Third generation cephalosporin resistance in clinical non-typhoidal Salmonella enterica in Germany and emergence of bla<sub>CTX-M</sub>-harbouring pESI plasmids

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**Abstract**

Non-typhoidal Salmonella enterica is an important gastrointestinal pathogen causing a considerable burden of disease. Resistance to third generation cephalosporins poses a serious threat for treatment of severe infections. In this study occurrence, phylogenetic relationship, and mechanisms of third generation cephalosporin resistance were investigated for clinical non-typhoidal S. enterica isolates in Germany. From 2017 to 2019, we detected 168 unique clinical S. enterica isolates with pheno-typic resistance to third generation cephalosporins in a nation-wide surveillance. Compared to previous years, we observed a significant ($P=0.0002$) and consistent increase in resistant isolates from 0.41% in 2005 to 1.71% in 2019. In total, 34 different serovars were identified, most often S. Infantis ($n=41; 24.4\%$), S. Typhimurium ($n=27; 16.1\%$), S. Kentucky ($n=21; 12.5\%$), and S. Derby ($n=17; 10.1\%$). Whole genome analyses revealed extended-spectrum β-lactamase (ESBL) genes as main cause for third generation cephalosporin resistance, and most prevalent were $\text{bla}_{\text{CTX-M-1}}$ ($n=55$), $\text{bla}_{\text{CTX-M-14}}$ ($n=25$), and $\text{bla}_{\text{CTX-M-65}}$ ($n=23$). There was no strict correlation between serovar, phylogenetic lineage, and ESBL type but some serovar/ESBL gene combinations were detected frequently, such as $\text{bla}_{\text{CTX-M-1}}$ and $\text{bla}_{\text{CTX-M-65}}$ in S. Infantis or $\text{bla}_{\text{CTX-M-14}}$ in S. Kentucky. The ESBL genes were mainly located on plasmids, including IncI, IncA/C variants, emerging pESI variants, and a novel $\text{bla}_{\text{CTX-M-1}}$-harbouring plasmid. We conclude that third generation cephalosporin resistance is on the rise among clinical S. enterica isolates in Germany, and occurrence in various S. enterica serovars is most probably due to multiple acquisition events of plasmids.

**DATA SUMMARY**

The authors confirm that all supporting data, code and protocols have been provided within the article. Illumina raw sequence reads were submitted to the European Nucleotide Archive (ENA) (https://www.ebi.ac.uk/ena/) and can be accessed under the bioproject PRJEB41165.

**INTRODUCTION**

Non-typhoidal Salmonella (S.) enterica (NTS) is an important agent of foodborne infections [1]. In Europe and Germany, salmonellosis, after campylobacteriosis, represents the second most reported gastrointestinal infection caused by bacteria, encompassing 87,923 cases and 13,693 cases, respectively, in 2019 [2, 3]. S. Typhimurium and S. Enteritidis are the predominant serovars causing human infections in Germany and account for ~60% of all reported cases (https://survstat.rki.de/). Further prevalent serovars like S. Infantis and S. Derby represented <5% of the reported cases [3]. Although in most cases salmonellosis is self-limiting, antibiotic treatment may be required for risk group patients and more severe disease, like bloodstream infections [4]. Antibiotics for treatment of salmonellosis comprise β-lactams, fluoroquinolones, macrolides, and trimethoprim-sulfamethoxazole [4]. Challengingly, rising numbers of multidrug-resistant (MDR - resistant to three or more antimicrobials) S. enterica have...
been observed in recent years [5]. Including a slight increase in resistance to third generation cephalosporins, which has been reported in antimicrobial resistance surveillance studies for clinical isolates, and isolates from food (including retail meat) and animals [5–9].

Resistance to third generation cephalosporins in *S. enterica* is mainly due to the production of extended-spectrum *β*-lactamases (ESBLs) or AmpC *β*-lactamas [9, 10], with CTX-M-1 as the most prevalent ESBL type identified in previous studies [5, 11]. ESBL and AmpC genes are often located on plasmids, which facilitate their spread via horizontal gene transfer (HGT) between enterobacterial species and genera [12, 13]. Interestingly, ESBL production correlates with certain serovars in *S. enterica*. Among serovars found in human infections, ESBL genes have been more frequently detected in *S. Typhimurium*, *S. Infantis* and *S. Kentucky* than in *S. Enteritidis* [5, 14]. Furthermore, recent analyses on ESBL-producing *S. Infantis* from broiler chickens, broiler meat, and humans revealed a clonal lineage harbouring an especially conjugative pESI-like plasmid [15]. This raises the concern for transmission of ESBL genes in *S. enterica* along the food chain from the primary production systems to clinical cases.

In the present study, we assessed the resistance of clinical NTS in Germany and further analysed whole genomes of third generation cephalosporin resistant *S. enterica* for determination of phylogeny, resistance determinants, and genetic backbones to gain knowledge about the spread of this resistance mechanism in Germany.

**METHODS**

**Bacterial strains, serotyping, and antibiotic susceptibility testing**

In the years 2017 – 2019, the National Reference Centre (NRC) for *Salmonella* and the Enteric bacterial Pathogens in Germany analysed 11,730 *S. enterica* isolates from human infections for pathogen surveillance. In the same time period 48,083 salmonellosis cases were notified to the German public health body. Knowledge about the distribution of antibiotic resistance patterns and the genetic factors of third generation cephalosporin resistant *S. enterica* in Germany, we found that third generation cephalosporin resistance is on the rise among clinical NTS isolates. Its occurrence in various *S. enterica* serovars is likely due to multiple acquisition events of plasmids highlighting its facilitated spread.

**Impact Statement**

Non-typhoidal *Salmonella enterica* (NTS) is one of the most important agents of foodborne infections worldwide. Although in most cases salmonellosis is self-limiting, antibiotic treatment may be required. However, antibiotic resistance in NTS has been a globally emerging problem, limiting treatment options of NTS infections and further presenting a considerable threat to the public health and food safety. Knowledge about the distribution of antibiotic resistance patterns and the genetic factors in *S. enterica* is therefore essential for appropriate treatment of these infections and for early intervention to prevent the spread of antibiotic resistance, starting at the level of food production. In a nation-wide surveillance of third generation cephalosporin resistant *S. enterica* in Germany, we found that third generation cephalosporin resistance is on the rise among clinical NTS isolates. Its occurrence in various *S. enterica* serovars is likely due to multiple acquisition events of plasmids highlighting its facilitated spread.

**Bacterial cultivation and whole genome sequencing**

 Müller Hinton broth was used for an overnight cultivation. DNA was subsequently extracted using the GenElute Bacterial Genomic DNA Kit (Sigma, St. Louis, Missouri, U.S.). DNA quantification was performed using the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.) according to manufacturer’s instructions. Sequencing libraries were generated using the Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, United States) and paired-end sequencing was performed using a MiSeq instrument with 2×300 MiSeq v3 reagent kit (Illumina, San Diego, CA, United States), HiSeq 2500 instrument with 2×150 with 2×300 HiSeq v2 RR reagent kit or NextSeq 550 instrument with 2×300 NextSeq v2.5 high reagent kit. Whole genome sequencing (WGS) was performed for 166 NTS isolates (Table S1, available with the online version of this article) and sequences were submitted to ENA (https://www.ebi.ac.uk/ena/) and can be accessed under the bioproject PRJEB41165.

**Bioinformatics analyses**

Quality of the raw sequence data was assessed using FastQC v0.11.5 (https://github.com/s-andrews/FastQC). To verify taxonomic read classification, raw data was analysed with Kraken v0.10.6 [17]. The assembly pipeline of the analysis platform SeqSphere+ v6.0.7 (Ridom, Münster, Germany) was applied for *de novo* assembly using SPAdes v3.12.0 with default parameter [18], including an additional FastQC and read trimming step [19].

Multilocus sequence typing (MLST) and core genome MLST (cgMLST) were performed using the *de novo* assembled contigs and Ridom SeqSphere+ v6.0.7 (Ridom; Münster, Germany). The EnteroBase *S. enterica* cgMLST v2 scheme...
template was applied. *In silico* serotyping, based on the *de novo* assembly genomes, was conducted, using the serotype prediction tool SISTR [20].

*De novo* assembled contigs were used for resistance gene and plasmid replicon type identification. For the detection of acquired antimicrobial resistance genes, the tool Abricate in combination with the NCBIres Database was used (https://github.com/tseemann/abricate, [21]). *In silico* replicon typing was performed to assign resistance gene contigs to plasmid replicon sequences with Abricate using the PlasmidFinder v2.0 database [22]. Identified contigs with bla gene and plasmid replicon sequence were further investigated by means of Gensearch Prime v2020.0.5 (Biomatters, Ltd., Auckland, New Zealand). In case of presence of a plasmid replicon sequence marker and a bla gene on an identical contig, these contigs were additionally checked for a possible chromosomal integration via blastn (database nucleotide collection (nr/nt)) and a subsequent Mauve alignment using S. Typhimurium str. LT2 as reference (NC_003197.2). If a chromosomal integration could be ruled out, bla gene carrying contigs were assigned to the respective identified plasmid replicon and considered as the bla resistance gene carrying contig. For phylogenetically closely related isolates with identical replicon and bla resistance gene patterns but exhibiting different *de novo* assembled contigs carrying bla gene and replicon sequence, a reconstruction strategy as described in Weber et al. was performed [23]. If multiple replicon sequences were identified in the genome sequence of an isolate and no replicon marker sequence could be addressed as genetic backbone for the bla gene or the *de novo* assembled bla gene carrying contigs exhibited not sufficient surrounding genetic environment, the bla gene carrying replicon was marked as ‘not determined’.

Plasmids carrying bla<sub>CTX-M-1</sub> (*n* = 22) and bla<sub>CTX-M-65</sub> (*n* = 15) in *S. Infantis* isolates were compared to variants of the emerging pESI plasmid [15]. In brief, contigs of these isolates were aligned and ordered according to the pESI plasmid backbone (accession no. CP052810). In silico replicon typing as described in Pietsch et al., Microbial Genomics 2021;7:000698, was performed, using the serotype prediction tool SISTR [20]. Resistance transfer experiments and molecular typing

Transfer of third generation cephalosporin resistance was tested by broth mating for three selected isolates that harboured bla<sub>CTX-M-1</sub> (18-04833), bla<sub>CTX-M-65</sub> (19-02625) and bla<sub>CTX-M-1</sub> (17-01817) using the sodium azide-resistant recipient strain *E. coli* J53 Azi’. Transconjugants were cultivated on LB agar with sodium azide (200 mg l<sup>-1</sup>) and ampicillin (30 mg l<sup>-1</sup>) and screened for resistance genes as previously described [11]. The plasmid sizes of bla gene positive transconjugants were determined by S1-nuclease restriction and pulsed-field gel electrophoresis [25]. Transconjugants and the isolates 18-00018 and 18-00680 were screened for resistance genes as previously described [11].

RESULTS

**Increasing proportion of third generation cephalosporin resistant *S. enterica* in Germany**

In the years 2017 – 2019, the German National Reference Centre for Salmonella and other Enteric Bacterial Pathogens (NRC) analysed 11 730 NTS isolates from human infections (2017 =3 399, 2018 =3 706, 2019 =4 625 isolates). Resistance to third generation cephalosporins was detected in 264 *S. enterica* isolates, of which most were collected in 2019 (2017: *n* =36, 2018: *n* =56, 2019: *n* =172). A substantial part of the isolates (*n* =100) from 2019 was attributed to a single infection cluster of *S. Typhimurium* in a regional setting. Four unique/representative isolates of this cluster which harboured certain changes (see details below) were included into the subsequent analyses. Consequently, the number of third generation cephalosporin resistant isolates considered for further analysis in 2019 was 76, resulting in a total of 168 study isolates for the years 2017–2019 (Table S1).

In 2019, a proportion of 1.71% of all analysed *S. enterica* were resistant to third generation cephalosporins, which was an increase compared to 1.06% in 2017. To analyse whether the increasing trend is seen in a larger time frame, NRC data from the years 2005 to 2011 [11] and 2012 to 2016 were integrated into this analysis, using identical selection criteria. Indeed, the rising trend of third generation cephalosporin resistance was corroborated for the entire time frame from 2005 to 2019. The proportion of third generation cephalosporin resistance consistently rose from 0.41% in 2005 to 1.71% in 2019 (Fig. 1) (*P* =0.0002; correlation coefficient *r* =0.8318; Spearman rank correlation). In addition to resistance to third generation cephalosporins, 44 of the 168 isolates (26.2%) also showed resistance to ciprofloxacin (Table S1) and 21 isolates (12.5%) exhibited a combined resistance to ampicillin, chloramphenicol and trimethoprim/sulfamethoxazole (Table S1). No isolate was resistant to meropenem.

The 168 third generation cephalosporin resistant isolates were assigned to 34 different serovars, of which *S. Infantis* (*n* =41), *S. Typhimurium* (*n* =27), *S. Kentucky* (*n* =21), *S. Derby* (*n* =17) and *S. Anatum* (*n* =9) were most prevalent (Table S1 and S2).

ESBL types CTX-M-1, CTX-M-14, and CTX-M-65 are the most abundant third generation cephalosporin resistance determinants

Whole genome sequence analyses including *in silico* detection of the resistance determinants revealed that for most of the isolates (*n* =163/166) the third generation cephalosporin resistance was mediated by ESBLs (*n* =147) or plasmid-borne AmpC β-lactamases (*n* =16) (Table S1). For three isolates
no β-lactamase genes were detected and analyses of porin genes revealed wild-type variants without a sign of porin loss. Therefore, the underlying mechanism of resistance of these three isolates remained unclear (Table S1). The most prevalent ESBL genes in the isolate collection were bla

= CTX-M-1 (n=55), followed by bla

= CTX-M-14 (S. Bovismorbificans, 17-00186 and 17-05354) (Table 1). The most commonly detected AmpC β-lactamase gene was bla

= CTX-M-65 (n=25), different variants) and bla

= CTX-M-14b , only one isolate harboured bla

= OXA-1 , one isolate bla

= CMY-4 , one isolate bla

= DHA-1 , and one isolate bla

= ACC-1 , and one isolate bla

= TEM-1 variants. Additionally, 38 isolates harboured plasmid-mediated quinolone resistance (PMQR) genes, mainly gene qnrS1 (n=25). Interestingly, four isolates were positive for variants of the mobile colistin resistance gene and the gene carrying replicon was observed (bla

= CTX-M-1, n=32, 18.8 %) revealed chromosomal integration of the ESBL genes. The chromosomal integration of bla

= CTX-M-14 in all S. Kentucky isolates was identical as described by Coipan et al: bla

= CTX-M-14 was flanked by an upstream ISEcpI element, downstream of the hcp1 gene in the type VI secretion system region of the genome [14]. In all S. Derby isolates the integration of bla

= CTX-M-1, with an upstream ISEcpI, as presumable mobilizing element, took place in a different genetic region than in S. Kentucky. Bla

= CTX-M-1 was integrated into the kdu1 gene, which was inferred from homology, causing a truncation of this pectin degradation pathway gene (accession no. AO379QY07).

For 26 isolates it was only possible to recover the direct genetic environment but not to attribute the respective replicon type.

While for some of the ESBL genes a correlation of resistance gene and the gene carrying replicon was observed (bla

= CTX-M-65 and IncFIB(K) (22/23), bla

= CTX-M-14b and chromosome (16/19), bla

= CMY-2 and IncI1 (6/13), bla

= DHA-1, and IncA/C (6/13)), other genes showed a more diverse genetic background. For example, bla

= CTX-M-1 was found on IncI1, IncHI2, IncN and IncQ plasmids.

Predominance of emerging pESI plasmids in S. Infantis harbouring bla

= CTX-M

Detailed comparative analyses of bla

= CTX-M-65 carrying plasmids revealed a high sequence identity with the previously described pESI-like plasmid identified in S. Infantis. Specifically, 22 of

Plasmids as the main carriers of genes encoding ESBLs and AmpC β-lactamases

For 137 of 163 of the ESBL/AmpC-positive isolates an attribution of the resistance gene to a respective genetic backbone / replicon was possible. The majority of the resistance genes was located on mobile genetic elements (n=105, 65.2 %), particularly on plasmids, irrespective of their hosting S. enterica serovar. However, a substantial number of isolates (n=32, 18.8 %) revealed chromosomal integration of the ESBL genes. The chromosomal integration of bla

= CTX-M-14 in all S. Kentucky isolates was identical as described by Coipan et al: bla

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= CTX-M-65 carrying plasmids revealed a high sequence identity with the previously described pESI-like plasmid identified in S. Infantis. Specifically, 22 of
23 bla<sub>CTX-M-65</sub> positive isolates showed high resemblance with the chimeric backbone of the pESI-plasmid p119944 (accession no. NZ_CP047882) and all belonged to the serovar S. Infantis. The non-matching isolate belonged to S. Indiana (18-00180) and the respective plasmid revealed no similarities to the pESI plasmid backbone. The integration of bla<sub>CTX-M-65</sub> usually takes place upstream of a previously described drug and heavy metal resistance gene carrying region and this was also observed for the S. Infantis isolates in this study [26]. Further analyses showed that the presence of bla<sub>CTX-M-1</sub> on a pESI-like plasmid backbone was observed only in two out of 15 S. Infantis isolates (17-01817 and 18-04022). We found that the integration of bla<sub>CTX-M-1</sub> took place in the conserved IncI1 backbone structure of pESI. The remaining 13 S. Infantis isolates however exhibited a high sequence identity (>95 %) with the IncI1 plasmid p15078279 (accession no. MK181566), which was previously identified in E. coli [27]. Additionally, in one isolate (18-04833) bla<sub>CMY-2</sub> was suspected to be located on a pESI-like plasmid. While confirmation of the insertion of bla<sub>CMY-2</sub> into the pESI based on short read sequencing data was not possible, the sequence data however suggested the presence of the full pESI-like backbone. Additional mating experiments and subsequent S1-nuclease restriction and pulsed-field gel electrophoresis revealed transferability of the bla<sub>CMY-2</sub> gene and its presence on an approx. 290 kb sized plasmid for the bla<sub>CMY-2</sub> positive transconjugant of 18-04833. In comparison, the size of the transferred bla gene carrying plasmids of bla positive transconjugants of other pESI-like plasmid carrying strains, such as S. Infantis 17-01817 (bla<sub>CTX-M-1</sub>) and 19-02625 (bla<sub>CTX-M-65</sub>), was approx. 290 kb and approx. 315 kb, respectively.

### Table 1. ESBL and AmpC β-lactamase genes identified in the third generation cephalosporin resistant clinical Salmonella isolates from Germany, 2017 – 2019.

| ESBL/AmpC gene | n | PMQR genes (n) | Mcr genes (n) | Majorly found in (n) |
|----------------|---|----------------|---------------|---------------------|
| bla<sub>CTX-M-1</sub> * | 55 | qnrS1 (4) |  | S. Infantis (15) S. Derby (17) |
| bla<sub>CTX-M-2</sub> | 1 | | | |
| bla<sub>CTX-M-3</sub> | 3 | | | |
| bla<sub>CTX-M-9</sub> | 3 | | | |
| bla<sub>CTX-M-14</sub> | 25 | qnrS1 (3) | mcr-1.1 (1), mcr-3.11 (2) | S. Kentucky (18) |
| bla<sub>CTX-M-15</sub> | 7 | qnrS1 (1) | | |
| bla<sub>CTX-M-32</sub> | 1 | | | |
| bla<sub>CTX-M-35</sub> | 14 | qnrS1 (6) | mcr-3.1(1) | |
| bla<sub>CTX-M-65</sub> | 23 | | | |
| bla<sub>BES-52</sub> | 2 | qnrB19 (1) | | |
| bla<sub>CMY-2</sub> | 13 | qnrS1 (10), qnrB2 (1), qnrA1 (1) | | S. Anatum (7) |
| bla<sub>CMY-2</sub> | 13 | qnrS1 (1), qnrB19 (4), qnrB6 (1) | | |
| bla<sub>CMY-4</sub> | 1 | qnrA1 (1) | | |
| bla<sub>ESX-1</sub> | 1 | qnrB4 (1) | | |
| bla<sub>ESX-1</sub> | | | | |
| bla<sub>ACC-1</sub> | 1 | | | |

*Includes four representative isolates originating from the 2019 S. Typhimurium infection cluster.

AmpC β-lactamase CMY-2 was the main determinant of resistance to third generation cephalosporins present in 13 isolates (mainly serovars S. Minnesota (n=4) and S. Typhimurium (n=3). The bla<sub>CMY-2</sub> gene was found to be located on plasmids of the replicon type IncI1 (n=6) and IncA/C replicon type (n=6) and for one isolate (19-00825) was not assignable. The plasmids of IncI1 replicon type showed a high sequence identity to two previously described variants: IncI1 belonging to the plasmid sequence type pST12 (accession no. CP012929) (n=4) and IncI1 belonging to pST2 (accession no. KT186369) (n=2) and shared >98%, respective >99% sequence identity with these reference plasmids. Isolates 17-05838 and 19-02072 resembled the backbone of IncA/C plasmids of type 1 (accession no. CP015835) [28], while the other four isolates (17-00273, 17-01659, 19-02661, 19-06333) showed a high similarity with a IncA/C reference plasmid (accession no. CP060509), which was identified in NTS isolates from Russia.

bla<sub>CMY-2</sub> harbouring plasmids share high sequence similarity with IncI1 and IncA/C plasmids from Europe
The *S. Typhimurium* infection cluster isolates from 2019 harboured a novel plasmid carrying *bla*\(_{\text{CTX-M-1}}\)

As depicted above, a substantial part of the *S. Typhimurium* isolates (n=100) of the year 2019 originated from a single infection cluster in a regional setting. Genome analyses of 36 of these isolates were performed and confirmed their close genetic relationship. The maximum distance in the cgMLST analysis were five alleles; the majority of the genomes depicted a distance of zero or one alleles (Fig. 3). The resistance gene content and plasmid replicon analyses showed almost identical
patterns (blaCTX-M-136/36, qnrS1:35/36, IncHI2 replicon type: 35/36). The identified resistance gene carrying plasmid showed no sequence similarity to previously published bla gene carrying plasmids. Interestingly, one isolate (19-05594) showed absence of the qnrS1 gene and revealed a unique plasmid replicon background (IncI1), indicating a potential independent plasmid acquisition event. Two further isolates (19-04536 and 19-04537) exhibited a slight alteration of the blaCTX-M-1 carrying plasmid, varying in IS element integration sites. One representative isolate from each group (19-04587, 19-06858, 19-04536: IncHI2 plasmid alteration; 19-05594: IncI1 plasmid alteration; 19-05594: IncI1 plasmid) was included in all here presented analyses (Figs 2 and 3).

No strict correlation of ESBL genes with certain serovars was observed, but some serovar/resistance gene combinations, such as S. Infantis with blaCTX-M-1 or blaCTX-M-49 or S. Kentucky with blaCTX-M-14b were detected most frequently.

cgMLST-based analysis revealed that the majority of the isolates showed no close phylogenetic relationship but clustered according to the serovar. However, among the serovars various (distinctly related) subclades were observed (Fig. 2, subclades shown for S. Anatum, S. Derby, S. Infantis, S. Kentucky, and S. Typhimurium). As expected, the four representative S. Typhimurium isolates from the infection cluster formed one cluster (sequence type ST19) (Figs 2 and 3), while the other S. Typhimurium isolates belonged to the ST34. Linking data on bla gene and plasmid content to phylogenetic information, a tendency regarding the presence of specific ESBL genes in certain serovars was observed. The most apparent correlations were seen for S. Infantis with blaCTX-M-1 and blaCTX-M-49, S. Typhimurium with blaCTX-M-1, S. Derby with blaCTX-M-1 and S. Kentucky with blaCTX-M-14b (Fig. 2).

In depth cgMLST analyses of the S. Infantis clade showed that these strains can be differentiated into at least two different subclades: subclade one exhibited the IncI1 blaCTX-M-1 carrying plasmid while subclade two is associated with blaCTX-M-65 and blaCTX-M-14b carrying pESI-like plasmids (Fig. S1). Additionally, one isolate (18-04833) exhibited blaCTX-M-3 presumably integrated into the pESI backbone. Little information on travel background of patients was available. However, for thirteen of the 41 S. Infantis isolates a definite exposition region was determined. Interestingly, especially isolates from patients from the subclade carrying the blaCTX-M-65 pESI-like plasmid exhibited travel history to South East Asia or South America (Figs 2 and S1).

DISCUSSION

The analyses of clinical NTS from Germany showed a trend towards increasing resistance proportions for third generation cephalosporins (Fig. 1). This observation is in accordance with the worldwide emergence of antibiotic resistance in NTS. European data for clinical NTS isolates (period 2018 – 2019) by EFSA and ECDC [5] uncovered high resistance rates for different classes of antibiotics, especially ampicillin, sulfonamides, and tetracyclines. Increasing resistance was further reported for fluoroquinolones, while resistance to third generation cephalosporins was still on low levels but with rising proportions in certain serovars [5]. In Germany, five serovars (S. Typhimurium, S. Derby, S. Kentucky, S. Infantis and S. Anatum) accounted for over 65% of the resistant isolates in this study [5, 29, 30]. S. Enteritidis, while being one of the most prevalent S. enterica serovars, remains mostly susceptible to third generation cephalosporins [31].

In the present study we identified ESBL and AmpCβ-lactamase genes as most important determinants of third generation resistance in clinical NTS. These observations are in accordance to reports from the EU, where in 2018/19 ESBLs were reported in 16 different serovars (among those S. Infantis, S. Kentucky, S. Typhimurium) and AmpC β-lactamases in ten different serovars, mostly in S. Anatum, S. Bredeny and S. Thompson [5]. The majority of isolates in this study harboured ESBL genes encoding CTX-M enzymes (n=132; 80.0%) with CTX-M-1, CTX-M-14 and CTX-M-65 as the most prevalent types. Similar results were found in studies on S. enterica and E. coli isolated from animals, especially poultry [11, 32, 33] and in reports from the EU, where, if tested by member states, the ESBL variants CTX-M-1, CTX-M-14/14b and CTX-M-9 were frequently reported [5]. In the present study blaCTX-M-1 was detected in various serovars, including S. Typhimurium, S. Infantis and S. Derby. However, blaCTX-M-14b and blaCTX-M-65 were almost exclusively found in S. Kentucky and S. Infantis, respectively. Detailed sequence analyses of the 23 blaCTX-M-65 carrying plasmids of the study isolates revealed a high sequence identity among them: out of the 23 isolates, 22 belonged to the serovar S. Infantis and showed an identical plasmid structure, which additionally was highly similar to the chimeric backbone of the pESI-plasmid p119944 (accession no. NZ_CP047882).
In recent studies the spread of third generation cephalosporin resistant 
S. Infantis in food-producing animals associated with the presence of a specific resistance plasmid type, the pESI-like megaplasmid, was reported [15, 34, 35]. This plasmid type/backbone was associated with the carriage of ESBL genes (blaCTX-M-65 and blaCTX-M-1), several other resistance genes (tet(A), sul1, dfrA1, dfrA14, adaA1) and heavy metal resistance genes [26]. The pESI-like megaplasmid was first identified by Aviv et al. in Israel [34], however, not yet harbouring third generation cephalosporin resistance genes. Subsequently findings of pESI harbouring third generation cephalosporin resistance genes were reported from Italy [15], the U.S. [28], Denmark, Netherlands and the UK [15, 34–36]. In 2018, Brown et al. observed the linkage of S. Infantis pESI-like plasmids carrying blaCTX-M-65 in the USA with travel activity to Latin America [37]. Despite too little information on the travel history of the patients in our study for statistical analyses, the present data partly indicate a correlation between travel activity (Asia and South America) and blaCTX-M-65 harbouring S. Infantis.

While the pESI plasmid with blaCTX-M-65 was highly prevalent in S. Infantis strains from Germany, only two of 15 S. Infantis strains exhibiting blaCTX-M-65 (17-01817 and 18-04022) harboured the resistance gene on the pESI plasmid. In Germany the occurrence of pESI was described in S. Infantis isolates from broiler farms since the 2010s [38]. However, these plasmids did not carry ESBL genes and were identified in S. Infantis strains of another lineage (ST2283) than the blaCTX-M-1 carrying isolates from this study (ST32). In our study one additional isolate of S. Infantis exhibited blaCTX-M-1 on a pESI-like plasmid, highlighting the potential of this particular plasmid backbone to acquire further resistance genes. Phylogenetic analysis revealed that isolating harbouring pESI with blaCTX-M-3, blaCTX-M-6 and blaCTX-M-1 are more related among each other than to the clade of the non-pESI blaCTX-M-1 carrying S. Infantis isolates. It can be speculated, that certain genetic factors enable the plasmid to establish in this specific S. Infantis clone. The presence of multiple post-segregational killing determinants on the plasmid may be of advantage for this process [34].

The majority of S. Infantis isolates with blaCTX-M-1 (13/15) exhibited a highly similar plasmid backbone to an IncI1 plasmid backbone. Identical or highly similar blaCTX-M-1 carrying plasmids were identified in the present study also in S. Derby, S. Enteritidis, S. Typhimurium, S. Stanley and S. Brandenburg. In contrast to the pESI plasmid, which appears to be more adapted to the S. Infantis host - but still can be transferred into other S. enterica serovars and E. coli [15, 39] - these non-pESI IncI1 plasmids exhibited a wider host range and were also reported in previous studies in E. coli [27].

Further resistance genes detected in this study were blaCTX-M-15 and blaCMY-2. ESBL type CTX-M-15, which is highly prevalent in E. coli from outpatient and hospitalized patients in Germany [40], was identified only in seven clinical S. enterica isolates of different serovars. AmpC β-lactamase CMY-2, which was detected in <1% of clinical E. coli isolates with third generation cephalosporin resistance in Germany [41] were detected in 12 S. enterica isolates in the present study (7.45 %). The observed higher proportions of CTX-M-1 and CMY-2 genes with comparable lower proportions of CTX-M-15 in our clinical S. enterica isolates may on the one hand indicate a possible transmission route between livestock and humans via the food chain. The active acquisition of plasmids via horizontal gene transfer along the food production chain seems rational, as identical resistance gene carrying plasmids were observed in S. enterica of different serovars. This is strengthened by the observation of identical resistance gene carrying plasmids in E. coli, Klebsiella pneumoniae and other Enterobacteriaceae species in Germany, Europe, and worldwide [42–44]. Furthermore, it has been reported that approx. 6% of the healthy population of Germany carries ESBL positive E. coli [45]. Ingestion of other ESBL-positive Enterobacteriaceae and transfer of resistance genes from commensal bacteria therefore might have an impact on the treatment options of severe salmonellosis, as the resistance genes may be acquired during treatment [46]. However, since CTX-M-15 is the most common ESBL type in E. coli/K. pneumoniae in the human population, these events might be limited in occurrence rather than being the main route for ESBL gene transmission.

We conclude that third generation cephalosporin resistance, is on the rise among clinical S. enterica isolates in Germany and that its occurrence in various S. enterica serovars is likely due to multiple acquisition events of plasmids. However, specific adaption processes, as observed for S. Infantis with pESI plasmids harbouring different blaCTX-M genes, could facilitate the spread of resistant S. enterica isolates.

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Author contributions
We describe author contributions to the paper using the CRediT taxonomy. Conceptualization: Conceptualization: M.P. and A.F. Data curation: M.P., S.S. and A.M. Formal Analysis: M.P. Funding acquisition: S.S. and A.F. Investigation: M.P., Y.P. and S.S. Methodology: M.P. Project administration: A.F. Resources: S.S., E.T. and A.F. Software: M.P. and E.T. Supervision: A.F. Validation: M.P., S.S. and A.F. Visualization: M.P. Writing – original draft: M.P. and A.F. Writing – review and editing: all authors.

Conflicts of interest
The authors have no conflicts of interest.

Ethical statement
The study does not include human subjects, human material, or human data. Solely, bacterial isolates were studied and were obtained as part of routine submissions to the National Reference Centre for Salmonella and other Enteric Bacterial Pathogens.
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