Analysis of extended-spectrum-β-lactamase-producing *Escherichia coli* isolates collected in the GERM-Vet monitoring programme

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Objectives: The aims of this study were (i) to detect extended-spectrum β-lactamase (ESBL) genes among 1378 *Escherichia coli* isolates from defined disease conditions of companion and farm animals and (ii) to determine the localization and organization of ESBL genes.

Methods: *E. coli* isolates from the German resistance monitoring programme GERM-Vet were included in the study. Plasmids were transferred by conjugation or transformation and typed by PCR-based replicon typing. ESBL genes were detected by PCR; the complete ESBL genes and their flanking regions were sequenced by primer walking. Phylogenetic grouping and multilocus sequence typing (MLST) were performed for all ESBL-producing *E. coli* isolates.

Results: Of the 27 ESBL-producing *E. coli* isolates detected, 22 carried bla<subCTX-M-1</sub> genes on IncN (n = 16), IncF (n = 3), IncI1 (n = 2) or multireplicon (n = 1) plasmids. A bla<subCTX-M-3</sub> gene was located on an IncN plasmid and a bla<subCTX-M-15</sub> gene was located on an IncI1 plasmid. A multireplicon plasmid and an IncHI1 plasmid harboured bla<subCTX-M-2</sub>. A bla<subTEM-52c</sub> gene was identified within Tn<sup>2</sup> on an IncI1 plasmid. The bla<subCTX-M</sub> genes located within the same or related genetic contexts showed differences due to the integration of insertion sequences. Various MLST types were detected, with ST10 (n = 7), ST167 (n = 4) and ST100 (n = 3) being the most common.

Conclusions: This study showed that the bla<subCTX-M-1</sub> gene is the predominant ESBL gene among *E. coli* isolates from diseased animals in Germany and a considerable structural heterogeneity was found in the regions flanking the bla<subCTX-M-1</sub> gene. Insertion sequences, transposons and recombination events are likely to be involved in alterations of the ESBL gene regions.

Keywords: ESBLs, diseased animals, plasmids, MLST

Introduction

Cephalosporins are widely used in human and veterinary medicine and cephalosporin resistance among Enterobacteriaceae is commonly due to the production of extended-spectrum β-lactamases (ESBLs). The first ESBLs derived from narrow-spectrum TEM and SHV β-lactamases; later on, CTX-M-type ESBLs occurred. The emergence of ESBL-producing Enterobacteriaceae isolates among companion and farm animals causes growing concern. Moreover, an animal reservoir for ESBL genes has been proposed. ESBLs of the CTX-M type are predominant in European Enterobacteriaceae isolates from humans and also from various companion, pet and food-producing animals. In Germany, CTX-M-15-producing *Escherichia coli* isolates belonging to the worldwide emerging O25:H4-ST131 clone from diseased dogs and a horse have been described. In addition, a bla<subCTX-M-15</sub> and two bla<subCTX-M-1</sub>-carrying plasmids from German *E. coli* isolates of canine (ST410 and ST1576) or porcine (ST1153) origin have been analysed.

The aims of this study were (i) to identify ESBL genes among 1378 *E. coli* isolates from defined disease conditions of companion and farm animals and (ii) to determine the localization and organization of these genes. In addition, ESBL-positive *E. coli* isolates were characterized by phylogenetic grouping and multi-locus sequence typing (MLST).

Materials and methods

**Bacterial isolates and susceptibility testing**

The 1378 *E. coli* isolates from swine, poultry, cattle, dogs, horses, cats, sheep and goats included in this study were collected in the German
national resistance monitoring programme GERM-Vet during the years 2006 to 2007. The numbers of isolates per animal species and defined disease condition are listed in Table 1. All isolates were tested for their susceptibility to 24 antimicrobial agents by broth microdilution according to the recommendations given in document M31-A3 of the CLSI.22 Furthermore, all isolates have been subjected to ESBL phenotypic confirmatory tests.22

### Molecular analyses

Plasmids were transferred either by conjugation or transformation into *E. coli* recipients HK225 or TOP10, respectively, with subsequent selection on Luria–Bertani agar supplemented with ampicillin (100 mg/L) or cefotaxime (1 mg/L). Transconjugants and transformants were tested for their antimicrobial susceptibility by disc diffusion and/or broth microdilution as recommended by the CLSI.22 The plasmid sizes were determined by S1 nuclease PFGE and the plasmids were typed by PCR-based replicon typing.21,23

The ESBL genes were identified using previously described PCR assays and sequence analysis of the amplicons.21,24 In order to obtain the complete ESBL genes and the flanking gene regions, primer walking was performed based on the sequenced PCR amplicons and using plasmid DNA from the respective transconjugants or transformants. Plasmids carrying *bla*<sub>CTX-M-1</sub>, *bla*<sub>CTX-M-3</sub> and *bla*<sub>CTX-M-15</sub> were digested with DraI and PstI and subsequently hybridized with a *bla*<sub>CTX-M-1</sub> DNA probe, which consisted of an internal *bla*<sub>CTX-M-1</sub> PCR amplicon. The *bla*<sub>CTX-M-2</sub>- and *bla*<sub>TEM-S2c</sub>-carrying plasmids were digested with BamHI, BglII and SacI (*bla*<sub>CTX-M-2</sub>) or EcoRI, EcoRV, PvuII and Clal (*bla*<sub>TEM-S2c</sub>), respectively. For the subsequent detection of these genes by Southern blot hybridization, a *bla*<sub>CTX-M-2</sub> group and a *bla*<sub>TEM</sub> DNA probe, respectively, each based on an internal PCR amplicon, were used. Integrons on *bla*<sub>CTX-M-2</sub>-carrying plasmids were amplified by PCR, cloned and sequenced.25

Sequence analysis was conducted using the software programs blastn and blastp (http://blast.ncbi.nlm.nih.gov/Blast.cgi), the Open Reading Frame (ORF) Finder program (http://www.ncbi.nlm.nih.gov/projects/gorf/) and the IS Finder program (http://www-is.biotoul.fr/is.html) (all web sites: date last accessed 19 September 2012). PCR-based phylogenetic grouping using a multiplex PCR and MLST was performed for *E. coli* isolates with an ESBL phenotype.26,27

### Nucleotide sequence accession numbers

The nucleotide sequences have been deposited in the European Molecular Biology Laboratory database under accession numbers HFS49088–HFS49095.

### Results

#### Presence and types of ESBL genes

Of the 1378 *E. coli* isolates tested, only 27 (1.96%) isolates showed an ESBL phenotype. The following ESBL genes were detected: *bla*<sub>CTX-M-1</sub> (*n* = 22), *bla*<sub>CTX-M-2</sub> (*n* = 2), *bla*<sub>CTX-M-3</sub> (*n* = 1), *bla*<sub>TX-M-15</sub> (*n* = 1) and *bla*<sub>TEM-S2c</sub> (*n* = 1). In all cases, the ESBL gene was located on a plasmid (Table 2). The ESBL-positive isolates were from swine (*n* = 12), cattle (*n* = 12), poultry (*n* = 2) and a horse (*n* = 1). No ESBL-producing isolates were identified among the *E. coli* isolates from dogs, cats, sheep and goats (Tables 1 and 2).

#### Plasmids carrying *bla*<sub>CTX-M-1</sub>

Sixteen *bla*<sub>CTX-M-1</sub> genes were located on replicon type N plasmids of ~40–50 kb in *E. coli* isolates from cases of porcine gastroenteritis/enteritis (*n* = 10), porcine urinary tract infection (*n* = 1), bovine gastro-enteritis/enteritis (*n* = 3), bovine urinary tract infection (*n* = 1) and from poultry suffering from septicemia (*n* = 1). Two *bla*<sub>CTX-M-1</sub> genes were located on IncI1 plasmids of ~83 or ~92 kb, respectively, in *E. coli* isolates from cattle suffering from gastrointestinal tract infections. Three replicon type F plasmids carrying *bla*<sub>CTX-M-1</sub> were detected, of which two had a size of ~70 kb and originated from *E. coli* isolates from bovine gastrointestinal tract infections and the remaining plasmid was ~50 kb.
### Table 2. Characteristics of the ESBL-producing E. coli isolates and their ESBL gene-carrying plasmids

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Animal species</th>
<th>Disease condition</th>
<th>Resistance pattern</th>
<th>Phylogenetic group</th>
<th>MLST type</th>
<th>ESBL gene</th>
<th>ESBL gene-carrying plasmid designation</th>
<th>PstI/DraI fragment (kb)</th>
<th>conjugation</th>
<th>replicon type(s)</th>
<th>approximate size (kb)</th>
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<th>Resistance pattern</th>
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<th>MLST type</th>
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<th>ESBL gene-carrying plasmid</th>
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<td>—</td>
<td>—</td>
<td>I1</td>
<td>83</td>
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<sup>a</sup>APR, apramycin; AMC, amoxicillin/clavulanic acid (2:1); BLA, β-lactams; CHL, chloramphenicol; ENR, enrofloxacin; FFN, florfenicol; GEN, gentamicin; NAL, nalidixic acid; SPT, spectinomycin; SXT, trimethoprim/sulfamethoxazole (19:1); TET, tetracycline; TMP, trimethoprim.

<sup>b</sup>Sequences from these plasmids have been deposited in the European Molecular Biology Laboratory database.
and had been obtained from a horse during a breeding hygiene sampling. One blaCTX-M-1-carrying plasmid, obtained from an E. coli from a pig suffering from gastritis/enteritis, had a size of ~160 kb and was positive for replicons I1, N and P (Table 2).

For a characterization of the blaCTX-M-1 gene region, these plasmids were digested separately by PstI and Dral and subsequent Southern blot hybridization with the blaCTX-M-1 probe showed that 13 of the 22 plasmids yielded same-sized PstI and Dral fragments of 4.5 kb each. These fragments corresponded to the fragments of the previously described blaCTX-M-1 gene region on the ~50 kb IncN plasmid pCTX246 of E. coli from porcine origin. This region contained a fragment of the insertion sequence ISecp1, truncated by an IS26 in the region upstream of the blaCTX-M-1 gene and in the downstream region the terminal part of orf477, followed by a partially deleted mrx gene, a complete mph(A) gene and a second IS26. This genetic context was located on 10 IncN plasmids ranging in size between 40 and 50 kb (pCTX1328, pCTX338, pCTX895, pCTX1637, pCTX1876, pCTX2049, pCTX2251, pCTX2255, pCTX4025 and pCTX4198), on two 70 kb IncF plasmids (pCTX2347 and pCTX2763) and on a 160 kb plasmid (pCTX99), which was positive for replicons I1, N and P. Moreover, two 44 and 46 kb IncN plasmids, pCTX1875 and pCTX3950, respectively, showed fragments of 9 kb (PstI) and 4.5 kb (Dral). The remaining seven plasmids, namely four IncN plasmids of 40–50 kb (pCTX1360, pCTX3780, pCTX1956 and pCTX4145), two IncI1 plasmids (pCTX1261 and pCTX3174) of 83 and 92 kb and the 53 kb IncF plasmid (pCTX2412) displayed individual PstI and Dral fragment patterns (Table 2).

The blaCTX-M-1 upstream region on the IncN plasmids pCTX1875, pCTX3950, pCTX1360, pCTX3780 and pCTX1956 showed 100% nucleotide sequence identity to pCTX246. The same was true for the downstream regions on pCTX1875, pCTX3950, pCTX1360 and pCTX3780, where the fragment of orf477 and a partially deleted mrx gene were identified. The downstream region on pCTX1956 differed distinctly, with an IS26 being inserted 37 bp downstream of blaCTX-M-1. In the blaCTX-M-1 upstream region on pCTX4145, an IS26 was detected 272 bp apart from blaCTX-M-1, whereas the IS26 on the aforementioned plasmids was located 294 bp apart from blaCTX-M-1. Downstream of blaCTX-M-1, the terminal 342 bp of orf477 were detected, followed by a partially deleted ecorIII gene. A complete ISecp1 was identified on the IncI1 plasmids pCTX1261 and pCTX3174, while on the IncF plasmid pCTX2412 an ISecp1-like insertion sequence, which differed slightly in size from ISecp1 in the non-coding region between the terminal inverted repeat and the tnpA gene, was detected. All three carried orf477 342 bp downstream of blaCTX-M-1. Further downstream on pCTX1261 and pCTX3174, the same truncated ORF was detected, whereas on pCTX2412, a tnpA-like gene was identified (Figure 1).

Five of the 22 blaCTX-M-1-carrying plasmids also conferred resistance to other antimicrobial agents. Plasmids pCTX2251 and pCTX4025 also conferred resistance to gentamicin, pCTX2347 and pCTX2763 to apramycin and gentamicin and pCTX99 to tetracycline and trimethoprim/sulfamethoxazole (Table 2).

Plasmid carrying blaCTX-M-3

The isolate harbouring the blaCTX-M-3-carrying plasmid originated from a case of porcine gastrointestinal infection. The conjugative plasmid had a size of ~68 kb and belonged to replicon type N. In the blaCTX-M-3 upstream region, the terminal 372 bp of ISecp1 were identified. This insertion sequence was disrupted by the integration of a reversely oriented IS911-like insertion sequence. The IS911-like insertion sequence showed 92% similarity to IS91 and the ISecp1 continued downstream of the IS91-like insertion sequence. As previously described for IS91, no target site duplication was identified and the right inverted repeat downstream of the transposase was 5′-GAAC-3′, while the left one was absent. In the downstream region of the blaCTX-M-3 gene, the terminal 149 bp of orf477 and one end of an IS26 were detected (Figure 1). This plasmid also conferred resistance to trimethoprim/sulfamethoxazole (Table 2).

Plasmid carrying blaCTX-M-15

The blaCTX-M-15-carrying plasmid was found in an E. coli isolate from a calf suffering from a gastrointestinal tract infection. The plasmid (pCTX1929) was transferred by transformation, had a size of ~150 kb and was positive for replicons FIA and FIB (Table 2). Sequence analysis identified the insertion sequence ISecp1 in the upstream region of blaCTX-M-15, while the downstream region comprised the terminal 342 bp of orf477 followed by a partially deleted transposase gene tnpA (Figure 1).

Plasmids carrying blaCTX-M-2

The two isolates with plasmids carrying blaCTX-M-2 originated from calves suffering from enteritis. One plasmid (pCTX3429) had a size of ~240 kb and the replicon type H11. The other one (pCTX2008) was ~140 kb in size and positive for replicons FIB, P and F. The BglII and SacI fragments carrying blaCTX-M-2 had the same size on both plasmids, but the BamHI fragments differed slightly in size. These results pointed towards the presence of a complex class 1 integron containing blaCTX-M-2, such as InS21 (accession no. AJ311891), but harbouring different gene cassettes between the 5′-conserved segment (CS) and the first 3′-CS. PCR amplification and sequencing of this area revealed a dfrA1 and an aadA1 gene cassette on pCTX2008 as well as a dfrA17 and an aadA5 gene cassette on pCTX3429. Sequence analysis of the upstream region of blaCTX-M-2 on pCTX2008 revealed the presence of orf513 and an orf3 downstream of blaCTX-M-2, which was also present in InS21 (Figure 1 and Table 2).

Plasmid carrying blaTEM-52c

The single blaTEM-52c-carrying plasmid, pCTX909, was from an E. coli isolate of poultry origin. The plasmid had a size of ~83 kb and belonged to replicon type I1. Sequence analysis of the immediate flanking regions identified a tnpR gene of transposon Tn2 in the blaTEM-52c upstream region and the right inverted repeat of Tn2 followed by a vagD gene in the blaTEM-52c downstream region. Furthermore, the plasmid fragments carrying the blaTEM-52c gene corresponded in size to those of transposon Tn2 on an IncI1 plasmid in the database (accession no. EF141186). Noteworthy to mention is that, according to Bailey et al., the transposon Tn2 is annotated incorrectly as Tn3 in the respective database entry (Figure 1 and Table 2).
Figure 1. Schematic presentation of the flanking gene regions of the \textit{bla}_{CTX-M} genes and the \textit{bla}_{TEM-52c} gene. The ORFs are shown as arrows, with the arrowhead indicating the direction of transcription. IS elements are shown as boxes. This figure appears in colour in the online version of \textit{JAC} and in black and white in the print version of \textit{JAC}. 

\cite{Schink2017}
**Phylogenetic grouping and MLST**

The 27 ESBL-producing *E. coli* isolates were assigned to four phylogenetic groups and 15 different sequence types. Eighteen *E. coli* isolates belonged to phylogenetic group A, comprising the sequence types ST10 (n = 7), ST167 (n = 4), ST100 (n = 3) as well as single isolates of ST23, ST83, ST1684 and the novel type ST2699. Six *E. coli* isolates, including ST648 (n = 2), ST57, ST362, ST925 and ST973 (one each), represented phylogenetic group D. Two *E. coli* isolates were positive for phylogenetic group B1 and were assigned to ST453 and the novel type ST2698. One *E. coli* isolate belonged to phylogenetic group B2 and had the sequence type ST131.

The 16 *E. coli* isolates harbouring IncN plasmids that carried blaCTX-M-1 were assigned to ST10 (n = 7), ST100 (n = 3), ST23, ST131, ST167, ST453, ST1684 and ST2699 (one each). *E. coli* isolates designated to ST83, ST648 and ST925 harboured blaCTX-M-1-carrying IncF plasmids and *E. coli* isolates of ST167 and ST362 harboured IncN plasmids with blaCTX-M-1. The multireplicon plasmid pCTX99 was detected in *E. coli* ST10. The blaCTX-M-3-carrying IncN plasmid pCTX2207 was also detected in an *E. coli* ST167 and blaCTX-M-15-carrying FIA-FIB plasmid pCTX1929 was present in *E. coli* ST648. *E. coli* ST57 and ST167 harboured the blaCTX-M-2-carrying plasmids pCTX2008 and pCTX3429, respectively. The *E. coli* isolate designated to the novel ST2698 harboured the blTEM-52c-carrying I1 plasmid, pCTX909 (Table 2).

**Discussion**

This study showed that (i) ESBL genes among *E. coli* from diseased animals in Germany are commonly located on plasmids that differ in size and replicon types, (ii) that blaCTX-M-1 was the most frequently detected ESBL gene and (iii) that a considerable structural heterogeneity was observed in the regions flanking the blaCTX-M-1 gene. The IS26-ISEcpl-blaCTX-M-1-orf477-Δmrx-mp(A)-IS26 structure was detected most frequently (figure 1) and has been identified previously on an IncN plasmid in a porcine *E. coli* from mastitis-metritis-agalactia syndrome in Germany. In the present study, this genetic environment of blaCTX-M-1 was seen on IncN but also on IncF plasmids and on a multireplicon plasmid in *E. coli* from swine and cattle suffering from gastrointestinal or urogenital tract infections. In *E. coli* isolates of human origin, an IncN1 plasmid carrying this structure and IncN plasmids with a similar structure were identified and the authors proposed the existence of an IS26 composite transposon. This theory is supported by the occurrence of the putative transpose on IncF plasmids. However, the presence of the IS26-ISEcpl-blaCTX-M-1-orf477-Δmrx-mp(A)-IS26 structure on different plasmids might also be due to interplasmid recombination events in which IS26 is involved. Furthermore, this resistance gene region seems to undergo alteration processes, which have been described during the analysis of pCTX168, an IncN plasmid from a canine *E. coli* isolate. This finding is supported by the observation in the present study that plasmids pCTX1875, pCTX3950, pCTX1360 and pCTX3780 from porcine, bovine and avian *E. coli* showed—despite the presence of an IS26-ISEcpl-blaCTX-M-1-orf477-Δmrx structure—different sized fragments carrying blaCTX-M-1 in Southern blot hybridization.

Novel blaCTX-M-1 flanking regions, which included different integration sites of the insertion sequence IS26, were identified on plasmids pCTX1956 and pCTX1445 (Figure 1). In the latter plasmid, the IS26 was located 272 bp upstream of blaCTX-M-1 instead of 294 bp as described by Diestra et al. IncN plasmids carrying identical or closely related blaCTX-M-1 gene regions, but also different plasmid backbones carrying identical blaCTX-M-1 gene regions, seem to be widely distributed among *E. coli* isolates from different host animals and disease conditions in Germany.

The IncI1 plasmids pCTX1261 and pCTX3174 and the IncF plasmid pCTX2412 showed similar ISEcpl-blaCTX-M-1-orf477 structures. Sequence analysis strongly suggested that the integration, which was most likely mediated by a one-sided ISEcpl transposition because of the 5 bp duplication at the integration site (5′-TTATA-3′, 5′-TCAGA-3′; Figure 1), occurred independently at different sites in the IncI1 and IncF plasmid backbones. The blaCTX-M-15 gene on pCTX1929 was embedded in the structure ISEcpl-blaCTX-M-15-orf477 inserted in a TnpA gene, which has been described for IncF plasmids before. IncI1 plasmids carrying blaCTX-M-15 gene has been reported in association with ISEcpl or IS26-ISEcpl in the upstream region and orf477 in the downstream region, but the genetic arrangement on pCTX2207 has not been reported so far, with the ISEcpl truncated by an IS91-like element and the IS26 truncating orf477. This underlines the important role of insertion sequences in the structural alteration of resistance gene regions.

Plasmids carrying complex class 1 integrons, containing blaCTX-M-2, have been described in *Salmonella* enterica, *Klebsiella pneumoniae*, *Morganella* morgani and *E. coli*. Class 1 integrons containing blaCTX-M-2 in combination with dfrA1 and adaA1 cassettes have been described on IncH2 plasmids in *S. enterica* from Belgian and Dutch origins, respectively, and seem to be widely distributed among different members of the family Enterobacteriaceae. Furthermore, the transmission of those plasmids between avian *E. coli* isolates from Belgium, but this is the first description of such a plasmid from German cattle.

IncI1 plasmids carrying blTEM-52c within transposon Tn2, like on pCTX909, have been described in *S. enterica* and *E. coli* isolates from poultry and humans in Belgium and the Netherlands, respectively, and seem to be widely distributed among different members of the family Enterobacteriaceae. Furthermore, the transmission of those plasmids between avian *E. coli* isolates and isolates of human origin was demonstrated in situ.

Most of the *E. coli* isolates from diseased livestock from Germany belonged to phylogenetic groups A and D, which is in accordance with the study of Valat et al. *E. coli* isolates with the sequence types ST10, ST23, ST131, ST167 and ST648 have been recently listed as lineages with a potential extended host range.
gastrointestinal tract infection to ST131, both harbouring bla\textsubscript{CTX-M-1}-Carrying IncN plasmids. These findings show that among ESBL-producing \textit{E. coli} isolates from German livestock, sequence types occur that have been identified in different hosts, including humans, and might have been transmitted between animals and humans. As the respective sequence types also harbour different plasmids and ESBL genes, the dissemination of these genes is not only due to clonal expansion but also to horizontal gene transfer. The fact that—besides the three \textit{E. coli} ST100 from cases of porcine gastrointestinal tract infections, which harboured bla\textsubscript{CTX-M-1} genes located on IncN plasmids—the remaining \textit{E. coli} isolates were assigned to different sequence types, including the novel types ST2698 from poultry and ST2699 from swine, supports this assumption.

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**Transparency declarations**

None to declare.

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ESBL-producing *E. coli* from animals


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