The Belgian National Reference Centre for Salmonella received 16,544 human isolates of Salmonella enterica between January 2009 and December 2013. Although 377 different serotypes were identified, the landscape is dominated by S. enterica serovars Typhimurium (55%) and Enteritidis (19%) in a ratio which is inverse to European Union averages. With outbreaks of Salmonella enterica serotypes Ohio, Stanley, and Paratyphi B variant Java as prime examples, 20 serotypes displayed significant fluctuations in this 5-year period. Typhoid strains account for 1.2% of Belgian salmonellosis cases. Large-scale antibiotic susceptibility analyses (n = 4,561; panel of 12 antibiotics) showed declining resistance levels in S. Enteritis and Typhimurium isolates for 8 and 3 tested agents, respectively. Despite low overall resistance to ciprofloxacin (4.4%) and ceftoxime (1.6%), we identified clonal lineages of Salmonella enterica serotypes Kentucky and Infantis displaying rising resistance against these clinically important drugs. Quinolone resistance is mainly mediated by serotype-specific mutations in GyrA residues Ser83 and Asp87 (92.2% not wild type), while an additional ParC_Ser801le mutation leads to ciprofloxacin resistance in 95.5% S. Kentucky isolates, which exceeds European averages. Plasmid-mediated quinolone resistance (PMQR) alleles qnrA1 (n = 1), qnrS (n = 9), qnrD1 (n = 4), and qnrB (n = 4) were found in only 3.0% of 533 isolates resistant to nalidixic acid. In ceftoxime-resistant isolates, we identified a broad range of Ambler class A and C β-lactamase genes (e.g., blα_SHV-12, blα_TEM-52, blαCTX-M-14, and blαCTX-M-15) commonly associated with members of the family Enterobacteriaceae. In conclusion, resistance to fluoroquinolones and ceftoxime remains rare in human S. enterica, but clonal resistant serotypes arise, and continued (inter)national surveillance is mandatory to understand the origin and routes of dissemination thereof.

Although there has been a steady decrease in the number of cases of human salmonellosis in the European Union, it remains the second most frequently reported zoonosis (20.4 confirmed cases per population of 100,000) and the most frequent cause of foodborne outbreaks (1). Nontyphoid salmonellosis is usually self-limiting and normally resolves without the need of antimicrobial agents, but life-threatening invasive infections may occur in vulnerable patients (2). The causative agent, Salmonella enterica subsp. enterica, is subdivided into 1,531 serovars based on antigenic differences in the lipopolysaccharide O antigen and two flagellin structures (3). These serotypes differ greatly in their natural reservoirs, their ability to provoke infections, and their resistance to antimicrobials (4, 5). The severity of inflicted disease also differs substantially among serotypes, with case fatality rates ranging over 100-fold and proportions of hospitalizations varying between 14 and 67% for nontyphoidal strains (6). Typhoidal Salmonella enterica serotypes Typhi and Paratyphi A and B are notorious pathogens, restricted to humans and causing 13.5 million annual episodes of typhoid fever, especially in low- and middle-income countries (7). In developed countries, outbreaks of typhoid fever account for <1% of all infections and are mainly associated with travel (8).

Invasive Salmonella infections mandate the need for chemotherapy. The increasing rates of resistance against traditional agents (aminopenicillins, trimethoprim-sulfamethoxazole, and chloramphenicol) caused a shift to fluoroquinolones (FQ) and third-generation cephalosporins (CSP) in empirical treatment (9, 10). In Europe, average resistance rates against ciprofloxacin (FQ) and ceftoxime (CSP) remain relatively low for S. enterica at <6% and <2%, respectively (1). However, a plethora of studies report high-level FQ and CSP resistance emerging in different parts of the world (11–15), and global travel and food trade increase the likelihood of acquiring infection from nondomestic sources. As such, it is crucial to closely monitor trends in resistance and the prevalence of mobile and non-mobile genetic determinants underlying resistance to these first-line drugs.

Genetic determinants of resistance against FQ and CSP are well documented. FQ resistance (FQR) is primarily associated with accumulation of chromosomal mutations in the targeted bacterial enzymes, i.e., DNA gyrase A and B (GyrA and GyrB) and DNA topoisomerase IV (ParC). Additional low-level resistance can be acquired by plasmid-encoded proteins (plasmid-mediated quinolone resistance [PMQR]) including Qnr-type proteins which protect the DNA gyrase from quinolones, the Aac(6’)-Ib-cr acetyltransferase, and OqxAB and QepA efflux pumps that extrude quinolones (16, 17). Resistance against CSP (CSPR) is predominantly associated with plasmid-borne β-lactamases, the most prevalent belonging to the Ambler class A extended-spectrum β-lactamases (ESBLs) of the CTX-M, TEM, and SHV families.
lies and the acquired class C cephalosporinases (AmpC) (18). These ESBLs are capable of hydrolyzing penicillins, cephalosporins (except cephamycins), and monobactams and are inhibited by clavulanic acid or cloxacillin (19). Although resistance to carbapenems, often considered the antibiotic of last resort (20), is extremely rare in S. enterica, isolates expressing acquired carbapenemases have been reported (21-23).

Here, we describe the serotype distribution and antimicrobial resistance patterns of all human Salmonella isolates sent to the Belgian Reference Centre for Salmonella (NRCS) between 2009 and 2013. By unraveling resistance determinants in isolates displaying reduced susceptibility to FQ and CSP, we compiled a blueprint of the current resistance landscape in Belgian human Salmonella isolates, which at the moment remains only marginally influenced by plasmid-encoded mechanisms.

MATERIALS AND METHODS

Bacterial strains, serotyping, and antimicrobial susceptibility testing. In Belgium, peripheral clinical laboratories collect Salmonella isolates from human patients and send them voluntarily to the Belgian Reference Centre for Salmonella (NRCS) for serotyping. The full collection of 16,544 S. enterica strains which were received between January 2009 and December 2013 was subjected to serotyping by slide agglutination with commercial antisera, according to the Kauffman-White scheme (24). A subset of 4,561 S. enterica strains (for selection criteria, see Table S1 in the supplemental material) was subjected to antimicrobial resistance typing using the disk diffusion (Kirby-Bauer) method and Mueller-Hinton agar as culture medium. They represent the most prevalent serological forms and the dualistic coefficient and no priority rules for the algorithm with BioNumerics 6.5 (Applied Maths).

For less common serotypes, clonal relatedness was determined by XbaI macrorestriction followed by pulsed-field gel electrophoresis (PFGE). The resulting fragments were interpreted according to the PulseNet protocol (37) using the BioNumerics software package. The dendrogram was constructed based on Dice coefficient of similarity and UPGMA algorithm with 1.5% band position tolerance.

RESULTS AND DISCUSSION

Serotype distribution. In the 5-year period spanning this study, the Belgian Reference Centre for Salmonella (NRCS) received 16,544 human S. enterica strains mainly isolated from feces (94.4%), blood (2.4%), and urine (1.5%) samples. The number of annually submitted strains remained fairly constant, varying between 3,182 and 3,668 isolates (Fig. 1A). In this vast collection, we identified 377 different serotypes (see Table S3 in the supplemental material). The landscape is clearly dominated by Salmonella typhimurium and Enteritidis which were retrieved in approximately 54.2% and 19.2% of all samples, respectively (Fig. 1B; see Table S3 in the supplemental material). Chi-square analysis (1) contrasts to the ratio of other European Union (EU)/European Economic Area (EEA) countries where Salmonella Typhimurium and Enteritis account for 25% and 44% of all reported serotypes, respectively (1). An explanation of this discrepancy can be found in the national vaccination program in layer flocks at the beginning of the millennium, which caused a drastic reduction in S. Typhimurium (2012) being the most significant (Table S3). The outbreak study (Fig. 1A). S. Typhimurium has a well-characterized ability to infect various species (38) and can survive for a long time in the environment (39), enhancing its ability to be one of the most common causes of salmonellosis. The observed ratio for Salmonella serotypes Typhimurium and Enteritidis (3-1) contrasts to the ratio in other European Union (EU)/European Economic Area (EEA) countries where Salmonella Typhimurium and Enteritis account for 25% and 44% of all reported serotypes, respectively (1). An explanation of this discrepancy can be found in the national vaccination program in layer flocks at the beginning of the millennium, which caused a drastic reduction in S. Enteritidis (40).

Other nontyphoid Salmonella strains are far less commonly encountered and account for a maximum of 2.1% isolates/year (Fig. 1B; see Table S3 in the supplemental material). Chi-square trend analysis (including Bonferroni’s correction) showed significant fluctuations in 20 serovars, with outbreaks of Salmonella serovars Ohio (2009), Paratyphi B dT+ (2009 to 2010), and Manhattan (2012) being the most significant (Table S3). The outbreak of Salmonella serotype Stanley in 2012 was traced back to Hungary by joint European Centre for Disease Prevention and Control (ECDC) efforts (41). Also notable is the stable, low prevalence of Salmonella serotype Newport (annually between 0.49 and 0.80%), which is comparable to the European average (1) but 10-fold lower than the global average (42, 43).
In the past 5 years, on average 1.23% of all Belgian salmonellosis cases involved typhoid infections (Fig. 1C). The large majority of cases involved Salmonella serotype Typhi. In 2013, only 0.09% of human Belgian Salmonella isolates were positively identified as Salmonella serotypes Paratyphi A and B.

**Serotype-dependent patterns of antimicrobial resistance.** Based on selection criteria formulated in Table S1 in the supplemental material, the susceptibility of 4,561 Salmonella isolates against a panel of 12 antibiotics was determined (Fig. 2). We found that 67.0% of all isolates were resistant against at least one antibiotic, while 32.4% were multidrug resistant (MDR). In total, 4.4% and 1.6% of all Salmonella strains are resistant to ciprofloxacin and oxyimino-CSPs cefotaxime and ceftazidime, respectively (Tables S4 and S5), which is comparable to recently published European data (i.e., 5.1 and 1.1%) (1). One must take into account that this might be an underestimation of the actual degree of FQR, as it now established that 5 μg ciprofloxacin will not reliably detect low-level resistance in Salmonella spp., justifying the switch to pefloxacin disks in routine screens from 2014 onwards (25).

Of the 2,435 S. Typhimurium strains tested, the majority displayed resistance against ampicillin (75.6%), streptomycin (55.2%), tetracycline (53.2%), and sulfonamides (60.1%), which is linked to the presence of Salmonella genomic island 1 (44). Although this particular phenotype remained stable during the last 5 years, resistance to nalidixic acid (NalR), trimethoprim-sulfamethoxazole, and sulfonamides declined significantly in this period (P < 0.01; Fig. 2). Clonal relatedness of 75 random isolates was investigated using 5-loci MLVA. We detected 44 different profiles clustering in 10 subgroups, with conservation of resistance profiles within each group although some exceptions are noticed (see Fig. S1A in the supplemental material). S. Enteritidis isolates are generally more susceptible to antibiotics (4) and show declining levels of resistance to 8/12 tested agents (Fig. 2). S. Dublin, one of the most invasive serotypes, generally displays resistance against only streptomycin and chloramphenicol. However, in 2013, a MDR isolate (S13BD03130) displaying resistance to cefotaxime was identified.

More disturbing trends appear in three specific serotypes. First, S. Hadar isolates are highly resistant to tetracycline, streptomycin, aminopenicillins, and nalidixic acid but remain sensitive to fluoroquinolones (FQ) and cephalosporins (CSP) (Fig. 2). Second, S. Infantis displays an unusually high incidence in clinical resistance against third-generation CSP, increasing from 0% in 2009 to 12.2% in 2013. Fifteen isolates revealed nine XbaI PFGE profiles (85.5% minimal similarity), with clear clustering of the CSP-resistant isolates in three specific profiles (see Fig. S1B in the supplemental material). Third, FQR in S. Kentucky isolates reaches 95.5% (Fig. 2), which is higher than the European average of 73.4% (45). Moreover, a clear trend toward complete resistance to aminopenicillins, tetracycline, aminoglycosides, and co-trimoxazole is observed in the 2009-2013 period, leaving third-generation CSP as the drug of choice (Fig. 2). Notably, one S. Kentucky isolate (11-4366) was pan-resistant against the entire panel of tested antibiotics.

All Belgian typhoid Salmonella isolates remain susceptible to CSP. S. Typhi isolates display above average resistance to pycrine synthesis inhibitors, while NalR is encountered in the majority of both S. Typhi (58/112) and Paratyphi A (27/32) isolates. Moreover, 25% of the S. Paratyphi A strains show intermediate ciprofloxacin resistance, which might complicate treatment outcome (8).

**Molecular mechanisms of FQ resistance.** Since it requires less chromosomal substitutions in the quinolone resistance-determining regions (QRDR) of gyrA, gyrB, and parC to establish NalR (46), this phenotype is widely used as indicator for upcoming FQR. Therefore, we screened NalR isolates in our collection (representing 16.4% of the 4,561 strains) for mutations in the QRDR of GyrA, GyrB, and ParC. Since it requires less chromosomal substitutions in the quinolone resistance-determining regions (QRDR) of gyrA, gyrB, and parC to establish NalR (46), this phenotype is widely used as indicator for upcoming FQR. Therefore, we screened NalR isolates in our collection (representing 16.4% of the 4,561 strains) for mutations in the QRDR of GyrA, GyrB, and ParC. First, we observed that not a single Salmonella NalR strain has a mutated GyrB protein, but a minority (8.1%) have wild-type GyrA (Fig. 3). Contrasting Asian studies reporting a plethora of mutations in both GyrA and ParC (47), the two main mutational GyrA hot spots in Belgian isolates are clearly Ser83 and Asp87, each displaying serotype-specific patterns. For example,
FIG 2 Antimicrobial resistance in human salmonellosis in Belgium, 2009 to 2013. Antibiotics are grouped according to class. The graphs show percentage of intermediate (gray) and full (black) resistance against the antibiotic in question per serotype (n) and per year. Linear trend analyses were performed using the STATCALC X2 module with the extended Mantel-Haenszel method (EpiInfo; CDC, Atlanta, GA). Significantly (P < 0.01) increasing and decreasing susceptibilities are indicated by red and green shading, respectively. Chloramph., chloramphenicol; Trimeth./Sulfameth., trimethoprim-sulfamethoxazole.
the Asp87Asn mutation dominates in S. serotype Hadar, while Ser83Phe is typical for S. serotype Paratyphi A. Notably, 50% of the S. Typhi GyrA proteins carry the Glu133Gly mutation not found in any other serovar (Fig. 3), shedding further doubt on which amino acid confines the wild-type GyrA_133 residue in S. Typhi (48). This mutation is often found in combination with a mutated Ser83 residue, but additional mutations in ParC are necessary to confine full FQ resistance, exemplified by S. Typhi isolate 09-02031 (see Table S4 in the supplemental material).

Many Salmonella serovars (including Newport, Kentucky, Infantis, and Paratyphi A) carry the Thr57Ser mutation in ParC, but this mutation has been shown not to be implicated in FQ resistance (49). Apart from this mutation, only 3.0% of the non-Kentucky strains carry a mutated ParC_Ser80 residue, which was solely found in S. Hadar (5.5%), Typhi (11.9%), and Typhimurium (10.7%) strains (Fig. 3). The FQ R phenotype of the Kentucky isolates can be explained by a triple QRDR mutation (GyrA_Ser83/Asp87 and ParC_Ser80) found in no less than 97.4% of the NalR isolates. The recently reported dissemination of this serotype in the European continent (1) has clearly reached Belgium, with poultry as the most likely vehicle for infection (22). Its clonal spread is reflected in the XbaI PFGE profiles of 7 randomly chosen isolates, which are >92% identical (see Fig. S1C in the supplemental material).

Next, we also screened 533 NalR isolates for the presence of PMQR alleles, finding positive hits in only 16 (3.0%) strains, which is comparable to other recent surveys (47, 49). As such, NalR might not effectively detect plasmid-mediated mechanisms. Dominant alleles were qnrS1 (n = 8), qnrD1 (n = 4), and qnrB (n = 4) (variations of qnrB), while qnrS2 and qnrA1 were identified once (see Table S4 in the supplemental material). Notably, statistical analysis showed that within our data set, qnr alleles are more prevalent among strain carrying wild-type QRDR (\( \chi^2 = 33.5; \text{df} = 1; P < 0.05 \)). Prime examples are strains 12-2428 and 12-2447, which are the sole S. Kentucky isolates with wild-type QRDR, yet carry either one (QnrD1) or two (QnrB1 and QnrD1) PMQR alleles. In this particular case, the additional presence of QnrB1 led to a 4-fold increase in MIC.

Resistance against \( \beta \)-lactam antibiotics. For 48 strains which fell below the respective EUCAST thresholds for CSP resistance, we set up a confirmatory ESBL screen using a combination disk diffusion test using 10 \( \mu \)g clavulanic acid (CLA). In all but six cases, addition of the \( \beta \)-lactamase inhibitor restored the susceptibility to the tested CSP (Fig. 4), phenotypically confirming the presence of Ambler class A ESBL genes (50). In contrast to all other tested strains, the CLA-insensitive strains displayed resistance against the 7-\( \alpha \)-methoxy group-containing cefoxitin (Fig. 4) which indicates the presence of Ambler class C-type cephalosporinases (51). In none of the Salmonella isolates in our study, resistance against the indicator carbapenem meropenem was detected.

In each cefotaxime-resistant isolate, we found at least one \( \beta \)-lactamase. The SHV-12 (n = 12) enzymes, always found in combination with at least one other ESBL (Fig. 4), have recently been reported in human S. enterica isolates in the United States, India, and United Kingdom (52–55). The most common alleles were \( \text{bla}_{\text{TEM}} \) and \( \text{bla}_{\text{CTX-M}} \) which were identified in 41 and 39 isolates, respectively. The relatively innocuous penicillinase

![FIG 3 Chromosomal mutations conferring fluoroquinolone (FQ) resistance. Sequencing results of the quinolone resistance-determining regions (QRDR) of 460 nalidixic acid-resistant (NalR) S. enterica isolates. For each serotype (number sequenced [n]), the percentage of isolates displaying a QRDR-related mutation in their gyrA or parC genes is shown. wt, wild-type.](http://aac.asm.org/)

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TEM-1 ($n$/H11005 29) was the most prevalent gene. Twelve strains carried the ESBL variant $\text{bla}_{\text{TEM-52}}$, specified by the E104K/G238S mutations and widely found in European isolates (56). Sequencing of the $\text{bla}_{\text{CTX-M}}$ alleles revealed the presence of the most globally widespread ESBLs, CTX-M-14 ($n$/H11005 7) and CTX-M-15 ($n$/H11005 7). The most prevalent allele, $\text{bla}_{\text{CTX-M-1}}$ ($n$/H11005 11), is the most common food animal-associated CTX-M enzyme in Europe and is circulating on IncN plasmids through Escherichia coli and Salmonella spp. from human, animal, and environmental sources (53, 57). In contrast, $\text{bla}_{\text{CTX-M-55}}$ has mainly been reported from Asian enterobacterial isolates (58). Other $\text{bla}_{\text{CTX-M}}$ genes identified in this study were confined to a specific serotype, like $\text{bla}_{\text{CTX-M-2}}$ to S. Paratyphi B (15) and $\text{bla}_{\text{CTX-M-9}}$ to S. Typhimurium (Fig. 4). Two isolates, S. Enteritidis 09-1903 and S. Typhimurium 09-3124 contained the $\text{bla}_{\text{CTX-M-61}}$ gene. This is, to our knowledge, the first description of this gene in S. enterica. Notable, five of the seven ESBL genes found in human Belgian Salmonella isolates (i.e., CTX-M-1, -2, -9, TEM-52, and SHV-12) were recently retrieved from fecal Salmonella isolates from healthy Belgian pigs and broiler chickens (58).

Last, although the cooccurrence of PMQR genes and CSP resistance has been reported (59), it remains a very rare phenomenon in human salmonellosis. In this study, only a single isolate of S. Infantis (S13BD03795) carried a plasmid containing both $qnrB$, CLA, clavulanic acid (10 µg); Fox, cefoxitin, MEM, meropenem.

FIG 4 Resistance of S. enterica against β-lactam antibiotics. Heat map of the resistance patterns of Salmonella isolates resistant against third-generation cephalosporins and indicator carbapenems, based on (double) disk diffusion tests and according to EUCAST guidelines. Red and orange indicate full and intermediate resistance, respectively. For each isolate, the sample identifier (Id.), Salmonella serotype, and identified ESBL alleles are shown. AB, antibiotic; CLA, clavulanic acid (10 µg); Fox, cefoxitin, MEM, meropenem.
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