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Clonal Emergence of Extended-Spectrum β-Lactamase (CTX-M-2)-Producing *Salmonella enterica* Serovar Virchow Isolates with Reduced Susceptibilities to Ciprofloxacin among Poultry and Humans in Belgium and France (2000 to 2003)

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Antibiotic treatment is not required in cases of *Salmonella enterica* gastroenteritis but is essential in cases of enteric fever or invasive salmonellosis or in immunocompromised patients. Although fluoroquinolones and extended-spectrum cephalosporins are the drugs of choice to treat invasive *Salmonella*, resistance to these antibiotics is increasing worldwide. During the period 2000 to 2003, 90 *Salmonella enterica* serovar Virchow poultry and poultry product isolates and 11 serovar Virchow human isolates were found to produce an extended-spectrum β-lactamase, CTX-M-2, concomitantly with a TEM-1 β-lactamase. The $bla_{\text{CTX-M-2}}$ gene was located on a large conjugative plasmid (>100 kb). Pulsed-field gel electrophoresis indicated a clonal relationship between the poultry and human isolates. All these isolates displayed additional resistance to trimethoprim-sulfamethoxazole and tetracycline as well as a reduced susceptibility to ciprofloxacin (MICs of between 0.5 and 1 μg/ml). CTX-M-2-producing *Salmonella* with a reduced susceptibility to fluoroquinolones constitutes a major concern, since such strains could disseminate on a large scale and jeopardize classical antibiotic therapy in immunocompromised patients.

Salmonellosis constitutes an important public health problem throughout the world. Nontyphoid *Salmonella* infections commonly cause self-limiting gastroenteritis, but severe infections, including bacteremia and meningitis, have also been reported (23). Antibiotic treatment is not required for straightforward *Salmonella* gastroenteritis but becomes essential to treat invasive salmonellosis (19, 28). Fluoroquinolones are the drugs of choice for an invasive extraintestinal infection in adults, whereas extended-spectrum cephalosporins (ESC) are preferentially used to treat salmonellosis in children (11). However, the use of these drugs is becoming compromised by the increasing development of ESC and quinolone resistance all over the world. Treatment failures due to in vivo acquisition of an extended-spectrum β-lactamase (ESBL) gene (31) or a reduced susceptibility to ciprofloxacin (1) in *Salmonella* are now well established.

Considering that the highest incidence of salmonellosis is among children <5 years old, resistance to ESC is of concern (39). *Salmonella* strains resistant to ESC have been reported since the late 1980s, and their numbers have increased ever since. Two major resistance mechanisms have been identified in *Salmonella*. In the first, the isolates express plasmid-mediated AmpC-like β-lactamases that hydrolyze the cephamycins, extended-spectrum cephalosporins, and monobactams. In the second mechanism, the *Salmonella* strains express an ESBL that is able to hydrolyze monobactams and oxyimino cephalosporins (such as cefotaxime and ceftazidime) but not the cephamycins (18). CTX-M β-lactamases (cefotaximases) are a relatively novel family of plasmid-mediated ESC and have been classified as Ambler class A. These enzymes generally possess higher levels of hydrolytic activity against cefotaxime than against ceftazidime and are inhibited by clavulanic acid, sulbactam, and tazobactam. They also provide resistance to cefepime. On the basis of their amino acid sequences, the CTX-M enzymes have been classified into five major phylogenetic branches (groups 1, 2, 8, 9, and 25) (4).

Here we report the emergence of ESBL (CTX-M-2)-producing *Salmonella enterica* serovar Virchow isolated in Belgium and in France from poultry, poultry products, and humans during the period 2000 to 2003. The ESBL-producing isolates were characterized by phage typing and pulsed-field gel electro-

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phoresis (PFGE), and the mechanisms of resistance to ESC were studied.

MATERIALS AND METHODS

Bacterial isolates. In Belgium, *Salmonella* isolates collected from human patients by peripheral clinical laboratories and isolates from meat and meat products (isolated by the official monitoring program of the Belgian Food Agency) are transferred to the National Reference Centre for *Salmonella* and *Shigella* for serotyping (approximately 10,000 to 12,000 strains/year). Isolates from poultry are collected in the framework of mandatory *Salmonella* monitoring programs, as described in European Directive 2003/99/EC, and are referred to the National Reference Laboratory for *Salmonella* in animals.

In France, the French National Reference Center for *Salmonella* receives human *Salmonella* isolates for serotyping through its network comprised of approximately 1,500 voluntary hospitals or private clinical laboratories, which together represent 30% of all French clinical laboratories. Approximately 12,000 strains are characterized per year through the French National Reference Center for *Salmonella*.

Serotyping and phage typing. Serotyping of *Salmonella* isolates was carried out by slide agglutination with commercial antisera by the Kauffmann-White scheme (26). Phage typing was performed according to the recommendations of the Health Protection Agency Service (Colindale, United Kingdom) (33).

Antimicrobial susceptibility testing. Antibiotic susceptibility was tested on a representative part of the population of *S. enterica* serovar Virchow isolated from humans between 2000 and 2003 (155 tested out of 570 isolated in Belgium and 190 tested out of 923 isolated in France). All strains isolated from poultry and poultry products were tested.

For the Belgian isolates, the susceptibility to 13 antibiotics was determined by the disk diffusion method (Kirby-Bauer) following recommendations of the CLSI (formerly NCCLS) (21). The antibiotics tested were ampicillin, amoxicillin plus clavulanic acid, cefotaxime, chloramphenicol, tetracycline, nalidixic acid, ciprofloxacin, streptomycin, kanamycin, gentamicin, sulfonamides, trimethoprim, and trimethoprim plus sulfamethoxazole. Bio-Rad (Nazareth, Belgium) disks were used for *Salmonella* isolated from humans, and Rosco tablets (International Medical, Brussels, Belgium) were used for *Salmonella* isolated from poultry products and poultry. Interpretation of inhibition zones was performed according to the CLSI criteria, and quality control was performed using the *Escherichia coli* ATCC 25922 reference strain.

Antimicrobial susceptibility testing for French isolates was performed as described previously (36) using French Antibiogram Committee guidelines.

Multidrug resistance was defined according to Enter-net (international surveillance network for enteric infections, http://www.hpa.org.uk/hpa/inter/index.htm) as resistance to four or more antimicrobials (34).

MIC determination. MICs were determined using Etest (AB Biodisk, Solna, Sweden) according to manufacturer's instructions and CSLI guidelines (20). The following antimicrobial agents were used: ampicillin, ticarcillin, ticarcillin-clavulanic acid, piperacillin, piperacillin-tazobactam, cefoxitin, cefotaxime, ceftazidime, cefepime, aztreonam, and ciprofloxacin. The ESBL phenotype was detected by using the ESBL detection Etest strip (E-testESBL; AB Biodisk, Solna, Sweden) and the double-disk synergy method.

Molecular characterization of β-lactamases. Total DNA was extracted using the InstaGene matrix kit (Bio-Rad) in accordance with the manufacturer's recommendations. CTX-M-specific PCR analysis was performed using forward primer CTX-M-F (5′-C(A/G)ATGTGCAG(C/T)ACCAGTAA-3′) and reverse primer CTX-M-R (5′-CGC (A/G)ATATC(A/G)TTGGTGGTG-3′), which amplify a 540-bp internal fragment of $bla_{\text{CTX-M}}$, as previously described (37). The PCR assay amplifying the entire coding sequence of $bla_{\text{CTX-M-2}}$ was performed using forward primer CTX-M-2-F (5′-ATGATGACTCAGAGCATTCG-3′) (located at positions 1 to 20 with respect to the CTX-M translational starting point) and reverse primer CTX-M-2-R (5′-TCAGAAACCGTGGGTTAC-3′) (located at positions 876 to 859). The forward primer TEM-F (5′-ATAAAATTCTTGAAGACGAAA-3′) and reverse primer TEM-R (5′-GACAGTTACCAATGCTTAATC-3′) were used for amplification of bla_{TEM} (37), and forward primer SHVfw (ATGCGTTATATTCGCCTGTG) and reverse primer SHVrev (TTAGCGTTGCCAGTGCTC) were used for amplification of bla_{SHV} (29). All PCR amplifications were carried out as previously described (37), except that the annealing temperature was 52°C for $bla_{\text{CTX-M}}$. The purified PCR fragments were sequenced on both strands by Genome Express (Meylan, France), using an ABI 3100 DNA sequencer (Applied Biosystems, Foster City, CA). The nucleotide sequence was analyzed with the Lasergene software (DNA Star, Madison, WI). The BLASTN program of NCBI (http://www.ncbi.nlm.nih.gov) was used for databases searches.

Preparation of crude extracts of β-lactamase and IEF. Crude extracts of β-lactamases from 10 CTX-M-2-producing strains (selected on the basis of the isolation year and their origin [human, poultry, or poultry product]) were obtained by sonication followed by 45 min of ultracentrifugation at 100,000 × *g* using a Beckman L8-55 M ultracentrifuge. Isoelectric focusing (IEF) was performed with a PhastSystem apparatus (Amersham-Pharmacia Biotech, Freiburg, Germany) as previously described (36).

Plasmid analysis. Plasmid transfer experiments were carried out in liquid medium with six selected isolates as previously described (37). These six isolates were selected on the basis of the isolation year and their origin. *E. coli* J5, which is resistant to rifampin, was used as the recipient strain. Transconjugants were selected on Drigalski agar supplemented with ceftiofur (10 mg/liter) and rifampin (250 mg/liter). Plasmid DNA was purified from *E. coli* transconjugants by an alkaline lysis procedure (32) and subjected to 0.8% agarose gel electrophoresis. DNA restriction fragment length polymorphisms were analyzed using agarose gel electrophoresis of plasmid DNA cleaved with EcoRI (Roche, Mannheim, Germany). Southern blotting using a $bla_{\text{CTX-M-2}}$ probe was performed as described previously (22).

PCR amplification and sequencing of QRDR regions of *gyrA*, *gyrB*, and *parC* genes. After amplification of a fragment of the *gyrA*, *gyrB*, and *parC* genes containing the quinolone resistance-determining region (QRDR) as previously described (12), the fragments were sequenced on a CEQ 2000 DNA sequencer (Beckman Coulter, High Wycombe, United Kingdom), using the DTSC-2 method. The sequences were compared and analyzed by Genestream software (developed by Institut de Génétique Humaine, Montpellier, France).

Pulsed-field gel electrophoresis. Genomic DNA suitable for PFGE was prepared according to the PulseNet method (www.cdc.gov/pulsenet) and digested with the restriction endonuclease XbaI (New England Biolabs, Leusden, Netherlands). *S. enterica* serovar Braenderup H9812 was used as a size marker. We have randomly selected cefotaxime-resistant isolates (33 among the 101 CTX-M-2-producing *S. enterica* serovar Virchow isolates) from different years and from different origins (poultry, poultry products, and animals). All human CTX-M-2-producing isolates (11) were included in the typing. Five other *S. enterica* serovar Virchow strains originating from national collections (cefotaxime susceptible or cefotaxime resistant [see Fig. 3]) were used in this study as comparison isolates (independent isolates). FingerprintingII Informatix software (Bio-Rad) was used to compare the PFGE profiles. The bands generated were analyzed by using the Dice coefficient and the unweighted-pair group method using average linkages, using a tolerance of 1%.

RESULTS

Epidemiological data. (i) Poultry flocks. In Belgium, the first ESC-resistant *S. enterica* serovar Virchow strains were isolated in 2000 from poultry (three strains, 2% of all serovar Virchow strains isolated that year). In 2001 and 2002, 11 (8% of all serovar Virchow strains isolated that year) and 9 (6.5% of all serovar Virchow strains isolated that year) *S. enterica* serovar Virchow strains that were resistant to cefotaxime were isolated, respectively. In 2003, a marked increase to 18% ESC resistance of all serovar Virchow strains isolated that year (45 out of 254 strains) was noted. The total number of serovar Virchow strains isolated from poultry also increased significantly during this period ($P < 0.05$). Over the period of 2000 to 2003, serovar Virchow was the most common *Salmonella* serovar isolated from poultry in Belgium. While in 1999 (when no ESC resistance was detected), a total of 76 serovar Virchow strains were isolated, 140, 138, 140, and 254 strains were isolated in the years 2000 to 2003, respectively (Fig. 1).

(ii) Food chain. Between 2002 and 2003, 22 cefotaxime-resistant *S. enterica* serovar Virchow strains were isolated from broiler filets at retail sites (5 strains), and from the skin and intestines of broilers at slaughterhouses (17 strains). The proportion of serovar Virchow in broiler meat also increased, from 7.3% of the total isolates in 1999 to 21% in 2003; the serovar Virchow strains became the most prevalent *Salmonella*

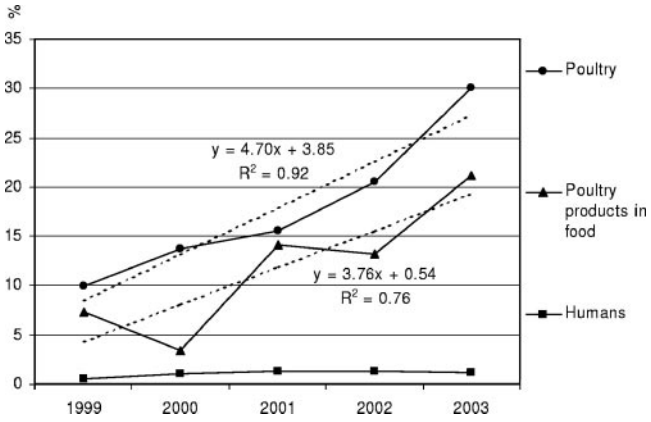

FIG. 1. Percentage of *S. enterica* serovar Virchow strains isolated in Belgium from humans, poultry, and poultry products between 1999 and 2003. The percentages of *S. enterica* serovar Virchow strains isolated from poultry and poultry products show a significant increase over the years ($P = 0.011$ for poultry and $P = 0.055$ for poultry products), following similar trends (slopes are not significantly different). The linear regression lines, equations, and r^2 coefficients are shown.

serovar in poultry meat, overtaking serovar Enteritidis (16% in 2003) and serovar Typhimurium (9% in 2003).

(iii) Humans. During the same period of time, 14 cephalosporin-resistant *S. enterica* serovar Virchow strains were isolated from stool samples of humans with gastroenteritis (two in 2001, three in 2002, and nine in 2003) in Belgium. In France, no ESC-resistant serovar Virchow strains of human origin were detected between 2000 and 2002, although 190 serovar Virchow strains were tested (Table 1). In 2003, three ESC-resistant strains were detected among the 100 serovar Virchow strains tested. These strains were recovered from three patients who suffered gastroenteritis but had no history of travel to Belgium. In both countries, the fraction of serovar Virchow among the total *Salmonella* isolated remained relatively stable in humans (approximately 1% in Belgium and 2% in France for the period 1999 to 2003) (Fig. 1). ESBL production was confirmed by double-disk synergy tests. While no data about the antibiotic regimens were available in Belgium, the three patients living in France were treated with ciprofloxacin or with extended-spectrum cephalosporins. All of the human cases arose sporadically in unrelated persons.

With the exception of the three human strains isolated in France, all of these ESBL-producing isolates were geographically clustered in northeast Belgium.

Antimicrobial susceptibility. In 2003, in Belgium, multidrug resistance to at least four unrelated antibiotics (35) was quite common among *S. enterica* serovar Virchow isolates (50% of human isolates) (Table 1); 63.6% of these isolates showed resistance to at least five antibiotics (ampicillin, tetracycline, trimethoprim, nalidixic acid, and sulfonamides) (Table 1). Nalidixic acid resistance in human strains doubled during the period 2000 to 2003 in Belgium (46% in 2000 and more than 84% in 2003).

Antimicrobial susceptibility was determined for all 104 ESBL-producing strains. All the isolates displayed coresistance to tetracycline, trimethoprim, nalidixic acid, and sulfonamides and showed a reduced susceptibility to ciprofloxacin (MICs of between 0.5 and 1 μg/ml).

The β-lactam susceptibility phenotype was determined for all the ESBL-producing strains. Table 2 shows characteristics of selected isolates. These strains were highly resistant to ampicillin (MICs of ≥256 μg/ml), cefotaxime (MICs of ≥256 μg/ml), cefepime (MICs of between 16 and 256 μg/ml), and to a lesser extent to aztreonam (MICs of between 16 and 64 μg/ml). The strains appeared to be partially susceptible or completely susceptible to ceftazidime (MICs of between 4 and 8 μg/ml) and susceptible to imipenem (MICs of between 0.25 and 0.5 μg/ml). The activities of piperacillin and, in some cases, of ticarcillin were partially restored by inhibitors such as tazobactam or clavulanic acid (data not shown). The synergy between cefotaxime and tazobactam or clavulanic acid confirmed the production of an ESBL. The higher levels of resistance to cefotaxime versus ceftazidime as well as the resistance to cefepime corresponded well with the resistance phenotype

TABLE 1. Distribution of antibiotic resistance phenotypes in *S. enterica* serovar Virchow strains isolated from humans in Belgium and France between 2000 and 2003

Phenotype[a]	No. (%) of resistant isolates							
	2000		2001		2002		2003	
	Belgium ($n = 13$)	France ($n = 50$)	Belgium ($n = 51$)	France ($n = 100$)	Belgium ($n = 47$)	France ($n = 40$)	Belgium ($n = 44$)	France ($n = 100$)
Susceptible	6 (46)	23 (46)	25 (49)	41 (41)	7 (15)	16 (40)	4 (9)	61 (61)
Nal	4 (31)	20 (40)	15 (29)	44 (44)	17 (36)	17 (42)	6 (14)	21 (21)
Amp Caz	1 (8)							
Amp Ctx Nal					1 (2)			
Amp Ctx Nal Tmp Sul Str					1 (2)			
Amp Ctx/Cro Nal Tmp Sul Tet			2 (4)		1 (2)		9 (20)	2 (2)
Amp Ctx/Cro Nal Tmp Sul Tet Str Kan								1 (1)
Other	2 (15)	7 (14)	8 (16)	15 (15)	20 (42)	7 (17)	25 (57)	15 (15)
Total	13 (100)	50 (100)	51 (100)	100 (100)	47 (100)	40 (100)	44 (100)	100 (100)

[a] Amp, ampicillin; Caz, ceftazidime; Ctx, cefotaxime; Cro, ceftriaxone; Nal, nalidixic acid; Str, streptomycin; Sul, sulfonamides; Tet, tetracycline; Tmp, trimethoprim; Kan, kanamycin. French Antibiogram Committee clinical breakpoints (in μg/ml; resistance and susceptibility, respectively): amoxicillin, >16 and ≤4; ceftazidime, >32 and ≤4; ceftriaxone, >32 and ≤4; nalidixic acid, >16 and ≤8; ciprofloxacin, >1 and ≤0.5; kanamycin, >16 and ≤8; gentamicin, >4 and ≤2; sulfonamides, >256 and ≤64; trimethoprim, >8 and ≤4; chloramphenicol, >16 and ≤8; tetracycline, >8 and ≤4.

TABLE 2. Susceptibilities, β-lactamase characterization, PFGE profiles, and phage types of selected *S. enterica* serovar Virchow isolates under study

Isolate	Yr	Origin/country	Etest MIC (mg/liter)[a]								Coresistance markers	PCR β-lactamase	β-lactamase pIs	Phage type	PFGE profile
			Amp	Pip	Pip + Tzb	Fox	Ctx	Caz	Caz + Ca	Fep	Imp	Cip					
1639-SA-00	2000	Poultry/Belgium	>256	>256	8	8	>256	8	1	32	0.25	1	Tet Nal Sul Tmp	bla_{TEM-1} $bla_{CTX-M-2}$	5.4, 7.9	PT37	Xb-Vir-1
01-2133	2001	Human/Belgium	>256	>256	6	8	>256	16	1	256	0.5	1	Nal Sul Tmp	bla_{TEM-1} $bla_{CTX-M-2}$	ND[b]	P31	Xb-Vir2
01-3494	2001	Human/Belgium	>256	>256	4	16	>256	8	1	32	0.5	1	Tet Nal Sul Tmp	bla_{TEM-1} $bla_{CTX-M-2}$	5.4, 7.9	PT37	Xb-Vir-1
142-SA-01	2001	Poultry/Belgium	>256	>256	8	8	>256	8	1	32	0.25	1	Tet Nal Sul Tmp	bla_{TEM-1} $bla_{CTX-M-2}$	5.4, 7.9	PT4	Xb-Vir-1
02-2928	2002	Human/Belgium	>256	>256	0.5	2	>256	8	1	>256	0.5	0.125	Tet Nal Sul Tmp	bla_{TEM-1} $bla_{CTX-M-2}$	5.4, 7.9	PT37	Xb-Vir-1
1642-SA-02	2002	Poultry/Belgium	>256	>256	8	8	>256	8	1	32	0.25	1	Tet Nal Sul Tmp	bla_{TEM-1} $bla_{CTX-M-2}$	ND	PT4	Xb-Vir-1
03-3832	2003	Human/France	>256	>256	8	4	>256	8	1	>32	0.25	0.5	Tet Nal Sul Tmp	bla_{TEM-1} $bla_{CTX-M-2}$	5.4, 7.9	PT4	Xb-Vir-1
03-5167	2003	Human/France	>256	>256	4	4	>256	8	0.5	32	0.25	0.5	Tet Nal Sul Tmp	bla_{TEM} $bla_{CTX-M-2}$	5.4, 7.9	PT4	Xb-Vir-1
03-664	2003	Human/Belgium	>256	>256	8	8	>256	8	0.5	>256	0.25	1	Tet Nal Sul Tmp	bla_{TEM-1} $bla_{CTX-M-2}$	5.4, 7.9	PT4	Xb-Vir-1
03-1333	2003	Human/Belgium	>256	>256	4	8	>256	4	0.5	>256	0.5	0.5	Tet Nal Sul Tmp	bla_{TEM-1} $bla_{CTX-M-2}$	ND	PT4	Xb-Vir-1
03-1436	2003	Human/Belgium	>256	>256	8	32	>256	8	0.5	32-256	0.25	0.5	Tet Nal Sul Tmp	bla_{TEM-1} $bla_{CTX-M-2}$	ND	PT4	Xb-Vir-1
03-11069	2003	Human/Belgium	>256	>256	8	8	>256	4	1	32	0.5	0.5	Tet Nal Sul Tmp	bla_{TEM-1} $bla_{CTX-M-2}$	ND	PT4	Xb-Vir-1
03-11111	2003	Human/Belgium	>256	>256	8	8	>256	8	1	>256	0.25	0.5	Tet Nal Sul Tmp	bla_{TEM-1} $bla_{CTX-M-2}$	ND	PT4	Xb-Vir-1
03-13397	2003	Human/Belgium	>256	>256	4	8	>256	4	1	16	0.25	1	Tet Nal Sul Tmp	bla_{TEM-1} $bla_{CTX-M-2}$	ND	PT4	Xb-Vir-1
03-1902	2003	Poultry product/ Belgium	>256	>256	4	4	>256	8	1	>256	0.5	1	Str Tet Nal Sul Tmp	bla_{TEM-1} $bla_{CTX-M-2}$	5.4, 7.9	PT4	Xb-Vir-1
03-12787	2003	Poultry/Belgium	>256	>256	8	8	>256	8	1	32	0.25	1	Tet Nal Sul Tmp	bla_{TEM-1} $bla_{CTX-M-2}$	5.4, 7.9	PT4	Xb-Vir-1
518-SA-03	2003	Poultry/Belgium	>256	>256	8	8	>256	8	1	32	0.25	1	Tet Nal Sul Tmp	bla_{TEM-1} $2bla_{CTX-M-2}$	5.4, 7.9	PT4	Xb-Vir-4

[a] Amp, ampicillin; Pip, piperacillin; Pip + Tzb, piperacillin plus tazobactam; Fox, cefoxitin; Ctx, cefotaxime; Caz, ceftazidime; Caz + Ca, ceftazidime plus clavulanic acid; Fep, cefepime; Imp, imipenem; Cip, ciprofloxacin.
[b] ND, not determined.

conferred by the CTX-M-type ESBLs in *Salmonella* strains as previously described (24).

All the ESBL-producing isolates also displayed a reduced susceptibility to ciprofloxacin (34) (MICs of between 0.5 and 1 μg/ml).

Determination of resistance genes for β-lactams and quinolone resistance mutations. In order to determine the nature of the β-lactamases produced, PCR amplifications specific for β-lactamase genes of the TEM, SHV, and CTX-M families were performed. TEM and CTX-M consensus PCR assays gave the expected PCR fragments (respectively, 1,080 bp and 540 bp) for all isolates tested. All SHV amplifications were negative. PCR product sequence analysis of 101 *S. enterica* serovar Virchow strains of the 104 isolates revealed 100% identity with the bla_{TEM-1} and $bla_{CTX-M-2}$ sequences (GenBank accession numbers AF427132 and AJ427469). Specific CTX-M-2 gene amplifications also confirmed the sequence analysis (data not shown). Two human Belgian isolates from 2002 harbored the bla_{TEM-52} or $bla_{CTX-M-3}$ gene, and one strain isolated in France in 2003 harbored the $bla_{CTX-M-9}$ gene. We further characterized only the CTX-M-2-producing strains.

To characterize the molecular basis of the reduced susceptibility to ciprofloxacin of the ESBL-producing strains, the QRDRs of the *gyrA*, *gyrB*, and *parC* genes of 15 representative isolates of different origins were subjected to PCR amplification and subsequent sequencing. For all of the isolates tested, only one mutation, leading to a Ser83-to-Phe substitution (TCC→TTC), was identified in *gyrA*.

IEF. IEF revealed the production of β-lactamases with isoelectric points of 5.4 and 7.9 in all isolates (Table 2), which confirmed that two enzymes, one of the TEM family (pI 5.4) and one of the CTX-M family (pI 7.9), might be responsible for the phenotypes.

Transfer of resistance and plasmid analysis. Resistance transfer experiments were carried out in liquid medium with *E. coli* J5 (Rif^r) as the recipient strain. *E. coli* transconjugants were obtained for all six selected *S. enterica* serovar Virchow isolates (142SA01, 1639SA00, 02-2928, 03-1902, 03-664, and 03-5167) of animal or human origin. All other resistance determinants, except nalidixic acid resistance, were cotransferred with the cefotaxime resistance determinant. Plasmid extraction from all transconjugants revealed a high-molecular-weight plasmid (>100 kb) (data not shown). EcoRI restriction analysis showed that all conjugative plasmids had very similar fingerprints (Fig. 2). Southern blot hybridization with a $bla_{CTX-M-2}$ probe confirmed the presence of the cefotaxime resistance gene on an approximately 6-kb EcoRI fragment (Fig. 2).

Phage typing. While most of the ESBL-producing strains were of phage type 4, several were of the closely related phage type 37, and one strain isolated from a human in 2001 was of phage type 31 (Table 2).

PFGE. XbaI macrorestriction and PFGE were used to determine the genetic relatedness of the cefotaxime-resistant *S. enterica* serovar Virchow isolates (Fig. 3A and B). Three distinct profiles were observed among the 33 cefotaxime-resistant isolates tested (Fig. 3B). Thirty-one of these isolates displayed the same restriction fragment profiles (X1), confirming the relatedness among the cefotaxime-resistant isolates. The second profile (differing by four bands) was represented by only one strain, isolated from a broiler in 2003 (X4). The third

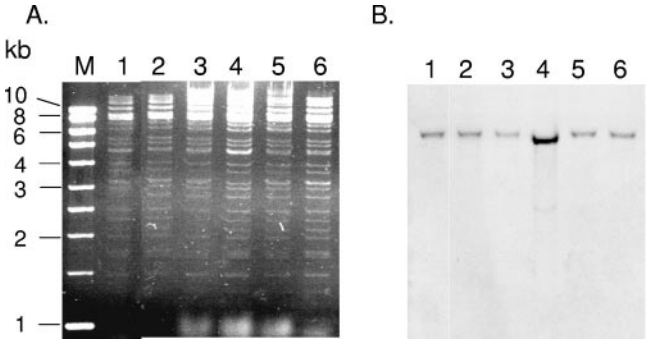

FIG. 2. Restriction analysis (EcoRI) (A) and Southern blot hybridization with a $bla_{CTX-M-2}$ probe (B) of plasmid DNAs isolated from *E. coli* transconjugants. Lanes M, markers (SmartLadder; Eurogentec, Seraing, Belgium); 1, *E. coli* transconjugant p142SA01-1; 2, *E. coli* transconjugant p1639SA00-1; 3, *E. coli* transconjugant p02-2928-1; 4, *E. coli* transconjugant p03-1902-1; 5, *E. coli* transconjugant p03-664-1; 6, *E. coli* transconjugant p03-5167-1. All lanes are from the same gel.

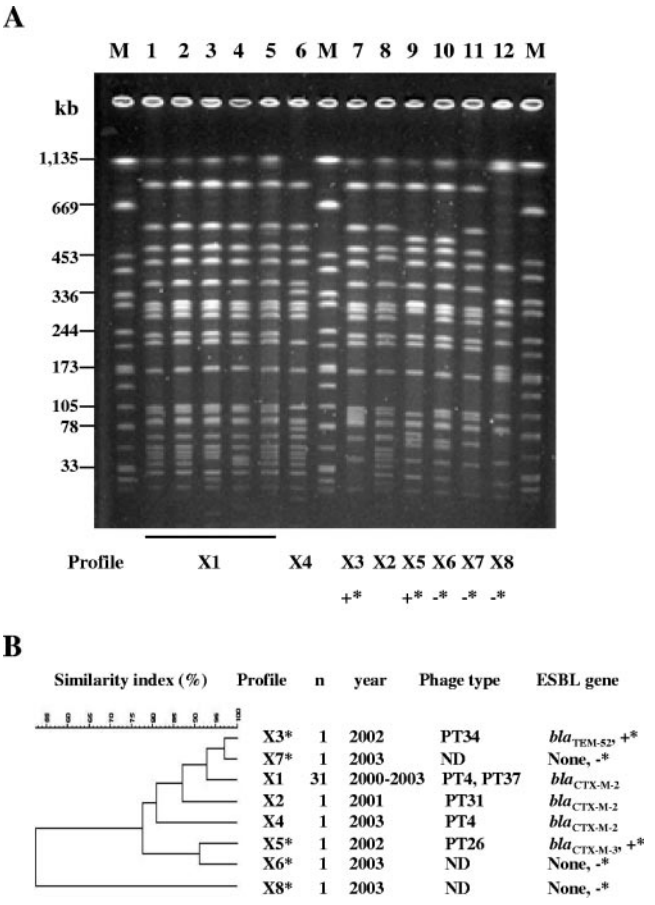

FIG. 3. (A) Representative XbaI PFGE patterns observed among CTX-M-2-producing *S. enterica* serovar Virchow isolates and among serovar Virchow isolates used as comparison isolates. Lane M, XbaI-digested DNA from *S. enterica* serovar Braenderup H9812, used as molecular size marker; lane 1, isolate 03-3832; lane 2, isolate 03-5167; lane 3, isolate 03-12787; lane 4, isolate 02-2928; lane 5, isolate 03-664; lane 6, isolate 518SA03; lane 7, comparison isolate 02-4352 (B/2002/H/TEM-52 ESBL, +*); lane 8, isolate 01-2133; lane 9, comparison isolate 02-4366 (B/2002/H/CTX-M-3 ESBL, +*); lane 10, comparison isolate 03-1324 (F/2003/H/nalidixic acid resistant, −*); lane 11, comparison isolate 03-3111 (F/2003/H/nalidixic acid and ampicillin resistant, −*); lane 12, comparison isolate 03-4256 (F/2003/H/pansusceptible, −*). F, France; B, Belgium; V, veterinary isolate; H, human isolate. −*, comparison isolate susceptible to cefotaxime; +*, comparison isolate resistant to cefotaxime. (B) Dendrogram generated by FingerprintingII Informatix software, showing the results of cluster analysis on the basis of PFGE fingerprinting. Similarity analysis was performed using the Dice coefficient, and clustering was by the unweighted-pair group method using average linkages. The different PFGE profiles (asterisks indicate profiles of comparison isolates), numbers of isolates, years of isolation, types of *bla* genes, and phage types are indicated. ND, not determined.

profile was observed in one human strain in 2001 (X2). The comparison isolates displayed profiles totally different from those shown by the CTX-M-2-producing isolates.

DISCUSSION

The present study documented the emergence of ESC-resistant *S. enterica* serovar Virchow strains with decreased susceptibility to ciprofloxacin in poultry flocks, poultry meat, and humans. The first ESBL (CTX-M-2)-producing resistant serovar Virchow isolate was detected in 2000 in Belgium in poultry. This clone might have been selected in poultry flocks, subsequently reaching the human population in 2001. This led the National Reference Centre for *Salmonella* and *Shigella* in 2003 to alert the national sanitary authority. Consequently, all Belgian hospitals and clinics were apprised of the emergence of such strains and informed about the possible problems for antibiotic treatment in cases of invasive salmonellosis with *S. enterica* serovar Virchow or *Salmonella* of group O7 (formerly C1).

All ESC-resistant human strains from Belgium caused self-limiting gastroenteritis. The two patients living in Paris were treated first unsuccessfully with extended-spectrum cephalosporins, followed by successful treatment with ciprofloxacin. With the exception of two strains isolated around Paris, all the ESBL (CTX-M-2)-producing strains were geographically clustered in northeast Belgium.

In this study, we report the clonal spreading of a strain carrying a large plasmid that harbored all resistance genes detected in the isolates with the exception of nalidixic acid resistance. During the last decade, the role of conjugative plasmids in the wide dissemination of antibiotic resistance genes has been increasingly reported in the literature (7, 13, 16).

While the origin of the first CTX-M-2-producing isolate remains unknown, the chronology of isolation of the resistant strains argues for a selection in poultry flocks and subsequent dissemination in humans via the food chain. Few studies have shown this way of spread of ESBL-producing *S. enterica* strains (25). Most commonly, the reports describe nosocomial outbreaks due to ESBLs in the genus *Salmonella* (14). The selection in poultry flocks might be attributable to the use of antimicrobial agents in these animals (5).

In Belgium, ceftiofur was approved in April 1990 for use by injection in 1-day-old chicks to prevent septicemia caused by *E. coli*. However, since maximum levels for ceftiofur residues in poultry meat were not established, the drug was withdrawn from the Belgian market on 1 January 2000. Presently, two ESC are still approved as therapeutic agents for systemic use in food-producing animals in Belgium: cefquinome (in 1998) and

ceftiofur (in 1997), which are approved for use in cattle and pigs but not for use in poultry.

The emergence of *Salmonella* isolates that are resistant to ESC has already been reported worldwide. This resistance is frequently attributed to the production of various plasmid-encoded extended-spectrum β-lactamases, including the TEM, SHV, PER, and CTX-M enzymes (24, 30). In the present study, the ESC-resistant isolates produced CTX-M-2 ESBL enzymes. In Belgium, two other *S. enterica* serovar Virchow strains (producing, respectively, TEM-52 and CTX-M-3 enzymes) were also isolated in humans during this period. In France in 2003, one and eight CTX-M-9-producing *S. enterica* serovar Virchow strains were isolated from a human and from poultry, respectively (37).

CTX-M β-lactamases usually confer high-level resistance to aminopenicillins (e.g., ampicillin or amoxicillin), carboxypenicillins (e.g., ticarcillin), ureidopenicillins (e.g., piperacillin), and narrow-spectrum cephalosporin. The susceptibilities of bacteria to 7-α-methoxy cephalosporins (cefoxitin) and carbapenems (e.g., imipenem), however, are unchanged (4). This group of enzymes is one of the most frequently encountered ESBL types in *Salmonella* spp. (15). The CTX-M-2 β-lactamase was first identified in *S. enterica* serovar Typhimurium in Buenos Aires, Argentina, in 1990 (3). More recently, 34 cefotaxime-resistant *S. enterica* serovar Typhimurium strains were identified in seven regions of Russia and Belarus (10). Their resistance to ESC was attributed to the production of CTX-M-4- and CTX-M-5-like β-lactamases, both belonging to the CTX-M-2 family (24). In 2002, CTX-M enzymes belonging to groups 1, 2, and 9 also emerged in human *E. coli* strains in Belgium (27).

In addition to ESBL production, all isolates displayed a decreased susceptibility to ciprofloxacin (MIC of between 0.5 and 1 μg/ml). This resistance was associated with one substitution in the GyrA protein at Ser83, which is the most frequently observed in *Salmonella* (9, 34). In members of the *Enterobacteriaceae*, resistance to quinolones is generally acquired in a two-step mutation process. A first mutation in the QRDR of the *gyrA* gene mediates full resistance to narrow-spectrum quinolones such as nalidixic acid and decreased susceptibility to fluoroquinolones. This is why the measurement of susceptibility to nalidixic acid is used as an indicator for the detection of low-level resistance to ciprofloxacin. A second mutation in one of the *gyrA*, *parC*, or *gyrB* genes mediates full resistance to fluoroquinolones (6). Another important mechanism of decreased susceptibility to fluoroquinolones may be active efflux due to overproduction of the AcrAB efflux pump. This mechanism was shown to play a major role in the high-level fluoroquinolone resistance of *S. enterica* serovar Typhimurium DT204 strains carrying multiple target gene mutations in *gyrA*, *gyrB*, and *parC* (2). Because the isolates in this study did not show full resistance to ciprofloxacin (MICs of between 0.5 and 1 μg/ml), the existence of an efflux mechanism was not investigated.

At the present time, ciprofloxacin is one of the most potent antimicrobials available for medical treatment of invasive gastrointestinal infections in adults (8). Even a decreased susceptibility to fluoroquinolones has become a major problem in medical practice. For example, Aarestrup et al. (1) reported several treatment failures in patients infected by *Salmonella* isolates due to a decreased susceptibility to fluoroquinolones (1). Generally, fluoroquinolones are not recommended to treat salmonellosis in young children due to the drugs' potential secondary effects on cartilage development. Recently, however, these drugs have been used successfully without secondary effects in both immunocompromised children and children suffering from multidrug-resistant gram-negative bacterial infections (including neonatal infections and multidrug-resistant enteric infections caused by *Salmonella* and *Shigella* spp.) (17). This result could be of importance for treatment of bacteremia due to ESBL-producing *S. enterica* serovar Virchow in children. The tendency of serovar Virchow to invade the bloodstream and to cause systemic disease in previously healthy children and infants was recently reported in the United Kingdom, Spain, Greece, and Australia (38); however, the reason for this phenomenon remains unclear. Because of the propensity of this serovar to cause invasive disease, resistance to therapeutically important antibiotics is of particular concern.

In conclusion, we have documented that *S. enterica* serovar Virchow producing CTX-M-2 ESBL was identified first in poultry flocks, then in poultry meat, and subsequently in humans in Belgium (and France) over the time period 2000 to 2003. The chronology of isolation of this strain suggests that these bacteria were transmitted to humans via the food chain, specifically by poultry meat. The appearance of decreased susceptibility to fluoroquinolones and resistance to extended-spectrum cephalosporins in animal, food, and human *Salmonella* isolates constitutes a major concern, since such strains could disseminate on a large scale and jeopardize classical antibiotic therapy. An efficient and vigilant surveillance system for antimicrobial drug resistance, especially in animal strains, is therefore essential to detect any trend in the short and long terms.

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