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 blaCTX-M-1 genes on IncN (n = 16), IncF (n = 3), IncI1 (n = 2) or multireplicon (n = 1) plasmids. A blaCTX-M-3 gene was located on an IncI plasmid and a blaCTX-M-15 gene was located on an IncF plasmid. A multireplicon plasmid and an IncHI1 plasmid harboured blaCTX-M-2. A blaTEM-52c gene was identified within Tn2 on an IncI1 plasmid. The blaCTX-M 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 blaCTX-M-1 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 blaCTX-M-1 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).1 The first ESBLs derived from narrow-spectrum TEM and SHV β-lactamases; later on, CTX-M-type ESBLs occurred.2 The emergence of ESBL-producing Enterobacteriaceae isolates among companion and farm animals causes growing concern.3,4 Moreover, an animal reservoir for ESBL genes has been proposed.5 ESBLs of the CTX-M type are predominant in European Enterobacteriaceae isolates from humans6 and also from various companion, pet and food-producing animals.7–19 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.20

In addition, a blaCTX-M-15- and two blaCTX-M-1-carrying plasmids from German E. coli isolates of canine (ST410 and ST1576) or porcine (ST1153) origin have been analysed.21 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 multilocus 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...
Table 1. Origin of the *E. coli* isolates used in the study

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<tr>
<th>Animal origin</th>
<th>gastrointestinal tract infection</th>
<th>urogenital tract infection</th>
<th>respiratory tract infection</th>
<th>septicemia</th>
<th>sudden death</th>
<th>yolk sac infection</th>
<th>infection of the musculoskeletal system</th>
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<td>152</td>
<td>45</td>
<td>193</td>
<td>132</td>
<td>49</td>
<td>15</td>
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<th>Animal origin</th>
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<th>septicemia</th>
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<th>yolk sac infection</th>
<th>infection of the musculoskeletal system</th>
<th>others</th>
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<td>Total</td>
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<td>152</td>
<td>45</td>
<td>193</td>
<td>132</td>
<td>49</td>
<td>15</td>
<td>26</td>
<td>1378 (27)</td>
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"*Unknwon (1) and polyesrosis (1)."  
"Hepatitis (1), polyesrosis (4), pericarditis (4) and peritonitis (4)."  
"Unknown (6), infection of the umbilical cord (1) and infection of the CNS (1)."  
"Unknown (1) and otitis externa (1)."  
"Unknown (1) and polyserositis (1)."  
"Infection of the CNS (1)."

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: *blaCTX-M-1* (n = 22), *blaCTX-M-2* (n = 2), *blaCTX-M-3* (n = 1), *blaCTX-M-15* (n = 1) and *blaTEM-52c* (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 *blaCTX-M-1***

Sixteen *blaCTX-M-1* genes were located on replicon type N plasmids of ~40–50 kb in *E. coli* isolates from cases of porcine gastrointestinal enteritis (n = 10), porcine urinary tract infection (n = 1), bovine gastritis/enteritis (n = 3), bovine urinary tract infection (n = 1) and from poultry suffering from septicaemia (n = 1). Two *blaCTX-M-1* 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 *blaCTX-M-1* 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>
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<tr>
<th>Isolate</th>
<th>Animal species</th>
<th>Disease condition</th>
<th>Resistance pattern a</th>
<th>Phyllogenetic group</th>
<th>MLST type</th>
<th>ESBL gene designation</th>
<th>PstI/DraI fragment (kb)</th>
<th>Conjugation</th>
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<td>11, N, P</td>
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<td>−</td>
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<th>Phylogenetic group</th>
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a 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.
b 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.21 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 IS26.21 This genetic context was located on 10 IncN plasmids ranging in size between 40 and 50 kb (pCTX328, pCTX338, pCTX895, pCTX1637, pCTX1876, pCTX2049, pCTX2251, 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 1.5 kb (Dral). The remaining seven plasmids, namely four IncN plasmids of 40–50 kb (pCTX1360, pCTX3780, pCTX1956 and pCTX4145), two IncI1 plasmids (pCTX1261 and pCTX1374) of 83 and 92 kb and the 53 kb IncF plasmid (pCTX2421) displayed individual PstI and Dral fragment patterns (Table 2).

The **blaCTX-M-1** upstream region on the IncN plasmids pCTX1875, pCTX3950, 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 pCTX1415, an IS26 was detected 272 bp apart from **blaCTX-M-1**. 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 **ecoRII** gene. A complete **ISEcp1** was identified on the IncI1 plasmids pCTX1261 and pCTX3780, while on the IncF plasmid pCTX2412 an **ISEcp1**-like insertion sequence, which differed slightly 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 pCTX3780, 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-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 ScaI 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**-like, 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 N. In the **blaTEM-52c** 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 IS911 and the **ISEcp1** continued downstream of the IS911-like insertion sequence. As previously described for IS911, no target site duplication was identified and the right inverted repeat downstream of the transposase was 5′-GAA-3′, while the left one was absent.28 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-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 IS911 and the **ISEcp1** continued downstream of the IS911-like insertion sequence. As previously described for IS911, no target site duplication was identified and the right inverted repeat downstream of the transposase was 5′-GAA-3′, while the left one was absent.28 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).
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}. 

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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 IncI1 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 F1A-F1B plasmid pCTX1929 was present in E. coli ST648. E. coli ST57 and ST648 harboured the blaCTX-M-2-Carrying plasmids pCTX2008 and pCTX3429, respectively. The E. coli isolate designated to the novel ST2698 harboured the blaTEM-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 a considerable structural heterogeneity was observed in the regions flanking the blaCTX-M-1 gene. The IS26-ΔISecp1-blaCTX-M-1-Δorf477-Δmrx-mpf(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 IncI1 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 transposon on IncF plasmids. However, the presence of the IS26-ΔISecp1-blaCTX-M-1-Δorf477-Δmrx-mpf(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-ΔISecp1-blaCTX-M-1-Δorf477-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 pCTX4145 (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 ISecp1-blaCTX-M-1-Δorf477 structures. Sequence analysis strongly suggested that the integration, which was most likely mediated by a one-sided ISecp1 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 ISecp1-blaCTX-M-15-Δorf477 inserted in a mrxA gene, which has been described for IncF plasmids before. The blaCTX-M-3 gene has been reported in association with ISecp1 or IS26-ΔISecp1 in the upstream region and Δorf477 in the downstream region, but the genetic arrangement on pCTX2207 has not been reported so far, with the ISecp1 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 morganii and E. coli. Complex class 1 integrons containing blaCTX-M-2 in combination with dfrA17 and adaA1 cassettes have been described on IncHI2 plasmids in S. enterica from Belgian poultry, but not on a multireplicon plasmid from cattle so far. IncHI1 plasmids carrying blaCTX-M-2 combined with dfrA17 and adaA5 cassettes in complex class 1 integrons have been reported recently in equine E. coli isolates from Belgium, but this is the first description of such a plasmid from German cattle.

IncI1 plasmids carrying blaTEM-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. This plasmid has been recently listed as lineages with a potential extended host spectrum genotype. In the present study, E. coli ST10 (n = 7) were identified among E. coli from swine and cattle suffering from gastrointestinal and urogenital tract infections and all of them harboured blaCTX-M-1 carrying IncN plasmids or the multireplicon plasmid pCTX99. In contrast, four E. coli ST167 from gastrointestinal tract infections of cattle and swine harboured different plasmid types and the ESBL genes blaCTX-M-1, blaCTX-M-3 or blaCTX-M-2. Among the isolates from cattle suffering from gastrointestinal infections, two had the sequence type ST648 and harboured IncF plasmids carrying blaCTX-M-1 and blaCTX-M-15, respectively. Furthermore, a poultry E. coli isolate from a case of septicaemia belonged to ST23 and one from porcine
gastrointestinal tract infection to ST131, both harbouring bla\textsubscript{CTX-M-1}\textsuperscript{-}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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