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Analysis of bla_{CTX-M}-Carrying Plasmids from *Escherichia coli* Isolates Collected in the BfT-GermVet Study▽

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In this study, 417 *Escherichia coli* isolates from defined disease conditions of companion and farm animals collected in the BfT-GermVet study were investigated for the presence of extended-spectrum β-lactamase (ESBL) genes. Three ESBL-producing *E. coli* isolates were identified among the 100 ampicillin-resistant isolates. The *E. coli* isolates 168 and 246, of canine and porcine origins, respectively, harbored $bla_{CTX-M-1}$, and the canine isolate 913 harbored $bla_{CTX-M-15}$, as confirmed by PCR and sequence analysis. The isolates 168 and 246 belonged to the novel multilocus sequence typing (MLST) types ST1576 and ST1153, respectively, while isolate 913 had the MLST type ST410. The ESBL genes were located on structurally related IncN plasmids in isolates 168 and 246 and on an IncF plasmid in isolate 913. The $bla_{CTX-M-1}$ upstream regions of plasmids pCTX168 and pCTX246 were similar, whereas the downstream regions showed structural differences. The genetic environment of the $bla_{CTX-M-15}$ gene on plasmid pCTX913 differed distinctly from that of both $bla_{CTX-M-1}$ genes. Detailed sequence analysis showed that the integration of insertion sequences, as well as interplasmid recombination events, accounted for the structural variability in the bla_{CTX-M} gene regions.

The production of β-lactamases is the predominant resistance mechanism against β-lactam antibiotics in *Enterobacteriaceae*. In contrast to narrow-spectrum β-lactamases, extended-spectrum β-lactamases (ESBLs) are able to hydrolyze the amide bond in the four-membered β-lactam ring of oxyimino-cephalosporins, such as cefotaxime (4). Nowadays, ESBLs are the major cause of clinical cephalosporin resistance among *Enterobacteriaceae* (24, 27). During recent years, ESBL-producing *Enterobacteriaceae* have been identified not only in the community (11, 20) but also in animals (2, 10, 16, 22, 34). Livermore et al. (20) stated that around the year 2000, dramatic shifts in the prevalence and types of ESBLs among *Enterobacteriaceae* occurred in Europe and that ESBLs of the CTX-M type have become predominant since then. In human isolates, the gene $bla_{CTX-M-15}$ has spread during recent years through the successful emergence of *E. coli* strains belonging to the multilocus sequence type ST131 (20). A recent study also showed that human pandemic O25:H4-ST131 *E. coli* carrying the $bla_{CTX-M-15}$ gene emerged among companion animals (14). Although different subtypes of ESBL genes seem to be prevalent in animals and humans, the predominant subtypes detected in members of the *Enterobacteriaceae* from food-producing animals, $bla_{CTX-M-1}$ and $bla_{CTX-M-2}$, were detected in four hospitals in Amsterdam, The Netherlands (1). Animals, especially food-producing animals, have been assumed to represent a reservoir for ESBL-producing bacteria (5), and this observation might suggest a transmission from the animal reservoir to the community. The spread of bla_{CTX-M} genes was shown to be due mainly to the dissemination of plasmids (10, 12, 24, 32). Thus, the investigation of plasmids is a key component for a better understanding of the dissemination and persistence of ESBL genes (6).

To date, little is known about the prevalence of bla_{CTX-M} genes in *Escherichia coli* strains from food-producing animals or companion animals in Germany. Therefore, the aims of the present study were as follows: (i) to investigate a representative collection of *E. coli* isolates from defined disease conditions of companion and farm animals for the presence of ESBL producers, (ii) to identify the types of ESBL genes present, (iii) to determine the location of the ESBL genes on plasmids or in the chromosomal DNA, and (iv) to sequence the ESBL gene region to gain insight into its organization.

MATERIALS AND METHODS

Bacterial isolates and susceptibility testing. The isolates included in this study originated from the BfT-GermVet monitoring study, which was carried out in Germany during the years 2004 to 2006 (30). In total, 1,626 bacterial isolates collected during these years from defined disease conditions of companion and farm animals have been tested for their susceptibility to 24 antimicrobial agents or combinations of antimicrobial agents by broth microdilution. This collection also included 417 *E. coli* isolates from diseased dogs/cats (n = 228), horses (n = 102), and swine (n = 87) (17). Among them, 100 *E. coli* isolates showed ampicillin resistance. They were from respiratory (n = 11), urinary/genital (n = 24), or gastrointestinal tract (n = 14) infections of cats/dogs, from urinary/genital tract infections, including mastitis-metritis-agalactia syndrome, of swine (n = 33), or from genital tract infections of horses (n = 18).

To identify potential ESBL producers, the 100 ampicillin-resistant *E. coli* isolates were subjected to an initial ESBL screening for growth in the presence of 1 μg/ml cefotaxime and subsequent ESBL phenotypic confirmatory tests as recommended by the Clinical and Laboratory Standards Institute (CLSI) (9).

Molecular analyses. The ESBL genes were identified by previously described PCR assays (13) and initially confirmed by sequence analysis of the amplicons. Multilocus sequence typing (MLST) was performed for the bla_{CTX-M}-positive *E. coli* isolates (36). Plasmids were transferred by conjugation or transformation as previously described (19), with subsequent selection on Luria-Bertani agar supplemented with ampicillin (100 μg/ml) or cefotaxime (1 μg/ml). Transconjugants and transformants were tested for their antimicrobial susceptibility by broth microdilution according to CLSI recommendations (9). Plasmids were subjected to PCR-based replicon typing (8), and plasmid sizes were determined by S1 nuclease pulsed-field gel electrophoresis (PFGE) using a Chef-DR III system (Bio-Rad, Munich, Germany). The DNA-containing agarose plugs were pre-

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TABLE 1. Characteristics of the three ESBL-producing *E. coli* isolates and their bla_{CTX-M}-carrying plasmids

Isolate	Animal species	Disease condition	Resistance pattern[a]	MLST	ESBL gene	ESBL-carrying plasmid	Replicon type	Approximate size (kb)	Colocated resistances[a]
168	Dog	Pneumonia	BLA, CHL, ENR, GEN, NEO, SMZ, SXT, TET	ST1576	$bla_{CTX-M-1}$	pCTX168	IncN	50	
246	Swine	Mastitis-metritis-agalactia syndrome	BLA, SMZ, SXT, TET	ST1153	$bla_{CTX-M-1}$	pCTX246	IncN	50	
913	Dog	Urinary tract infection	BLA, CHL, ENR, GEN, NEO, SMZ, SXT, TET	ST410	$bla_{CTX-M-15}$	pCTX913	IncF	50	GEN, TET

[a] BLA, β-lactams; CHL, chloramphenicol; ENR, enrofloxacin; GEN, gentamicin; NEO, neomycin; SMZ, sulfamethoxazole; SXT, sulfamethoxazole-trimethoprim (19:1); TET, tetracycline.

pared as recommended in the PulseNet protocol (29), and the slices were digested with S1 nuclease (Fermentas, St. Leon-Rot, Germany) as described previously (18). The pulse times were increased from 1 to 25 s over 17 h at a voltage of 6 V and an angle of 120°. *Salmonella enterica* serovar Braenderup H9812 digested with XbaI (Fermentas) and the MidRange PFG marker I (New England BioLabs, Frankfurt, Germany) were used as size markers.

The flanking regions of the bla_{CTX-M} genes were sequenced by primer walking using either the plasmid DNA obtained from the respective transformants and transconjugants or cloned plasmid fragments. For the latter approach, the cloning vector pBluescript(SK+) or pCR-BluntII-TOPO (both from Invitrogen, Groningen, The Netherlands) was used. Sequence comparisons were carried out using the software programs blastn and blastp (http://blast.ncbi.nlm.nih.gov/Blast.cgi), the ORF Finder (open reading frame finder) program (http://www.ncbi.nlm.nih.gov/gorf/gorf.html), and the IS finder program (http://www-is.biotoul.fr/is.html) (all last accessed 18 February 2011).

Nucleotide sequence accession numbers. The nucleotide sequences of the $bla_{CTX-M-1}$ genes, the $bla_{CTX-M-15}$ gene, and their flanking regions have been deposited in the EMBL database under accession numbers FN806788, FN806790, and FR828676.

RESULTS AND DISCUSSION

Presence and types of ESBL genes. According to their cefotaxime resistance and the subsequent ESBL phenotypic confirmatory tests, three ESBL-producing *E. coli* isolates were identified among the 100 ampicillin-resistant isolates (Table 1). Comparative analysis of the zone diameters of ceftazidime (30 μg) and ceftazidime-clavulanic acid (30/10 μg), as well as cefotaxime (30 μg) and cefotaxime-clavulanic acid (30/10 μg), showed a ≥5-mm increase in the zone diameter of either antimicrobial agent tested in combination with clavulanic acid versus its zone diameter when tested alone (9). PCR and sequence analysis confirmed that isolate 913 harbored a $bla_{CTX-M-15}$ gene while the remaining two isolates, 168 and 246, had a $bla_{CTX-M-1}$ gene.

Analysis of the $bla_{CTX-M-15}$ gene region of isolate 913. *E. coli* 913 was obtained in 2004 from the urine sample of a dog suffering from a urinary tract infection. It showed high MIC values for cefalothin (≥64 μg/ml), cefazolin (≥64 μg/ml), cefoperazone (≥32 μg/ml), ceftiofur (≥32 μg/ml), and cefquinome (≥32 μg/ml) and for the non-β-lactams chloramphenicol (128 μg/ml), enrofloxacin (≥32 μg/ml), gentamicin (64 μg/ml), neomycin (32 μg/ml), sulfamethoxazole (≥2,048 μg/ml), trimethoprim-sulfamethoxazole (≥64/1,216 μg/ml), and tetracycline (≥128 μg/ml). The isolate exhibited the MLST type ST410, which belongs to clonal complex 23. *E. coli* isolates of this clonal complex and harboring $bla_{CTX-M-15}$ genes have been found to be present among human patients in the United States (31) and in Brazil (26) and very recently in clinical and food samples from Spain (21). In isolate 913, the $bla_{CTX-M-15}$ gene was located on a plasmid of approximately 50 kb, designated pCTX913, which was transferable by transformation. Replicon typing confirmed that this plasmid belonged to the incompatibility group IncF. The plasmid also conferred resistance to gentamicin and tetracycline.

Sequencing of the $bla_{CTX-M-15}$ flanking regions revealed the presence of IS*Ecp1* in the upstream region. Downstream of the $bla_{CTX-M-15}$ gene, the terminal 342 bp of a reading frame for a hypothetical protein of 182 amino acids (aa), previously identified on the $bla_{CTX-M-3}$-carrying plasmid pCTX-M360 from *Klebsiella pneumoniae* (38), were detected. This segment was followed by a truncated transposase gene, *tnpA*, previously found on the bla_{TEM-1}-carrying transposon Tn*3* (Fig. 1). At the junction of both sequences, a potential recombination site was seen which might have served for a recombination event which led to the truncation of the reading frames of both the hypothetical protein and the Tn*3*-associated transposase.

A database search revealed that identical sequences downstream of the $bla_{CTX-M-15}$ gene were present in a wide variety of $bla_{CTX-M-15}$-carrying plasmids from *E. coli* (3, 13, 15, 32, 37) and *K. pneumoniae* (33) but also *Enterobacter cloacae* (GenBank accession no. HM204572, 219230) and *Serratia liquefaciens* (GenBank accession no. HM470254). Some of these plasmids were distinctly larger than pCTX913, belonged to different replicon types (including multireplicon types), and conferred additional resistance properties (3, 32, 37). This comparison showed that identical $bla_{CTX-M-15}$ genes are present in diverse plasmid backgrounds in different members of the *Enterobacteriaceae* from human and animal sources in different countries. Such an observation underlines the enormous flexibility of resistance plasmids to undergo structural changes which might render them resistant to different selective pressures by acquisition of additional resistance genes and able to replicate in different hosts by acquisition of additional replicons.

Analysis of the $bla_{CTX-M-1}$ gene regions of isolates 168 and 246. Isolate 168 was obtained in 2004 from a case of canine pneumonia. It showed high MIC values of amoxicillin combined with clavulanic acid (≥64/32 μg/ml), cefalothin (≥64 μg/ml), cefazolin (≥64 μg/ml), cefoperazone (≥32 μg/ml), ceftiofur (≥32 μg/ml), and cefquinome (≥32 μg/ml) and of chloramphenicol (128 μg/ml), enrofloxacin (≥32 μg/ml), gentamicin (64 μg/ml), neomycin (32 μg/ml), sulfamethoxazole (≥2,048 μg/ml), trimethoprim-sulfamethoxazole (≥64/1,216

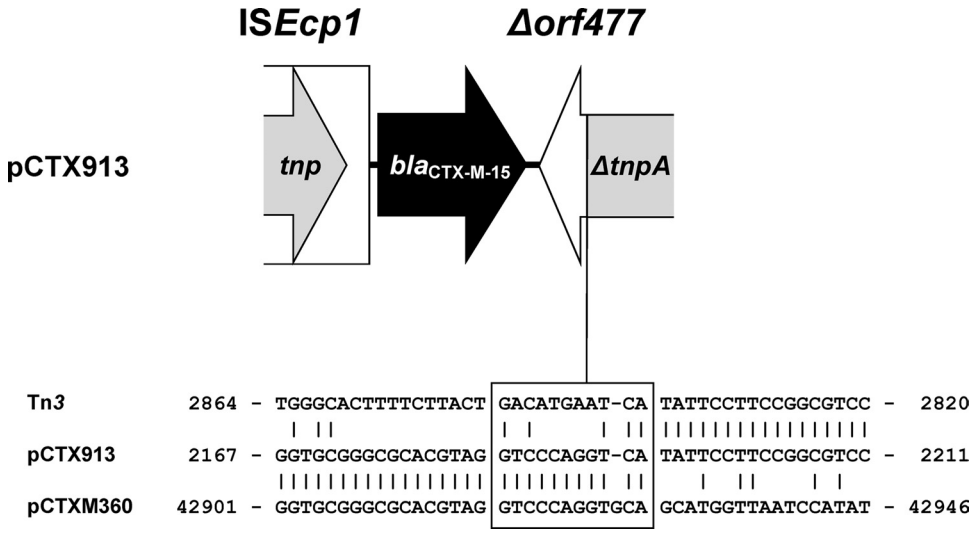

FIG. 1. Schematic presentation of the flanking gene regions of the $bla_{CTX-M-15}$ gene. The open reading frames are shown as arrows, with the arrowhead indicating the direction of transcription. IS elements are shown as boxes. The recombination site between the Tn*3*-associated *tnpA* gene and *orf477* is shown below the map of the $bla_{CTX-M-15}$ gene region. The sequences depicted in this alignment are from Tn*3* (GenBank accession no. V00613), pCTX913 (FR828676), and pCTXM360 (EU938349). Vertical bars indicate identical bases compared to the pCTX913 sequence. The area where the crossover most likely occurred is indicated by a box.

μg/ml), and tetracycline (≥128 μg/ml). Isolate 246 was identified in 2004 from a cervix swab of a sow suffering from the mastitis-metritis-agalactia syndrome. It showed high MIC values of cefalothin (≥64 μg/ml), cefazolin (≥64 μg/ml), cefoperazone (≥32 μg/ml), ceftiofur (≥32 μg/ml), and cefquinome (≥32 μg/ml) and of sulfamethoxazole (≥2,048 μg/ml), trimethoprim-sulfamethoxazole (≥64/1,216 μg/ml), and tetracycline (≥128 μg/ml).

Each of the two *E. coli* isolates belonged to a novel MLST type, namely, ST1576 (isolate 168) and ST1153 (isolate 246). The $bla_{CTX-M-1}$-bearing plasmids, designated pCTX168 and pCTX246, were approximately 50 kb in size and proved to be closely related in their BglII, DraI, KpnI, PstI, PvuI, and SspI fragment patterns. None of them conferred other resistance properties besides β-lactam resistance. Plasmid pCTX168 was transferable by conjugation, whereas plasmid pCTX246 was transferable by transformation. PCR-based replicon typing identified both plasmids as positive for IncN.

The genetic organization of the $bla_{CTX-M-1}$ gene regions of plasmids pCTX168 and pCTX246 is shown in Fig. 2. The upstream region of the $bla_{CTX-M-1}$ genes was similar in the two plasmids. It consisted of a 214-bp fragment of the insertion sequence IS*Ecp1*, including the right terminal repeat, which was truncated by the integration of an IS*26* element. In plasmid pCTX168, the IS*26* element and the region immediately downstream of it were completely sequenced, whereas it was sequenced only in part in pCTX246. Downstream of the IS*26* element in pCTX168, a small reading frame for a hypothetical protein of 65 aa was found. The same reading frame was also detected on the IncN plasmid R46 (NC_003292). Immediately upstream of this open reading frame, an incomplete reading frame for a protein was detected, of which the C-terminal 186 aa corresponded exactly to the Mrr protein of the IncN plasmid pKOX105 from *Klebsiella oxytoca* (7).

In the $bla_{CTX-M-1}$ downstream region, a truncated *mrx* gene was identified in both plasmids. This gene is part of the *mph*(A)-*mrx*-*mphR*(A) gene cluster, which is involved in macrolide resistance if complete (25). The truncation of the *mrx* gene is most likely the result of an interplasmid recombination event (Fig. 2). Sequence analysis identified two plasmids, pECL0701 from *Enterobacter cloacae* (GenBank accession no. FJ966096) and pTZ3509 from *E. coli* (25), which carried a complete *mph*(A)-*mrx*-*mphR*(A) gene cluster and displayed striking similarity to the sequences detected in plasmids pCTX168 and pCTXM246. A 12-bp region in plasmids pCTX168 and pCTXM246, which exhibited 75% identity to the pECL0701 sequence and 66.7% identity to the pTZ3509 sequence, was considered the recombination site. The part further upstream of the Δ*mrx* gene differed distinctly between the two plasmids. On plasmid pCTX246, a complete *mph*(A) gene and another IS*26* element, which is located in the same orientation as the IS*26* element in the $bla_{CTX-M-1}$ upstream region, were detected. In contrast, the *mph*(A) gene on plasmid pCTX168 was truncated by the integration of a segment homologous to plasmids pSE11-3 (28) and 55989p (35) from *E. coli* (Fig. 2). Further sequencing revealed an open reading frame for a putative protein of 133 aa without known function and part of a Tn*3*-associated resolvase gene. Another potential recombination site was identified, which might explain the truncation of the *mph*(A) gene in plasmid pCTX168.

These results showed that the structural variability of the $bla_{CTX-M-1}$ gene region resulted from interplasmid recombination events and the integration of insertion sequences. The gene $bla_{CTX-M-1}$ in combination with the IS*26*-ΔIS*Ecp1*-structure and the truncated *mph*(A)-*mrx*-*mphR*(A) gene cluster, which is followed by a second IS*26* element, linked to IncN plasmids, was recently found in a German human clinical *E. coli* ST131 isolate, proposing a novel composite transposon with both IS*26* elements being flanked by 8-bp direct repeats (5′-TTACCGGT-3′) (13). An identical sequence was located

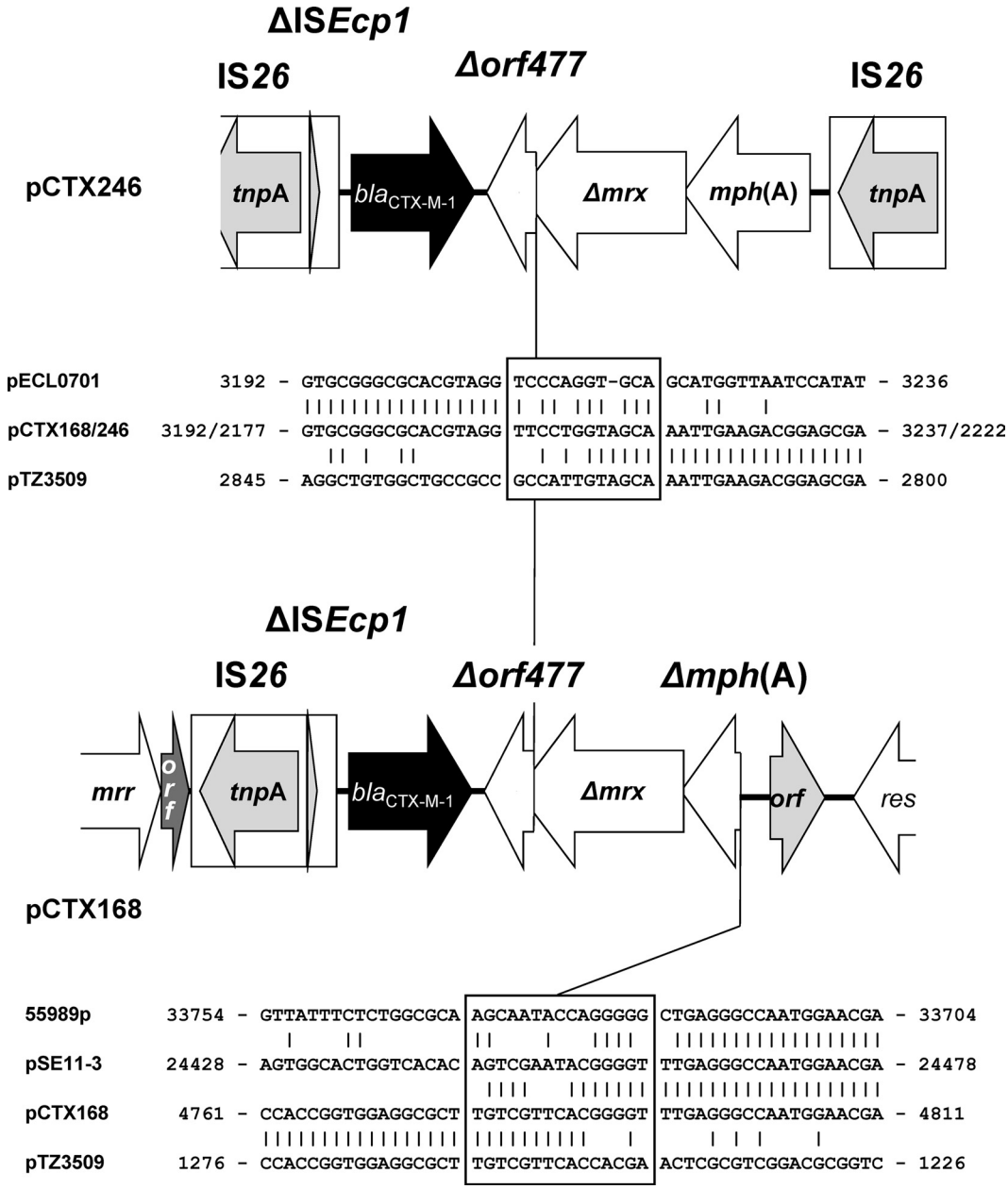

FIG. 2. Schematic presentation of the flanking gene regions of the $bla_{CTX-M-1}$ genes in plasmids pCTX246 and pCTX168. The open reading frames are shown as arrows, with the arrowhead indicating the direction of transcription. IS elements are shown as boxes. The recombination site between the *mrx* gene and *orf477* is shown between the two maps, while the recombination site that played a role in the truncation of the *mph*(A) gene in pCTX168 is shown below the map of pCTX168. The sequences depicted in these two alignments are from pCTX168 (GenBank accession no. FN806788), pCTX246 (FN806790), pECL0701 (FJ966096), pTZ3509 (AB038042), pSE11-3 (AP009243), and 55989p (CU928159). Vertical bars indicate identical bases compared to the pCTX246 and/or pCTX168 sequences or bases that are identical between the pSE11-3 and 55989p sequences. The areas where the crossovers most likely occurred are indicated by boxes.

upstream of the right-hand IS*26* in pCTX246, too, while in pCTX168 the direct repeat was found downstream of the IS*26*. This observation underlines the structural relatedness of these $bla_{CTX-M-1}$ gene regions, which have not been found in animal *E. coli* isolates from Germany so far. IncN plasmids carrying the $bla_{CTX-M-1}$ gene have been described to be transmitted between Danish farm personnel and pigs in different *E. coli* lineages (23). The finding of $bla_{CTX-M-1}$ genes with similar genetic environments on IncN plasmids in different human, porcine, and canine *E. coli* isolates from Germany points toward a plasmid transfer among *E. coli* isolates from human and animal sources and a common gene pool for transferable ESBL genes.

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