Extended-Spectrum Cephalosporin-Resistant Salmonella enterica serovar Heidelberg Strains, the Netherlands

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Extended-spectrum cephalosporin-resistant Salmonella enterica serovar Heidelberg strains (JF6X01.0022/XbaI.0251, JF6X01.0326/XbaI.1966, JF6X01.0258/XbaI.1968, and JF6X01.0045/XbaI.1970) have been identified in the United States with pulsed-field gel electrophoresis. Our examination of isolates showed introduction of these strains in the Netherlands and highlight the need for active surveillance and intervention strategies by public health organizations.

Salmonella enterica serovar Heidelberg is among the most prevalent causes of human salmonellosis in the United States and Canada but has been reported infrequently in Europe (1–3). Although most nontyphoidal Salmonella infections are self-limiting and resolve within a few days, Salmonella ser. Heidelberg tends to provoke invasive infections (e.g., myocarditis and bacteremia) that require antimicrobial drug therapy (4). To treat systemic nontyphoidal Salmonella infections, third-generation cephalosporins are preferred drugs for children or for adults with fluoroquinolone contraindications (5). Resistance to third-generation cephalosporins is increasing in S. enterica infections, mainly because of production of plasmid-mediated extended-spectrum or AmpC β-lactamases (6).

Resistance to extended-spectrum cephalosporins (ESCs) among Salmonella Heidelberg strains found in human infections, food-producing animals, and poultry meat indicates zoonotic and foodborne transmission of these strains and potential effects on public health (7,8). Unlike in Canada and the United States, few ESC-resistant Salmonella Heidelberg strains have been documented in Europe (9–13). However, increased occurrence of ESC resistance in S. enterica infections and decreased susceptibility to fluoroquinolones compromise the use of these drugs and constitute a serious public health threat (6,14).

Few data are available regarding prevalence of ESC-resistant Salmonella Heidelberg isolates in Europe, their underlying antimicrobial drug resistance gene content, and genetic platforms (i.e., plasmids and insertion sequence [IS] elements) associated with resistance genes. We attempted to determine the occurrence and molecular characteristics of Salmonella Heidelberg isolates recovered from human patients, food-producing animals, and poultry meat in the Netherlands during 1999–2013.

The Study

During 1999–2013, the Netherlands National Institute of Public Health and the Environment collected 437 Salmonella Heidelberg isolates from human infections (n = 77 [17.6%]), food-producing animals (n = 138 [31.6%]), poultry meat (n = 170 [38.9%]), and other sources (n = 52 [11.9%]). From this collection, we selected 200 epidemiologically unrelated isolates for further analysis (Table; online Technical Appendix, http://wwwnc.cdc.gov/EID/article/22/7/15-1377-Techapp.pdf).

MICs for antimicrobial agents were determined with the broth microdilution method (online Technical Appendix) and showed a higher frequency of multidrug non–wild-type susceptibility phenotype in isolates from poultry meat (n = 44 [68.8%]) than in isolates from food-producing animals (n = 14 [31.8%]) and human infections (n = 16 [19.5%]). Most human infections exhibited wild-type MICs to most antimicrobial agents tested (Table).

Of the 200 Salmonella Heidelberg isolates in the study, 47 (23.5%) were ESC resistant. ESC resistance in Salmonella Heidelberg isolates increased from 33.3% in 2011 to 60.0% in 2012 to 75.0% in 2013, after which Salmonella Heidelberg was the predominant serotype in ESC-resistant Salmonella isolates in the Netherlands (Figure 1).

These isolates showed MICs for cefotaxime and ceftazidime of 2 to >4 mg/L and 4 to >16 mg/L, respectively; non–wild-type susceptibility to fluoroquinolones was 87.2%. The emergence of isolates with decreased
susceptibility to these first-line antimicrobial drugs limits effective treatment options for potential human infections.

ESC typing of the 47 isolates, performed by microarray analysis followed by PCR and sequencing (online Technical Appendix), revealed the presence of the \( bla_{CMY-2} \) gene in 41 ESC-resistant Salmonella Heidelberg isolates that exhibited an AmpC \( \beta \)-lactamase phenotype. The other 6 isolates exhibited an extended-spectrum \( \beta \)-lactamase phenotype and encoded \( bla_{CTX-M-2} \) \( (n = 4) \), \( bla_{CTX-M-1} \) \( (n = 1) \), or \( bla_{CTX-M-14} \) \( (n = 1) \) genes (Figure 2).

We assessed the genetic relatedness of the 47 cephalosporin-resistant Salmonella Heidelberg isolates by using the standardized XbaI–pulsed-field gel electrophoresis (PFGE) (online Technical Appendix), which identified 2 major PFGE types: XbaI.1968 and XbaI.1973 (PFGE numbers assigned by the European Centre for Disease Prevention and Control, Solna, Sweden). Of the 47 isolates, 26 (55.3%) belonged to XbaI.1968 and 5 (10.6%) belonged to XbaI.1973. Forty-one of the isolates were \( bla_{CMY-2} \) carriers, 31 (75.6%) of which belonged to these 2 PFGE types; 10 (24.4%) were distributed equally among other PFGE types.

### Table. Characteristics of Salmonella enterica serovar Heidelberg isolates recovered from human infections, food-producing animals, poultry meat, and other sources, the Netherlands, 1999–2013

| Source                      | 1999–2001 | 2002–2004 | 2005–2007 | 2008–2010 | 2011–2013 |
|-----------------------------|-----------|-----------|-----------|-----------|-----------|
| Human infections            |           |           |           |           |           |
| No. isolates studied        | 13        | 10        | 22        | 23        | 15        |
| Resistance phenotypes (no.) | Amp (1),  | AmpSmxStr (1), | AmpSmxTmpNalCip (1), | AmpFotTazStr (1), | Col (1),  |
|                             | AmpCol (1), | AmpTetSmx (1), | AmpTet (1), | AmpFotTazStr (1), | Col (10), |
|                             | AmpSmxNal (1), | SMX (3), Str (1), | NalCip (2), | SMX (1), | Str (5), |
|                             | AmpFotTaz (1), | TetSmxNal (1), | Tet (1), | TetSmxNalCip (1), | TetCol (1), |
|                             | AmpSmxNalCip (1), | WT (3), | WT (14), | TetSmxNalCip (1), | TetSmxTmp |
|                             | Str (1), | Str (5), | Str (1), | Str (1), | Str (8) |
|                             | WT (2)    | WT (3)    | WT (5)    | WT (1)    | WT (8)    |
| No. ESCR isolates           | 0         | 0         | 1         | 0         | 0         |
| Food-producing animals      |           |           |           |           |           |
| No. isolates studied        | 5         | 16        | 5         | 7         | 13        |
| Resistance phenotypes (no.) | NalCip (1), | Amp (3),  | AmpSmxTmpNalCip (1), | AmpFotTazNalCip (1), | AmpFotTaz |
|                             | WT (4)    | AmpSmxTmpNalCip (1), | AmpFotTazNalCip (1), | AmpFotTazNalCip (1), | Col (4)   |
|                             |           | AmpStr (2), | AmpFotTazNalCip (1), | AmpFotTazNalCip (1), | Col (2),  |
|                             |           | NalCip (5), | AmpFotTazNalCip (1), | AmpFotTazNalCip (1), | TetSmxNal |
|                             |           | SMX (1), Str (1), | AmpFotTazNalCip (1), | AmpFotTazNalCip (1), | CipStrKan |
|                             |           | WT (3)    | AmpFotTazNalCip (1), | AmpFotTazNalCip (1), | Col (1),  |
|                             |           |           | AmpFotTazNalCip (1), | AmpFotTazNalCip (1), | Col (2),  |
|                             |           |           | AmpFotTazNalCip (1), | AmpFotTazNalCip (1), | TetSmxCip |
|                             |           |           | AmpFotTazNalCip (1), | AmpFotTazNalCip (1), | StrKan (1), |
|                             |           |           | AmpFotTazNalCip (1), | AmpFotTazNalCip (1), | WT (2)    |
| No. ESCR isolates           | 0         | 0         | 0         | 2         | 5         |
| Poultry meat                |           |           |           |           |           |
| No. isolates studied        | 3         | 3         | 15        | 6         | 40        |
| Resistance phenotypes (no.) | AmpTetSmxTmpNalCip (1), | AmpSmxSm (1), | AmpFotTazSmxTmpNalCip (1), | AmpFotTazSmxTmpNalCip (1), | AmpFotTaz |
|                             | SmxSm (1), | SMX (1), | SmxSm (1), | SmxSm (1), | SmxSm (1) |
|                             | WT (1)    | WT (2)    | WT (8)    | WT (1)    | WT (8)    |
|                             |           |           |           |           |           |
| No. ESCR isolates           | 0         | 0         | 0         | 3         | 35        |
| Other                       |           |           |           |           |           |
| No. isolates studied        | 0         | 1         | 0         | 6         | 4         |
| Resistance phenotypes (no.) | WT (1)    | Str (1), StrCol (2) | Col (2), NalCip (1), | Col (1), Str (1), StrCol (2) | NalCip (1), |
|                             |           |           |           |           |           |
| No. ESCR isolates           | 0         | 0         | 0         | 0         | 1         |

*Amp, ampicillin; Cip, ciprofloxacin; Chl, chloramphenicol; Col, colistin; ESCR, extended-spectrum cephalosporin-resistant; Fot, cefotaxime; Gen, gentamicin; Kan, kanamycin; Nal, nalidixic acid; Smx, sulfamethoxazole; Str, streptomycin; Taz, ceftazidime; Tet, tetracycline; Tmp, trimethoprim; WT, wild type.
introduction of 4 epidemic clones of ESC-resistant *Salmonella* Heidelberg strains in the Netherlands (JF6X01.0022/\* XbaI.0251, JF6X01.0326/XbaI.1966, JF6X01.0258/\* XbaI.1968, and JF6X01.0045/XbaI.1970). To raise awareness and determine whether related ESC-resistant *Salmonella* Heidelberg isolates had been observed in other European countries, the Epidemic Intelligence Information System (European Centre for Disease Prevention and Control) issued an alert on September 18, 2014.

We successfully transferred plasmids carrying extended-spectrum or AmpC β-lactamases from ESC-resistant *Salmonella* Heidelberg isolates to the recipient *E. coli* DH10B strain (online Technical Appendix). PCR-based Inc/Rep typing and multilocus or double-locus sequence typing (ST) of the plasmids revealed that the *bla*\_CMY-2 or *bla*\_CTX-M genes were located on plasmids for 46 (97.8%) of the 47 isolates. ESC-resistant *Salmonella* Heidelberg isolates encoding *bla*\_CMY-2 on IncI1/ST12 plasmids were associated predominantly with the XbaI.1968 (n = 26 [78.8%]) PFGE type; those encoding *bla*\_CMY-2 on IncA/C plasmids were associated with XbaI.1973 (n = 5 [71.4%]). Isolates encoding *bla*\_CTX-M-2 on IncHII2P/ST2, *bla*\_CTX-M-1 on IncI1/ST49, and *bla*\_CTX-M-12 on IncI1/ST80 plasmids were associated with XbaI.1964, XbaI.1963, and XbaI.1966, respectively (Figure 2).

The *bla*\_CMY-2 gene was present in 12 different PFGE types and was carried on plasmids of 2 different incompatibility groups (IncI1/ST12 and IncA/C) or on the chromosome. This gene’s diverse genetic background suggests that emergence of the *bla*\_CMY-2-producing *Salmonella* Heidelberg strain in the Netherlands results not only from expansion of a single clone but from multiclonal dissemination of the strain and horizontal transfer of plasmids encoding the *bla*\_CMY-2 gene. IncI1/ST12 and IncA/C plasmids have been associated with the *bla*\_CMY-2 gene in *Salmonella* Heidelberg isolates in the United States and Canada (8,15).

We analyzed a subset of ESC-resistant *Salmonella* Heidelberg isolates to determine the size and conjugation frequency of plasmids carrying extended-spectrum and AmpC β-lactamases. We also assessed a subset of *Salmonella* Heidelberg isolates (n = 17) for each PFGE type, including isolates for each type if they showed variation in extended-spectrum and AmpC β-lactamase genes or in gene location. This assessment sought to detect the upstream presence of resistance genes (*bla*\_CTX-M and *bla*\_CMY) of frequently encountered insertion sequences (ISEcp1, ISCR1, and IS26) (Figure 2; online Technical Appendix).

We attribute the increase of ESC-resistant *Salmonella* Heidelberg isolates in the Netherlands to the frequent occurrence of isolates carrying IncI1/ST12 plasmids encoding *bla*\_CMY-2 in food-producing animals and poultry products imported from Brazil. Isolates from imported poultry products are associated predominantly with PFGE types XbaI.1968 and XbaI.1973 (Figure 2). A similar introduction of ESC-resistant *Salmonella* Heidelberg strains in Ireland was associated with imported poultry meat from Brazil (R. Slowey, pers. comm.). Although ESC-resistant *Salmonella* Heidelberg strains are rarely reported in Europe, their introduction through imported poultry meat could pose a public health risk; Brazil is among the world’s leading countries for exporting poultry meat.

**Conclusions**

Most ESC-resistant *Salmonella* Heidelberg isolates in our study had profiles (XbaI.0251, XbaI.1966, XbaI.1968, and XbaI.1970) indistinguishable from those of previous epidemic types (JF6X01.0022, JF6X01.0326, JF6X01.0258, and JF6X01.0045) that caused outbreaks and showed potential for bloodstream infections (16). Our identification of clonal clusters shared by ESC-resistant *Salmonella* Heidelberg strains in food-producing animals or poultry meat that can cause human infections underscores the risk for potential zoonotic or foodborne transmission of these strains to humans.

Although we observed a frequent occurrence of ESC-resistant *Salmonella* Heidelberg isolates in poultry products, no human infections linked to these contaminated products have been yet documented in the Netherlands. Nevertheless, the risk of potential zoonotic or foodborne transmission of ESC-resistant *Salmonella* Heidelberg strains highlights the necessity for active surveillance and intervention strategies by public health organizations.

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References
1. European Centre for Disease Prevention and Control. Technical document. EU protocol for harmonised monitoring of antimicrobial resistance in human Salmonella and Campylobacter isolates. Stockholm: The Centre; 2014.
2. Public Health Agency of Canada. National Enteric Surveillance Program (NESP). Annual summary 2012. Ottawa (Ontario, CA): The Agency; 2014.
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3. Centers for Disease Control and Prevention. National Antimicrobial Resistance Monitoring System: enteric bacteria. 2012 Human isolates final report. Atlanta: The Centers; 2014.

4. Hoffmann M, Zhao S, Petten ioutil J, Luo Y, Monday SR, Abbott J, et al. Comparative genomic analysis and virulence differences in closely related Salmonella enterica serotype Heidelberg isolates from humans, retail meats, and animals. Genome Biol Evol. 2014;6:1046–68. http://dx.doi.org/10.1093/gbe/evu079

5. Achesson D, Hohmann EL. Nontyphoidal salmonellosis. Clin Infect Dis. 2001;32:263–9. http://dx.doi.org/10.1086/318457

6. Miriagou V, Tassios PT, Legakis NJ, Tzouvelekis LS. Expanded-spectrum cephalosporin resistance in non-typhoid Salmonella. Int J Antimicrob Agents. 2004;23:547–55. http://dx.doi.org/10.1016/j.ijantimicag.2004.03.006

7. Dutil L, Irwin R, Finley R, Ng LK, Avery B, Boerlin P, et al. Cef tiofur resistance in Salmonella enterica serovar Heidelberg from chicken meat and humans, Canada. Emerg Infect Dis. 2010;16:48–54. http://dx.doi.org/10.3201/eid1601.1009729

8. Folster JP, Pecic G, Singh A, Duval B, Rickert R, Ayers S, et al. Characterization of extended-spectrum cephalosporin-resistant Salmonella enterica serovar Heidelberg isolated from food animals, retail meat, and humans in the United States 2009. Foodborne Pathog Dis. 2012;9:638–43. http://dx.doi.org/10.1089/fpd.2012.1130

9. Aarestrup FM, Hasman H, Olsen I, Sorensen G. International spread of bla<sub>CTX-M-15</sub>-mediated cephalosporin resistance in a multiresistant Salmonella enterica serovar Heidelberg isolate stemming from the importation of a boar by Denmark from Canada. Antimicrob Agents Chemother. 2004;48:1916–7. http://dx.doi.org/10.1128/AAC.48.5.1916-1917.2004

10. Miriagou V, Filip R, Coman G, Tzouvelekis LS. Expanded-spectrum cephalosporin-resistant Salmonella strains in Romania. J Clin Microbiol. 2002;40:4334–6. http://dx.doi.org/10.1128/JCM.40.11.4334-4336.2002

11. González-Sanz R, Herrera-León S, de la Fuente M, Arroyo M, Echeita MA. Emergence of extended-spectrum β-lactamases and AmpC-type β-lactamases in human Salmonella isolated in Spain from 2001 to 2005. J Antimicrob Chemother. 2009;64:1181–6. http://dx.doi.org/10.1093/jac/dkp361

12. Batchelor M, Hopkins KL, Threlfall EJ, Clifton-Hadley FA, Stallwood AD, Davies RH, et al. Characterization of AmpC-mediated resistance in clinical Salmonella isolates recovered from humans during the period 1992 to 2003 in England and Wales. J Clin Microbiol. 2005;43:2261–5. http://dx.doi.org/10.1128/JCM.43.5.2261-2265.2005

13. Burke L, Hopkins KL, Meunier D, de Pinna E, Fitzgerald-Hughes D, Humphreys H, et al. Resistance to third-generation cephalosporins in human non-typhoidal Salmonella enterica isolates from England and Wales, 2010–12. J Antimicrob Chemother. 2014;69:977–81. http://dx.doi.org/10.1093/jac/dkt469

14. Piddock LJ. Fluoroquinolone resistance in Salmonella serovars isolated from humans and food animals. FEMS Microbiol Rev. 2002;26:3–16. http://dx.doi.org/10.1111/j.1574-6976.2002.tb00596.x

15. Andrysiak AK, Olson AB, Tracz DM, Dore K, Irwin R, Ng LK, et al.; Canadian Integrated Program for Antimicrobial Resistance Surveillance Collaborative. Genetic characterization of clinical and agri-food isolates of multi drug resistant Salmonella enterica serovar Heidelberg from Canada. BMC Microbiol. 2008;8:89. http://dx.doi.org/10.1186/1471-2180-8-89

16. Centers for Disease Control and Prevention. Investigation update: multistate outbreak of human Salmonella Heidelberg infections linked to “kosher broiled chicken livers” from Schreiber Processing Corporation. 2012 Jan 11 [cited 2015 Jul 23]. http://www.cdc.gov/salmonella/2011/chicken-liver-1-11-2012.html

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Technical Appendix

Materials and Methods

Bacterial Strains and Identification

During 1999–2013, the Dutch National Institute of Public Health (RIVM) collected 30,472 *Salmonella* isolates from various surveillance programs on patients with salmonellosis and from farms, slaughterhouses, and retail markets. The isolates originated from human infections (n = 17,363), food-producing animals (n = 6,136), poultry meat (n = 1,260) and other sources (n = 5,713). Using micronitration, RIVM performed serotyping based on somatic (O) and flagellar (H) antigens according to the latest version of the White-Kaufmann-Le Minor scheme (1). Recovered *Salmonella* isolates were stored at −80°C in Peptone Broth supplemented with 30% (v/v) glycerol for further analysis.

We selected 200 isolates from the 437 *Salmonella enterica* serovar Heidelberg isolates received at RIVM during 1999–2013. Only the first isolate per patient was included, and to avoid epidemiologically clustered isolates in the selection, only 1 isolate was included per sample type (i.e., human infection, food-producing animal, poultry meat, or others) and origin (i.e., hospital, institute, laboratory, farm, company, or surveillance program) per 14-day period.

Antimicrobial Susceptibility Testing

The susceptibility of the isolates to antimicrobial agents was assessed by broth microdilution, as described by the International Standard Organization (standard 20776–1:2006), by using microtiter trays with a custom-designed, dehydrated panel of antimicrobial drugs (EUMVS, Sensititre, Thermo Fischer, Basingstoke, UK). The antimicrobial agents tested included ampicillin, cefotaxime, ceftazidime, ciprofloxacin, chloramphenicol, colistin, florfenicol, gentamicin, kanamycin, nalidixic acid, streptomycin, sulfamethoxazole, trimethoprim, and tetracycline. *Escherichia coli* strain ATCC 25922 and *Enterococcus*
*faecalis* strain ATCC 29212 were used as quality controls. For interpretation, we used epidemiologic cutoff values recommended by the European Committee on Antimicrobial Susceptibility Testing (http://mic.eucast.org). Multidrug non–wild-type phenotype was defined as non–wild-type MICs to ≥1 antimicrobial agents from ≥3 antimicrobial classes. Production of extended-spectrum or AmpC β-lactamases was evaluated by a combined disc test that used discs of cefotaxime and ceftazidime with (30/10 μg) and without clavulanic acid (30 μg) and a disc of cefoxitin (30 μg) for all isolates; this process satisfied the phenotypic criteria indicative of extended-spectrum cephalosporinase production, as recommended by the European Centre for Disease Prevention Control (2).

**Characterization of Resistance Determinants**

We assessed the presence of genes conferring the extended-spectrum cephalosporinase-resistant phenotype. DNA was extracted by using the DNeasy Blood and Tissue kit (QIAGEN, Hilden, Germany) according to the manufacturer’s recommendations. All isolates putatively producing extended-spectrum or AmpC β-lactamases were screened for a broad spectrum of extended-spectrum and AmpC β-lactamase gene families by using the Check-MDR CT-101 array platform (Check-Points, Wageningen, the Netherlands) according to the manufacturer’s recommendations. The presence of extended-spectrum or AmpC β-lactamase genes was confirmed by PCR and subsequent sequencing as described (3). The nucleotide and deduced amino acid sequences were compared with sequences in the Lahey clinic database (http://www.lahey.org/Studies).

**Clonal Analysis**

All isolates carrying extended-spectrum or AmpC β-lactamases (n = 47) and isolates randomly selected on the basis of year and source of isolation (n = 64) were analyzed for genetic relatedness by pulsed-field gel electrophoresis (PFGE) of *XbaI*-digested genomic DNA by using a CHEF DR-III apparatus (Bio-Rad Laboratories, Hercules, CA, USA), according to the standardized protocol of PulseNet (4). *XbaI*-digested genomic DNA from *S. enterica* ser. Braenderup strain H9812 was used as a molecular reference marker (5). Image normalization and construction of similarity matrices were carried out by using BioNumerics, version 6.6 (Applied Maths, Sint-Martens-Latem, Belgium). Bands were assigned manually, and dendrograms were generated by employing the Unweighted Pair Group Method with Arithmetic mean based on the Dice similarity index, by using 1% optimization and 1% band tolerance as position tolerance settings.
PFGE fingerprints of the isolates were submitted to The European Surveillance System molecular surveillance service of the European Centre for Disease Prevention and Control database, which assigned pattern names. PFGE fingerprints were subsequently compared with those from the PulseNet database. An alert was issued on September 18, 2014, through the European Epidemic Intelligence Information System for the Food and Waterborne Diseases and Zoonoses network to raise awareness and determine whether related extended-spectrum cephalosporinase-resistant *S. enterica* ser. Heidelberg isolates had been observed in other member countries of the European Epidemic Intelligence Information System.

**Plasmid Analysis**

The replicon types were characterized for all plasmids carrying extended-spectrum or AmpC β-lactamases. Purified plasmid DNA was transformed into DH10B cells by electroporation (Invitrogen, Van Allen Way, CA USA) under the following conditions: 1.25 kV/cm, 200 Ω, 25 μFar (6). Transformants were selected on Luria-Bertani agar plates supplemented with cefotaxime (1 mg/L). PCR-based replicon typing was conducted on the transformants to determine the replicon type of the plasmid by using the PBRT KIT—PCR-based replicon typing (DIATHEVA, Fano, Italy); plasmid multilocus or double-locus sequence typing (pMLST or pDLST) were used to further subtype IncI1 and IncHI2 plasmids as previously described (7,8). A subset of transformants (n = 16) was selected according to PFGE profile of the parental strain, replicon type of the plasmid, and antimicrobial-resistance determinant. These plasmids were subjected to S1-PFGE for accurate determination of molecular sizes (9). If no transformants were obtained, the chromosomal location of the extended-spectrum or AmpC β-lactamase genes was confirmed by I-CeuI PFGE of total bacterial DNA, followed by Southern blot hybridization, as described (10).

**Conjugation Experiments**

The transferability of the extended-spectrum cephalosporinase-resistant phenotype by conjugation was assessed for the subset of *S. enterica* ser. Heidelberg isolates described above. Plasmid-free rifampin-resistant *E. coli* E3110 was used as a recipient strain for liquid-mating assays in a ratio of 1:1. Filter-mating assays were attempted for strains for which no transconjugants were obtained by liquid mating. For both liquid- and filter-mating assays, the donor and recipient strains in mid-exponential phase were co-incubated for 4 hours without agitation at 37°C. Transconjugants were selected on MacConkey agar supplemented with a combination of rifampin (100 mg/L) and cefotaxime (1 mg/L). Positive transconjugants were
confirmed by PCR amplification for the resistance determinant. All mating assays were conducted in triplicate. The conjugation frequency was calculated as the number of transconjugants per donor cell.

**Analysis of Regions Upstream of Resistance Determinants**

The association of \( \text{bla} \text{CTX-M} \) and \( \text{bla} \text{CMY} \) genes with frequently encountered insertion sequences (ISEcp1, ISCR1, and IS26) was assessed for a subset of \( S. \text{enterica} \) ser. Heidelberg isolates (\( n = 17 \)) representing each unique PFGE profile and the variation in extended-spectrum or AmpC \( \beta \)-lactamase gene type and its location (chromosome or plasmid replicon type). This association was investigated with PCR by using forward primers specific for ISEcp1, ISCR1, or IS26 and a reverse primer for \( \text{bla} \text{CTX-M} \) or \( \text{bla} \text{CMY} \) genes, as described (11). Subsequently, sequence analysis confirmed the amplicons obtained.

**References**

1. Grimont P, Weil F. Antigenic formulae of the \( \text{Salmonella} \) serovars. Paris: Pasteur Institute; 2007.
2. European Centre for Disease Prevention and Control. EU protocol for harmonised monitoring of antimicrobial resistance in human \( \text{Salmonella} \) and \( \text{Campylobacter} \) isolates. Stockholm: The Centre; 2014.
3. Dierikx CM, van Duijkeren E, Schoormans AH, van Essen-Zandbergen A, Veldman K, Kant A, et al. Occurrence and characteristics of extended-spectrum-\( \beta \)-lactamase- and AmpC-producing clinical isolates derived from companion animals and horses. J Antimicrob Chemother. 2012;67:1368–74. http://dx.doi.org/10.1093/jac/dks049
4. Ribot EM, Fair MA, Gautom R, Cameron DN, Hunter SB, Swaminathan B, et al. Standardization of pulsed-field gel electrophoresis protocols for the subtyping of \( \text{Escherichia coli} \) O157:H7, \( \text{Salmonella} \), and \( \text{Shigella} \) for PulseNet. Foodborne Pathog Dis. 2006;3:59–67. http://dx.doi.org/10.1089/fpd.2006.3.59
5. Hunter SB, Vauterin P, Lambert-Fair MA, Van Duyne MS, Kubota K, Graves L, et al. Establishment of a universal size standard strain for use with the PulseNet standardized pulsed-field gel electrophoresis protocols: converting the national databases to the new size standard. J Clin Microbiol. 2005;43:1045–50. http://dx.doi.org/10.1128/JCM.43.3.1045-1050.2005
6. Hordijk J, Wagenaar JA, Kant A, van Essen-Zandbergen A, Dierikx C, Veldman K, et al. Cross-sectional study on prevalence and molecular characteristics of plasmid mediated \( \text{ESBL/AmpC}- \)producing \( \text{Escherichia coli} \) isolated from veal calves at slaughter. PLoS One. 2013;8:e65681. http://dx.doi.org/10.1371/journal.pone.0065681
7. García-Fernández A, Chiaretto G, Bertini A, Villa L, Fortini D, Ricci A, et al. Multilocus sequence typing of IncI1 plasmids carrying extended-spectrum beta-lactamases in *Escherichia coli* and *Salmonella* of human and animal origin. J Antimicrob Chemother. 2008;61:1229–33. http://dx.doi.org/10.1093/jac/dkn131

8. García-Fernández A, Carattoli A. Plasmid double locus sequence typing for IncHI2 plasmids, a subtyping scheme for the characterization of IncHI2 plasmids carrying extended-spectrum beta-lactamase and quinolone resistance genes. J Antimicrob Chemother. 2010;65:1155–61. http://dx.doi.org/10.1093/jac/dkq101

9. Barton BM, Harding GP, Zuccarelli AJ. A general method for detecting and sizing large plasmids. Anal Biochem. 1995;226:235–40. http://dx.doi.org/10.1006/abio.1995.1220

10. Liu SL, Hessel A, Sanderson KE. Genomic mapping with I-Ceu I, an intron-encoded endonuclease specific for genes for ribosomal RNA, in *Salmonella* spp., *Escherichia coli*, and other bacteria. Proc Natl Acad Sci U S A. 1993;90:6874–8. http://dx.doi.org/10.1073/pnas.90.14.6874

11. Kiiru J, Butaye P, Goddeeris BM, Kariuki S. Analysis for prevalence and physical linkages amongst integrons, ISEcp1, ISCR1, Tn21 and Tn7 encountered in *Escherichia coli* strains from hospitalized and non-hospitalized patients in Kenya during a 19-year period (1992-2011). BMC Microbiol. 2013;13:109. http://dx.doi.org/10.1186/1471-2180-13-109