	5.9	TEM-4	G, TM, N	P4	A	A2
$MMAS_7$	1997	Stools	5.9	TEM-4	G, TM, N	P4	A	A2
$MMAS_8$	1997	Stools	5.9	TEM-4	G, TM, N	P1	A	A1
$MMAS_{10}$	1995	Stools	5.9	TEM-4	G, TM, N	P1	A	A1
$MMAS_{13}$	1997	Stools	5.9	TEM-4	G, TM, N	P5	A	A2
$MMAS_{14}$	1997	Stools	5.9	TEM-4	G, TM, N	P5	A	A2
$MMAS_{15}$	1997	Stools	5.9	TEM-4	G, TM, N	P2	A	A2
$MMAS_{16}$	1997	Stools	5.9	TEM-4	G, TM, N	P3	A	A2
$MMAS_{17}$	1997	Stools	5.9	TEM-4	G, TM, N	P3	A	A2
$MMAS_{19}$	1998	Stools	5.9	TEM-4	G, TM, N	P1	A	A1
$MMAS_{21}$	1998	Stools	5.9	TEM-4	G, TM, N	P1	A	A1
$MMAS_{22}$	1998	Stools	5.9	TEM-4	None	P7	B	B
$MMAS_{23}$	1998	Stools	5.9	TEM-4	G, TM, N	P1	A	A1
$MMAS_{24}$	1998	Stools	5.9	TEM-4	G, TM, N	P1	A	A1
$MMAS_{26}$	1998	Blood	5.9	TEM-4	G, TM, N	P1	A	A1
$MMAS_{27}$	1998	Blood	5.9	TEM-4	G, TM, N	P1	A	A1
$MMAS_{28}$	1998	Stools	5.9	TEM-4	G, TM, N	P1	A	A1
$MMAS_{29}$	1998	Blood	5.9	TEM-4	G, TM, N	P1	A	A1
$MMAS_{30}$	1999	Stools	5.9	TEM-4	N, G, TM, N	P6	A	A1
$MMAS_{31}$	1999	Stools	5.9,7.6	TEM-4, SHV-2a	G, TM, N, TP, S	P6	A	A1
$MMAS_{32}$	1999	Stools	5.9	TEM-4	G, TM, N	P6	A	A1
$MMAS_{33}$	1999	Stools	5.9	TEM-4	G, TM, N	P6	A	A1
$MMAS_{35}$	1999	Stools	5.9,7.6	TEM-4, SHV-2a	G, TM, N, TP, S	P6	A	A1
$MMAS_{37}$	1999	Stools	5.9,7.6	TEM-4, SHV-2a	G, TM, N, TP, S	P6	A	A1
$MMAS_{39}$	1999	Urinary tract	5.9	TEM-4	G, TM, N	P1	A	A1
$MMAS_{40}$	1999	Pus	7.8	ACC-1a	G, TM, N, TP, S	P8	C	C

[a] G, gentamicin; TM, tobramycin; N, netilmicin; TP, trimethoprim; S, sulfonamides.

for ESBL production by the double-disk synergy (DDS) method. The disks were placed 25 mm from center to center.

Conjugation experiments. *Escherichia coli* strain K-12 (resistant to nalidixic acid) and *E. coli* J53-2 (resistant to rifampin) were used as recipients in transfer experiments. Overnight cultures of donor and recipient strains in TS broth at 37°C were mixed on MH agar at a ratio of 1:1 (200 μl of donor culture and 200 μl of recipient culture). The mixture was spread with Pasteur pipettes and incubated at 37°C for 18 h. Colonies from the mixed culture were suspended in 5 ml of sterile water, and 200 μl of the suspension was plated on Drigalski agar containing rifampin at 250 μg/ml or nalidixic acid at 50 μg/ml and cefotaxime at 2.5 μg/ml. Growing colonies were subjected to the DDS test to confirm the presence of ESBL transconjugants. All of the transconjugants were tested for their susceptibility to all of the antibiotics used for the donors.

Plasmid DNA analysis. Plasmid DNA was extracted from *Salmonella* serotype Mbandaka isolates and their transconjugants by the method of Kado and Liu (29). The resulting DNA preparation was submitted to electrophoresis on horizontal slab gels containing 0.8% agarose (Bio-Rad SA). Plasmids with known molecular sizes—pIP112 (100 kb), pIP173 (128 kb), pCFF04 (85 kb), and pBK-CMV (4.4 kb) (Stratagene, Amsterdam, The Netherlands)—were used for size estimation. Electrophoresis was carried out at 100 V for 3 h. The slab gels were stained in ethidium bromide solution (10 μg/ml) for 10 min, followed by immersion in distilled water for 45 min. The slab gels were placed under UV light, and the bands were analyzed by using a computer-based program (Gel Doc 1000; Bio-Rad SA).

PCR fingerprinting. Template DNA was extracted from each isolate by using a commercial genomic DNA purification kit (QIAampDNA mini kit; Qiagen, Courtaboeuf, France). For rep-PCR (final volume, 100 μl), the primers were REP1R-Dt (5′-IIINCGNCGNCATCNGGC-3′) and REP2-Dt (5′-NCGNCTT ATCNGGCCTAC-3′), used as a pair. We used 50 pM each of primers REP1R-Dt and REP2-Dt, 5 U of *Taq* DNA polymerase (Amersham Pharmacia Biotech, Saclay, France), and 10 μl of PCR buffer. The rep-PCR parameters were as follows: initial denaturation at 95°C for 3 min; 40 cycles of denaturation at 92°C for 30 min, annealing at 40°C for 1 min, and extension at 65°C for 8 min; and a final extension at 65°C for 15 min. For ERIC-PCR, we used only one primer, ERIC2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′). The PCR parameters were identical to those used for rep-PCR, except for the annealing temperature (52°C).

PCR amplicons were resolved on 1% (wt/vol) agarose (Bio-Rad SA) containing ethidium bromide (0.5 μg/ml) by horizontal electrophoresis in Tris-borate-EDTA buffer. Gels were visualized under UV light and photographed with a computer-controlled image analyzer (Gel Doc 1000).

IEF of β-lactamases. Crude β-lactamase preparations were obtained by sonication. Briefly, 50 ml of overnight cultures at 37°C in TS broth were centrifuged at 5,000 rpm for 5 min. The supernatants were eliminated, and the cells were resuspended in sterile distilled water (weight/weight). The bacterial suspensions were disrupted by sonication, twice for 30 s each at 40 Hz (Vibracell 300; Bioblock, Ilkirch, France). Enzyme activity was detected before electrophoresis by applying crude extracts to nitrocefin disks (Biomerieux SA). The extracts were subjected to isoelectric focusing (IEF) on pH 3.5 to 10 Ampholine (Amersham) polyacrylamide gels (32). TEM-25 and TEM-2 (*Salmonella* serotype Mbandaka CF1509; pIs, 5.3 and 5.6, respectively), TEM-3 (pCFF04; pI, 6.3), ACC-1 and TEM-1 (*Klebsiella pneumoniae* SLK54; pIs, 7.8 and 5.4, respectively), and CTX-M-9 and TEM-1 (*E. coli* RAJ; pIs, 8.2 and 5.4, respectively) were used as pI markers. Electrophoresis was performed at 14°C, 400 V, 6 W, and 15 mA for 18 h. β-Lactamase activity was detected by the chromogenic nitrocefin test by overlaying the gels with filter paper soaked with nitrocefin solution (0.5 mg/ml).

Molecular characterization of β-lactamase-encoding genes. Genomic DNA was extracted (QIAampDNA mini kit). Specific primers were used for PCR. Primers OT3 and OT4 were used to amplify the putative bla_{TEM} genes (5). The putative bla_{SHV} genes were amplified with the corresponding primers, namely, OS5 and OS6 (6). The internal primers ACC upper and ACC lower were used to amplify the internal fragments of the bla_{ACC} gene (34). PCR assays were performed with a final volume of 100 μl containing 4 U of *Taq* DNA polymerase and 50 pM each primer. The PCR parameters were as follows: initial denatur-

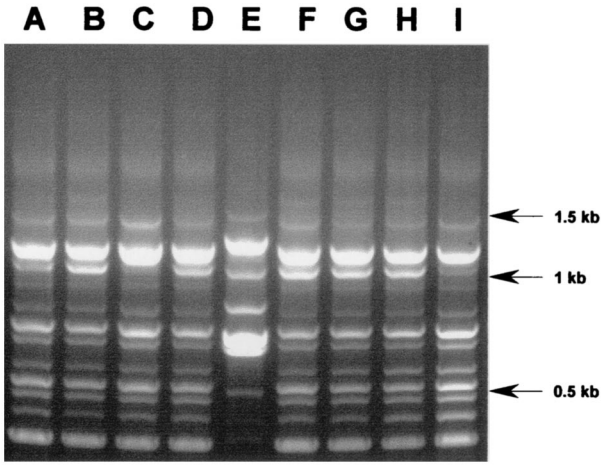

FIG. 1. PCR fingerprint patterns obtained with primer ERIC2 for selected Tunisian clinical isolates of *Salmonella* serotype Mbandaka resistant to broad-spectrum cephalosporins. Lane A, isolate $MMAS_1$ (pattern A1); lane B, isolate $MMAS_2$ (pattern A1); lane C, isolate $MMAS_5$ (pattern A2); lane D, isolate $MMAS_{21}$ (pattern A1); lane E, isolate $MMAS_{22}$ (pattern B); lane F, isolate $MMAS_{23}$ (pattern A1); lane G, isolate $MMAS_{29}$ (pattern A1); lane H, isolate $MMAS_{31}$ (pattern A1); lane I, isolate $MMAS_{32}$ (pattern A2).

ation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 30 s, and extension at 72°C for 1 min; and a final extension at 72°C for 7 min.

The amplified *bla* genes were sequenced by using two independent PCR products along both strands (44) with PCR primers as sequencing primers on an ABI 373A DNA sequencer (Applied Biosystems, Foster City, Calif.). The nucleotide sequences and deduced protein sequences were analyzed with the BLAST and ClustalW programs (2, 45).

RESULTS AND DISCUSSION

Identification of *Salmonella* isolates. The 31 isolates were identified as *Salmonella* by biochemical characterization and as *Salmonella* serotype Mbandaka by serotyping (O 6,7, Z_{10}, e,n,z_{15}).

Antimicrobial susceptibility and transferability of resistance. All of the isolates except for $MMAS_{40}$ were resistant to amoxicillin, cephalothin, ticarcillin, piperacillin, cefuroxime, cefotaxime, ceftazidime, and ceftriaxone. They were susceptible to β-lactam– β-lactamase inhibitor combinations and to cefoxitin and imipenem. The DDS test was positive for all of these isolates. $MMAS_{40}$ was highly resistant to the amoxicillin-clavulanic acid and tazobactam-piperacillin combinations. $MMAS_{40}$ was also highly resistant to ceftazidime and fully susceptible to cefepime (almost all of the other isolates showed intermediate susceptibility), cefoxitin, and imipenem. These β-lactam resistance phenotypes were always associated with gentamicin, tobramycin, and netilmicin resistance. Six isolates ($MMAS_{30}$, $MMAS_{31}$, $MMAS_{32}$, $MMAS_{35}$, $MMAS_{37}$, and $MMAS_{40}$) were resistant to the trimethoprim-sulfonamide combination. All of the isolates were fully susceptible to nalidixic acid, ofloxacin, ciprofloxacin, rifampin, and chloramphenicol. Our findings and those reported over the last 10 years in Tunisia and other countries worldwide show the emergence of resistance to broad-spectrum cephalosporins in *Salmonella* isolates (1, 4, 7–11, 16, 18–23, 25, 27, 31, 33, 35, 41–43, 46–49). Such isolates are also often resistant to chloramphenicol and co-trimoxazole. Fluoroquinolones are the last family of antibiotics to remain active against these isolates, but they are not yet approved for use in children.

The transferability of broad-spectrum cephalosporin resistance by conjugation has been frequently observed among the *Enterobacteriaceae*. This phenomenon is incriminated in the diffusion of this resistance, which is generally encoded by large transferable plasmids. Resistance to β-lactams, aminoglycosides, and co-trimoxazole is readily transferable by conjugation to *E. coli*. A single large plasmid (molecular size of between 110 and 140 kb) was detected in each transconjugant (data not shown).

Characterization of broad-spectrum cephalosporin resistance. In IEF studies, all but one of the isolates ($MMAS_{40}$) produced a β-lactamase with a pI of 5.9. Three isolates

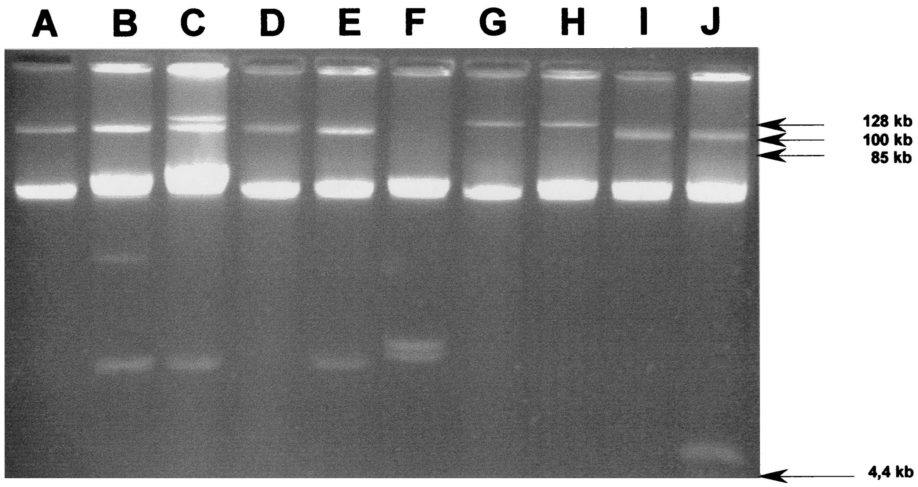

FIG. 2. Plasmid patterns of selected Tunisian clinical isolates of *Salmonella* serotype Mbandaka resistant to broad-spectrum cephalosporins. Lane A, isolate $MMAS_1$ (pattern P1); lane B, isolate $MMAS_2$ (pattern P2); lane C, isolate $MMAS_3$ (pattern P3); lane D, isolate $MMAS_5$ (pattern P1); lane E, isolate $MMAS_7$ (pattern P4); lane F, isolate $MMAS_{22}$ (pattern P7); lane G, isolate $MMAS_{30}$ (pattern P6); lane H, isolate $MMAS_{32}$ (pattern P6); lane I, isolate $MMAS_{39}$ (pattern P1); lane J, isolate $MMAS_{40}$ (pattern P8).

($MMAS_{31}$, $MMAS_{35}$, and $MMAS_{37}$) produced an additional β-lactamase with a pI of 7.6. $MMAS_{40}$ differed from the other isolates, producing a β-lactamase with a pI of 7.8 (data not shown) (Table 1). Similar results were obtained for the transconjugants.

bla_{TEM} amplification was obtained with primers OT3 and OT4 for all of the isolates and their transconjugants that produced a β-lactamase with a pI of 5.9 in IEF (i.e., except for $MMAS_{40}$). With primers OS5 and OS6, the bla_{SHV} gene was amplified only for $MMAS_{31}$, $MMAS_{35}$, and $MMAS_{37}$ and their transconjugants that produced an additional band of β-lactamase activity at pI 7.6. DNA sequencing of PCR products was performed only for clinical isolates. Analysis of deduced amino acid sequences showed that bla_{TEM} encoded a TEM-4 β-lactamase (pI 5.9) and that bla_{SHV} encoded an SHV-2a β-lactamase (pI 7.6). The β-lactam resistance patterns, together with the positive DDS test results for 30 of 31 isolates, suggested the presence of class A ESBLs (15). SHV-2, one of the most frequently described ESBLs worldwide (28, 38, 40), was previously described for *S. enterica* and notably for a nosocomial outbreak in Tunisia (25). On the other hand, this was the first reported identification of the TEM-4 ESBL in Tunisian *Salmonella* isolates. TEM-4 was first reported for *E. coli* in France (37) and subsequently for *E. coli* and *K. pneumoniae* in Spain (17). This enzyme was recently described for an isolate of *Salmonella* collected during a French national survey in 1998 (18); unfortunately, no information on the origin of this isolate was available. This report was also the second description of two different ESBLs in salmonellae (7). The last isolate, $MMAS_{40}$, had a particular β-lactam resistance phenotype—resistance to β-lactam–β-lactamase inhibitor combinations and susceptibility to cefepime—suggesting the presence of an AmpC-type enzyme (39). Susceptibility to cefoxitin and a pI of 7.8 suggested an ACC-type β-lactamase (34). This suggestion was confirmed by sequencing of the PCR product obtained with the ACC upper and ACC lower primers. Analysis of the deduced amino acid sequence showed the presence of the ACC-1a enzyme in this isolate. ACC-1 was first described in Germany and then in France during a multiresistant *K. pneumoniae* outbreak in an intensive care unit following the admission of a patient transferred from Sfax in Tunisia (34). A subsequent investigation in a Sfax hospital revealed the presence of the ACC-1 enzyme in many isolates of the *Enterobacteriaceae*, including *K. pneumoniae*, *Proteus mirabilis*, and *S. enterica* serotype Livingstone (43). All of these results suggest that the ACC-1 β-lactamase is probably widespread in Tunisia.

Epidemiological characteristics. We used PCR fingerprinting to evaluate the relatedness of the *Salmonella* serotype Mbandaka isolates. Primer ERIC2 was used to amplify the enterobacterial repeat intergenic consensus sequence, yielding three patterns, A, B, and C. One pattern (A) contained two subtypes, A1 and A2 (lanes C and I versus lanes A, B, D, F, G, and H in Fig. 1), which differed by only one band of about 1 kb. Fingerprinting with combined primers REP1R-Dt and REP2-Dt also generated three patterns (data not shown). These observations suggested that the isolates belonged to three clonal *Salmonella* populations (Table 1). One population predominated, comprising 26 isolates producing TEM-4 and 3 isolates producing both TEM-4 and SHV-2. The last two fingerprints each corresponded to one isolate ($MMAS_{22}$ and $MMAS_{40}$, producing TEM-4 and ACC-1, respectively).

The 31 *Salmonella* isolates yielded eight plasmid profiles (Table 1) comprising between one and three bands (Fig. 2). The estimated molecular sizes of the plasmids were between 5 and 140 kb. The most predominant profile (P1) was shared by 13 isolates and corresponded to a single large plasmid of about 110 kb. The second profile (P6) (only one plasmid of about 130 kb) was shared by six isolates. The third profile, P3, shared by four isolates, was characterized by two large plasmids of about 110 and 140 kb. Plasmid profiles P2, P4, and P5 were each represented by two isolates and differed from P1 by the additional presence of one or two small plasmids (less than 20 kb). Profile P7, which corresponded only to isolate $MMAS_{22}$, was unusual, being characterized by the absence of a large plasmid and the presence of two small plasmids. Profile P8 ($MMAS_{40}$) corresponded to the presence of a large plasmid of about 110 kb and a small plasmid of about 5 kb.

Epidemiological results based on PCR fingerprinting and plasmid analyses showed that 29 of the 31 isolates (with the exception of $MMAS_{22}$ and $MMAS_{40}$) belonged to the same clonal population, despite their different plasmid contents. The plasmid profiles of this clonal population distinguished at least three subclonal populations. The first corresponded to the predominant P1-like profiles (P1, P2, P4, and P5), characterized by a large conjugative plasmid (110 kb); the isolates yielding these profiles were recovered throughout the 5-year study period. Profile P3 corresponded to two isolates recovered in 1997. Profile P6 corresponded to six isolates recovered in 1999, three of which produced SHV-2. The last two isolates, $MMAS_{22}$ and $MMAS_{40}$, were unusual: no conjugative transfer was obtained with $MMAS_{22}$, which harbored only two small plasmids, and $MMAS_{40}$ was the only isolate to produce ACC-1.

In conclusion, our study confirms that *S. enterica* isolates are an important reservoir of genes encoding resistance to broad-spectrum cephalosporins in Tunisia. Most such genes encode ESBLs derived from TEM- or SHV-type β-lactamases (28, 38, 40). More recently, other enzymes were described; these belonged to Ambler class A (3), such as CTX-M-2 (10, 23, 26) (derived from the chromosome-encoded β-lactamase of *Kluyvera ascorbata*), CTX-M-3 (7), and PER-type enzymes (11, 46), or were derived from chromosomal class C β-lactamases, such as CMY-2 (19, 20, 31, 35, 47–49), DHA-1 (21), and ACC-1 (43), originating from *Citrobacter freundii*, *Morganella morganii*, and *Hafnia alvei*, respectively (9, 34, 39). These multiresistant isolates of *Salmonella* are often responsible for nosocomial outbreaks (23, 25, 42, 46) or for animal-to-human transmission (20, 48).

Salmonellae are ubiquitous, being found in aquatic environments, animals, and humans. Acquisition of new resistance genes by this genus could be facilitated by the simultaneous presence in the environment of naturally resistant *Enterobacteriaceae* or gram-negative bacilli and by traces of antimicrobial agents used in human and veterinary medicine. There is an increasingly urgent need to restrict the use of antimicrobial agents in animals (24) and humans alike and to control the disposal of these drugs in the environment.

ACKNOWLEDGMENTS

This work was partially financed by grants from the Ministère de la Recherche (Réseau Beta-Lactamase) and from the Faculté de Médecine Saint-Antoine.

We thank D. Sirot for the gift of *S. enterica* serotype Mbandaka CF1509.

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