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Characteristics of extended-spectrum cephalosporin-resistant *Escherichia coli* and *Klebsiella pneumoniae* isolates from horses

An T.T. Vo [a,c], Engeline van Duijkeren [a,*], Ad C. Fluit [b], Wim Gaastra [a]

[a] *Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, PO Box 80165, 3508 TD Utrecht, The Netherlands*
[b] *Eijkman-Winkler Institute, University Medical Center Utrecht, The Netherlands*
[c] *Faculty of Animal Husbandry and Veterinary Medicine, NongLam University, Vietnam*

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Abstract

The aim of the present study was to contribute to the knowledge on extended-spectrum β-lactamases (ESBL's), AmpC β-lactamases and integrons in Enterobacteriaceae isolated from horses, which is still limited. The susceptibility of 1581 clinical isolates from animals to ceftiofur was tested. Most of these isolates (n = 1347) originated from horses. Seven ceftiofur-resistant equine isolates (four *Escherichia coli* and three *Klebsiella pneumoniae*) were identified and all seven were multidrug-resistant. These isolates were further studied for the presence of ESBL's, AmpC β-lactamases and class 1 integrons. The potential for the horizontal transfer of resistance genes among these clinical isolates was also studied. ESBL-type resistance genes were found in five isolates, AmpC-type genes in one isolates and integrons in six isolates. Nucleotide sequence analysis revealed that the isolates carried the $bla_{\text{CTX-M-1}}$, $bla_{\text{CMY-2}}$, $bla_{\text{TEM-1}}$ and/or $bla_{\text{SHV-1}}$ genes. This is the first report describing the in vitro conjugal transfer of the $bla_{\text{CTX-M-1}}$ genes from a clinical *E. coli* isolate to *Salmonella* isolates. Gene cassettes encoding resistance to aminoglycosides (*aadA1*, aad*A2* and *aadA5*), and trimethoprim (*dfrA1, drfA12 and dfrA17*) were found on the integrons present in the isolates. The cassette arrays of the *dfrA17-aadA5* and *dfrA1-aadA1* genes in the two integrons of a single *E. coli* isolate have not yet been described before. To our knowledge this is the first report on ESBL's and AmpC β-lactamases in equine *E. coli* and *Klebsiella* isolates.

Keywords: Extended spectrum β-lactamases; AmpC; Enterobacteriaceae; Horse; Antibiotic resistance

1. Introduction

The introduction of the third-generation cephalosporins (TGC) in the early 1980s improved clinical practice in both human and veterinary medicine. Unfortunately, soon after their introduction resistance to extended-spectrum cephalosporins (ESC) was

* Corresponding author. Tel.: +31 30 253 3603; fax: +31 30 253 3199.
E-mail address: E.duijkeren@vet.uu.nl (E. van Duijkeren).

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detected (Bradford, 2001). ESC resistance has been studied in detail in Enterobacteriaceae isolated from humans and to a lesser extent in isolates from food-producing animals (Philippon et al., 2002; Paterson and Bonomo, 2005). Data on ESC-resistant Enterobacteriaceae from companion animals and horses is limited (Miriagou et al., 2004).

Commonly, TGC resistance in Enterobacteriaceae relates to the production of extended-spectrum β-lactamases (ESBL) (Ambler et al., 1991) or AmpC β-lactamases (Philippon et al., 2002). ESBLs confer resistance to the penicillins, cephalosporins and aztreonam and are usually inhibited by β-lactamase inhibitors. AmpC β-lactamases have an even broader resistance spectrum including the cephamycins and are not blocked by β-lactamase inhibitors (Philippon et al., 2002). ESC-resistant strains are often also resistant to fluoroquinolones (Tolun et al., 2004; Paauw et al., 2006), or contain integrons encoding antimicrobial resistance (Wang et al., 2003; Paauw et al., 2006), which even further narrows the treatment options. Integrons are gene capture systems that contain genetic determinants for recognition and incorporation of mobile gene cassettes (Hall and Stokes, 1993). Among three distinct classes of integrons described in clinical Enterobacteriaceae isolates, class 1 integrons are the most significant. If resistance determinants are located on self-transmissible plasmids, the chance that resistance spreads to other bacteria is highly increased.

In the present work the antimicrobial resistance characteristics of seven TGC-resistant *Escherichia coli* and *Klebsiella pneumoniae* isolates from horses were determined. Our focus was on genes responsible for resistance to TGC and resistance genes associated with class 1 integrons. Subsequently, the potential of in vitro self-transfer of the resistance elements between the seven TGC-resistant isolates and different multidrug-resistant (MDR) clinical *Salmonella* isolates was investigated.

2. Materials and methods

2.1. Bacterial isolates

In the present study, the criterion for isolate selection was resistance to ceftiofur. In the study

Table 1
Characteristics of extended-spectrum cephalosporin-resistant Enterobacteriaceae isolates

ID number	Historical data of the animals				Isolation date	Resistance patterns	ESBL		AmpC		Other β-lactamase genes	Integron	
	Species	Age	Sex	Specimen			DDT	genes	ADT	gene		Size (bp)	Gene cassettes
E1	Horse	10 years old	M	pus	11-2003	A(Ac)CeCefCtxSGKCTSuTp	+	CTX-M-1	–	–	TEM-1	1700	*dfrA17, aadA5*
E2	Horse	17 years old	F	stomach	12-2003	A(Ac)CeCefCaz(Ctx)SGTNorSuTp	–	–	+	CMY-2	TEM-1	1700	*dfrA17, aadA5*
E3	Horse	1 month old	F	synoviae	05-2004	A(Ac)CefCtxSGKCTSuTp	+	CTX-M-1	–	–	–	1700/1650	*dfrA17, aadA5/dfrA1, aadA1*
E4	Horse	9 years old	F	uterus	05-2005	A(Ac)CeCefG	+	unidentified	–	–	–	–	–
K1	Horse	23 years old	M	pus	04-2004	A(Ac)CeCefCtxSGKCTSuTp	+	CTX-M-1	–	–	TEM-1, SHV-1	2000	*dfrA12, orfF, aadA2*
K2	Horse	1 month old	F	feces	04-2004	A(Ac)CeCefCtxSGKCTSuTp	+	CTX-M-1	–	–	TEM-1, SHV-1	2000	*dfrA12, orfF, aadA2*
K3	Horse	1 month old	M	feces	05-2004	A(Ac)CeCefCtxSGKCTSuTp	+	CTX-M-1	–	–	SHV-1	2000	*dfrA12, orfF, aadA2*

Antimicrobials in brackets, intermediate resistance. Abbreviations used: E, *Escherichia coli*; K, *Klebsiella pneumonia*; S, *Salmonella* Braenderup; M, male; F, female; A, ampicillin; Ac, amoxicillin/clavulanate; Ce, cephalexin; Cef, ceftiofur; Caz; ceftazidime; Ctx, cefotaxime; S, streptomycin; G, gentamicin; K, kanamycin; T, tetracycline; C, chloramphenicol; Nor, norfloxacin; Su, sulfamethozole; Tp, trimethoprim; na, not available; DDT, double disk test; ADT, AmpC disk test; –, not found.

Table 2
In vivo conjugation experiments between resistant clinical isolates of Enterobacteriaceae

ESC resistant isolates			mating	MDR *Salmonella* isolates				Transconjugants found		
Name	Species	Criteria		Name	Sero-phage type	R-phenotype	Criteria	Identified as	Resistance phenotype	ESC-resistance determinant
E1	*E. coli*	$Caz^{R}Nor^{S}$	×	S57	Typhimurium UT	AAcSGKCTSuTpNor	$Caz^{S}Nor^{R}$	*S.* Typhimurium S57 × E1	A(Ac)CefCtxSGKCTSuTpNor	CTX-M-1
E1	*E. coli*	$Caz^{R}Nor^{S}$	×	S58	Typhimurium UT	AAcSGKCTSuTpNor	$Caz^{S}Nor^{R}$	*S.* Typhimurium S58 × E1	A(Ac)CefCtxSGKCTSuTpNor	CTX-M-1
E1	*E. coli*	$Caz^{R}Nor^{S}$	×	S60	Typhimurium 507	AAcSGKCTSuTpNor	$Caz^{S}Nor^{R}$	*S.* Typhimurium S60 × E1	A(Ac)CefCtxSGKCTSuTpNor	CTX-M-1
E3	*E. coli*	$Caz^{R}Nor^{S}$	×	S57	Typhimurium UT	AAcSGKCTSuTpNor	$Caz^{S}Nor^{R}$	–	–	–
E3	*E. coli*	$Caz^{R}Nor^{S}$	×	S58	Typhimurium UT	AAcSGKCTSuTpNor	$Caz^{S}Nor^{R}$	–	–	–
E3	*E. coli*	$Caz^{R}Nor^{S}$	×	S60	Typhimurium 507	AAcSGKCTSuTpNor	$Caz^{S}Nor^{R}$	–	–	–
K1	*K. pneumoniae*	$Caz^{R}Nor^{S}$	×	S57	Typhimurium UT	AAcSGKCTSuTpNor	$Caz^{S}Nor^{R}$	–	–	–
K1	*K. pneumoniae*	$Caz^{R}Nor^{S}$	×	S58	Typhimurium UT	AAcSGKCTSuTpNor	$Caz^{S}Nor^{R}$	–	–	–
K1	*K. pneumoniae*	$Caz^{R}Nor^{S}$	×	S60	Typhimurium 507	AAcSGKCTSuTpNor	$Caz^{S}Nor^{R}$	–	–	–
K2	*K. pneumoniae*	$Caz^{R}Nor^{S}$	×	S57	Typhimurium UT	AAcSGKCTSuTpNor	$Caz^{S}Nor^{R}$	–	–	–
K2	*K. pneumoniae*	$Caz^{R}Nor^{S}$	×	S58	Typhimurium UT	AAcSGKCTSuTpNor	$Caz^{S}Nor^{R}$	–	–	–
K2	*K. pneumoniae*	$Caz^{R}Nor^{S}$	×	S60	Typhimurium 507	AAcSGKCTSuTpNor	$Caz^{S}Nor^{R}$	–	–	–
K3	*K. pneumoniae*	$Caz^{R}Nor^{S}$	×	S57	Typhimurium UT	AAcSGKCTSuTpNor	$Caz^{S}Nor^{R}$	–	–	–
K3	*K. pneumoniae*	$Caz^{R}Nor^{S}$	×	S58	Typhimurium UT	AAcSGKCTSuTpNor	$Caz^{S}Nor^{R}$	–	–	–
K3	*K. pneumoniae*	$Caz^{R}Nor^{S}$	×	S60	Typhimurium 507	AAcSGKCTSuTpNor	$Caz^{S}Nor^{R}$	–	–	–
E2	*E. coli*	$Caz^{R}K^{S}$	×	S103	Blockley	SKNaT	$Caz^{S}K^{R}$	–	–	–
E2	*E. coli*	$Caz^{R}K^{S}$	×	S161	Typhimurium 510	SGKTSuTp	$Caz^{S}K^{R}$	–	–	–
E2	*E. coli*	$Caz^{R}K^{S}$	×	S283	Rissen	ASKCTSuTp	$Caz^{S}K^{R}$	–	–	–
E2	*E. coli*	$Caz^{R}K^{S}$	×	S15	Typhimurium 90	ASGKNaTSuTp	$Caz^{S}K^{R}$	*E. coli* E2 × S15	A(Ac)(Caz)Cef(Ctx)SGKTSuTpNor	CMY-2
E2	*E. coli*	$Caz^{R}K^{S}$	×	S305	Typhimurium RDNC	ASGKNaTSuTp	$Caz^{S}K^{R}$	*E. coli* E2 × S305	A(Ac)(Caz)Cef(Ctx)SGKTSuTpNor	CMY-2
E2	*E. coli*	$Caz^{R}K^{S}$	×	S309	Typhimurium 507	ASGKNaTSuTp	$Caz^{S}K^{R}$	*E. coli* E2 × S309	A(Ac)(Caz)Cef(Ctx)SGKTSuTpNor	CMY-2
E4	*E. coli*	$Caz^{R}K^{S}$	×	S103	Blockley	SKNaT	$Caz^{S}K^{R}$	–	–	–
E4	*E. coli*	$Caz^{R}K^{S}$	×	S161	Typhimurium 510	SGKTSuTp	$Caz^{S}K^{R}$	–	–	–
E4	*E. coli*	$Caz^{R}K^{S}$	×	S283	Rissen	ASKCTSuTp	$Caz^{S}K^{R}$	–	–	–
E4	*E. coli*	$Caz^{R}K^{S}$	×	S15	Typhimurium 90	ASGKNaTSuTp	$Caz^{S}K^{R}$	–	–	–
E4	*E. coli*	$Caz^{R}K^{S}$	×	S305	Typhimurium RDNC	ASGKNaTSuTp	$Caz^{S}K^{R}$	–	–	–
E4	*E. coli*	$Caz^{R}K^{S}$	×	S309	Typhimurium 507	ASGKNaTSuTp	$Caz^{S}K^{R}$	–	–	–

Abbreviations used: ESC, extended-spectrum cephalosporin; MDR, multi drug resistance; R, resistance; E, *Escherichia coli*; K, *Klebsiella pneumonia*; UT, untypeable; RDNC, reaction does not conform to any recognized phage types; A, ampicillin; Ac, amoxicillin/clavulanate; Ce, cephalexin; Cef, ceftiofur; Caz; ceftazidime; Ctx, cefotaxime; S, streptomycin; G, gentamicin; K, kanamycin; T, tetracycline; C, chloramphenicol; Nor, norfloxacin; Su, sulfamethozole; Tp, trimethoprim; antimicrobials in brackets, intermediate resistance.

period from 2003 to 2005, 1581 isolates were tested for their susceptibility to ceftiofur at the veterinary microbiology diagnostic center (VMDC) of Utrecht University. Most isolates ($n = 1347$, 85%) originated from horses. Thirty-three ceftiofur-resistant bacteria were present, most of which were not Enterobacteriaceae. Only seven epidemiologically unrelated Enterobacteriaceae isolates were found and included in the present study (four *E. coli* and three *K. pneumonia* isolates E1–E4 and K1–K3). The isolates were cultured from different horses coming from different regions in The Netherlands (Table 1). Species and serovars were identified by conventional methods at the VMDC and at the RIVM (the Dutch National Institute of Public Health and the Environment). The *Salmonella* isolates, used in the conjugation experiments (see below) were from a *Salmonella* collection obtained from humans and animals in Vietnam (Vo et al., 2006c). Only norfloxacin- or kanamycin-resistant and ceftazidime-susceptible isolates were selected (Table 2). A clinical *E. coli* isolate with a confirmed FOX β-lactamase was used as positive control in the AmpC *β-lactamase* genes determination.

2.2. Antimicrobial susceptibility testing

Susceptibility of the isolates to antimicrobials was tested by a disk diffusion assay using Iso-sensitest agar (Oxoid, UK) and Neo-sensitab discs (Rosco, Denmark) based on the procedure recommended by the Dutch Committee on guidelines for susceptibility testing CRG (Commissie Richtlijnen Gevoeligheidsbepalingen (CRG), 2000). The antimicrobials used were ampicillin (30 μg), amoxicillin/clavulanic acid (30/15 μg), cephalexin (30 μg), ceftazidime (30 μg), ceftiofur (30 μg), cefotaxime (30 μg), streptomycin (100 μg), gentamicin (40 μg), kanamycin (100 μg), tetracycline (80 μg), norfloxacin (10 μg), chloramphenicol (60 μg), sulphonamides (240 μg), and trimethoprim (5.2 μg). To determine the appropriate concentration of antibiotics for the conjugation experiments, a microbroth dilution method was used for the donors and the recipients with the antimicrobials kanamycin, norfloxacin, and ceftazidime (Sigma, Germany) according to the guidelines of the clinical and laboratory standards institute (CLSI, formerly the national committee for clinical laboratory standards–NCCLS) (National Committee for Clinical Laboratory Standards, 2001). *E. coli* ATCC 25922 and *E. coli* ATCC 35218 were used as reference strains.

2.3. Extended spectrum beta-lactamase (ESBL) investigation

2.3.1. Double disk test

The seven isolates were tested for the phenotypic presence of ESBL. The test is based on the synergy between clavulanate and the indicator cephalosporins as described (Jarlier et al., 1988; Emery and Weymouth, 1997).

2.4. Genetic characterization of ESBL genes

The same isolates were analysed for the presence of TEM, SHV, OXA and CTX-M type ESBLs by PCR (Bradford, 1999; Olesen et al., 2004; Hasman et al., 2005; Paauw et al., 2006). Template DNA was prepared by the whole bacteria cell boiled lysate method (Levesque et al., 1995). PCR products of the expected size were extracted from agarose gel using the Qiaquick Gel Extraction kit (Qiagen, Germany). The purified PCR products were sequenced on an ABI 3730 sequencer (Foster, USA). Nucleotide sequences were analyzed using Clone Manager Suite (version 8). Mutations were determined by consulting sequence data in the GenBank database and on website http://www.lahey.org/study. *E. coli* 09A488, *K. pneumoniae* 09A018 and *Enterobacter cloacae* 03773 which harbour TEM-1, SHV and CTX-M-9 type β-lactamase, respectively, were included as positive controls.

2.5. AmpC-type β-lactamases investigation

2.5.1. AmpC disk test

The isolates were also investigated for the presence of AmpC-type β-lactamases. In this test Tris-EDTA is used to permeabilise the bacterial cell wall which releases the β-lactamases into the external environment (Black et al., 2005).

2.5.2. Genetic characterization of plasmid-mediated AmpC β-lactamase genes

The presence of the AmpC β-lactamase genes frequently found in Enterobacteriaceae was tested by

multiplex PCR using the MOX (for detecting $bla_{\text{MOX-1, -2, CMY-1, -8–11}}$ genes), CIT (for detecting $bla_{\text{LAT-1–4, CMY-2–7, BIL-1}}$ genes), DHA (for detecting $bla_{\text{DHA-1, -2}}$ genes), ACC (for detecting bla_{AAC} gene), EBC (for detecting $bla_{\text{MIR-1, ACT-1}}$ genes and FOX (for detecting $bla_{\text{FOX-1–5b}}$ genes) primers as described (Perez-Perez and Hanson, 2002). The nucleotide sequence of the amplicons was determined to confirm the identity of the β-lactamase gene in question. The procedures for purification of PCR products, sequencing, and sequence analysis were the same as used for the ESBL genes. Sequences were analysed by searching the GenBank database. *E. coli* pNU2936, pMG247 (a gift from Dr G.A. Jacoby) carrying MOX-1 and DHA-1 β-lactamase, respectively, were used as positive controls.

2.6. Class 1 integron detection and characterization of gene cassettes

The detection of class 1 integrons and the genetic characterization of inserted gene cassettes were performed as described (Vo et al., 2006a). The obtained nucleotide sequences of the gene cassettes were analyzed with the Clone Manager Suite by consulting the GenBank database via the BLAST network service.

2.7. Nucleotide accession numbers

The sequences of the ESBL genes detected in this study, the $bla_{\text{CTX-M-1}}$ from *E. coli* isolate E1, the $bla_{\text{CMY-2}}$ from *E. coli* isolate E2, and the $bla_{\text{CTX-M-1}}$ from *K. pneumoniae* isolate K2, are listed in the GenBank database under accession numbers DQ663489, DQ663490, and DQ663591, respectively. The sequences of the 1.65 and 1.7 kb-amplicons containing the *dfrA1-aadA1* and *dfr17-aadA5* genes from *E. coli* isolate E3 have been assigned the GenBank accession numbers DQ663487 and DQ663488.

2.8. Conjugation assay

A conjugation experiment was performed to study whether resistance determinants can be transferred between different Enterobacteriaceae and whether ESC-resistant isolates can play a role as donor or recipient in the horizontal transfer of antibiotic resistance. To find suitable acceptor and donor bacteria for the mating experiments, nine MDR *Salmonella* isolates were selected as mentioned in Section 2 under Bacterial isolates. The scheme of the conjugation experiments, in which the MDR *Salmonella* strains and the ESC-resistant *E. coli* or *Klebsiella* isolates could either act as donor or recipient, is shown in Table 2. Conjugation was carried out using the broth mating method (Vo et al., 2006b) except that the MacConkey agar was supplemented with a combination of ceftazidime (8 μg/ml) and kanamycin (256 μg/ml) or a combination of ceftazidime (8 μg/ml) and norfloxacin (32 μg/ml) to select for transconjugants. The transconjugants were identified as *Salmonella* or *E. coli* using the API 20E system (bioMerieux, France). *Salmonella* transconjugants were serotyped by slide agglutination with polyvalent antisera and antisera specific for the O antigens of *Salmonella* (Staten Serum Institute, Denmark). Transconjugants were also assayed for their susceptibility to 15 antimicrobial agents and the antibiograms were compared with those of the donors and recipients. The presence of the ESC resistance genes in the transconjugants was demonstrated by PCR.

2.9. Plasmid profiling and southern hybridization

Transconjugants, donors and recipients were subjected to plasmid analysis using a modified alkaline lysis method (Kado and Liu, 1981) to determine which plasmid carrying ESC genes had been transferred. Subsequently, southern blot hybridization (Maniatis et al., 1982) with bla_{ACC}, $bla_{\text{CTX-M}}$ or bla_{CMY} probes was performed to detect the target genes. *S.* Typhimurium phage type 13 containing five plasmids of different sizes (180, 82, 39, 5.5 and 4.4 kbp) was used as a standard for the determination of plasmid sizes. Hybridization took place at 60 °C for the bla_{CMY} probes and at 55 °C for the $bla_{\text{CTX-M}}$ probe. The presence of any hybridized targets was detected using the alkaline phosphate-conjugated antibody DNA detection kit (Roche, Germany) and NTB/BCIB (bromo-4-chloro-3′-indolyphosphate p-toluidine salt/nitro-blue tetrazolium chloride). Between hybridizations the membrane was stripped as described by the manufacturer.

3. Results

3.1. Resistance phenotype

All seven ceftiofur-resistant *E. coli* and *K. pneumoniae* isolates were multidrug-resistant. The phenotypic resistance patterns of the isolates are documented in Table 1. Seven isolates were positive in the phenotypic tests for ESC, six isolates in the double disk test for ESBL confirmation and one isolate in the AmpC disk test (Table 1).

3.2. β-lactamase genes

ESBL genes were detected in five of the six isolates positive in the double disk test (Table 1). The presence of TEM-, SHV- and CTX-M type ESBL genes was detected by PCR and confirmed by nucleotide sequencing. The CTX-M-1 gene, was detected in two *E. coli* isolates (E1 and E3) and three *K. pneumoniae* isolates (K1, K2, and K3). One *E. coli* isolate (E4) was considered an ESBL producer with an unidentified mechanism since this isolate was positive in the double disk test but neither TEM type-, SHV-type-, CTX-M type-, nor OXA-type-ESBL genes could be detected. One isolate tested positive in the AmpC disk test and was positive for the CMY group of β-lactamases in the PCR. Nucleotide sequencing followed by a BLAST search confirmed the identity of the gene encoding the AmpC-type β-lactamase. The $bla_{\text{CMY-2}}$ gene was found in the *E. coli* isolate E2. In addition to the genes encoding for ESC-resistance, TEM-1 and SHV-1 β-lactamase genes were present in five out of seven isolates. In two *K. pneumoniae* isolates, three genes encoding β-lactamases (TEM-1, SHV-1, and CTX-M-1) were detected (Table 1).

3.3. Class 1 integron and resistance gene cassettes

Six integron-carrying isolates were identified by int-PCR. The results are summarized in Table 1. Three types of class 1 integrons were found. Gene cassettes encoding resistance to aminoglycosides (*aadA1*, *aadA2* and *aadA5*) or to trimethoprim (*dfrA1*, *dfrA12* and *dfrA17*) were present in these integrons. Remarkably, the presence of the two class 1 integrons (amplicons of 1650 and 1700 bp with *dfrA1-aadA1* and *dfrA17-aadA5*, respectively) was detected in an *E. coli* isolate (E3) cultured from a one month-old foal.

3.4. Horizontal transfer of integrons and resistance determinants

Resistance determinants could be exchanged between *Salmonella* isolates and *E. coli* isolates but not between *Salmonella* and *K. pneumoniae*. In the 30 conjugations performed, 9 transconjugants were obtained (Table 2).

(i) TGC-resistant *E. coli* as a donor; *Salmonella* Typhimurium as a recipient: the recipients from conjugation experiment of *E. coli* isolate E1 and *S.* Typhimurium S57, S58 or S60 were identified as *S.* Typhimuirum with resistance characteristics shown in Table 2. *E. coli* isolate E1 could transfer its $bla_{\text{CTX-M-1}}$ gene to *S.* Typhimurium S57, S58 and S60 since these genes were detected by PCR in the donor (E1) and the three transconjugants but not in the recipients. Phenotypic resistance to ceftazidime was confirmed in the three transconjugants. Plasmid profiles of the donor, the recipients and the transconjugants were similar (see Appendix B supplementary figure).

(ii) *Salmonella* Typhimurium as a donor; ESC-resistant *E. coli* as a recipient: The recipients from conjugation experiment of *E. coli* isolate E2 and *S.* Typhimurium S15, S305 or S309 were identified as *E. coli* with resistance characteristics shown in Table 2. *E. coli* isolate E2 which carries the $bla_{\text{CMY-2}}$ gene obtained a kanamycin resistance determinant probably from the donors (S15, S305 and S309) since the transconjugants *E.coli* (E2 × S15, E2 × S305, E2 × S309) were phenotypically resistant to both ceftazidime and kanamycin. No difference was observed between the plasmid profile of the *E. coli* recipient E2 and those of the *E. coli* transconjugants (E2 × S15, E2 × S305, E2 × S309).

4. Discussion

The presence of ESBL and AmpC type genes encoding resistance to extended-spectrum cephalosporins among Enterobacteriaceae isolates from horses

(including horses of one month old) is an alarming finding. In The Netherlands, extended-spectrum cephalosporins (ceftiofur, cefquinome) are prescription-only medicines approved for the treatment of infections of the respiratory tract and pododermatitis interdigitalis in cattle and pigs (Bureau Diergeneesmiddelen, 2005), but they were not licensed for horses during the study period. However, ceftiofur is used off-label for the treatment of equine infections.

To the best of our knowledge, the presence of the cassette arrays in two class 1 integrons (one which yields a CS-amplicon of 1700 bp containing the *dfrA17-aadA5* genes and one yielding a CS-amplicon of 1650 bp containing the *dfrA1-aadA1* genes) in a single *E. coli* isolate has not been described before. Interestingly, the gene cassettes of these two integrons contain distinct genes but encode resistance against the same antibiotics: the *dfrA1* and *dfrA17* genes both against trimethoprim; the *aadA1* and *aadA5* genes both against streptomycin and spectinomycin. Besides the genes encoding resistance to extended-spectrum cephalosporins, almost all (six out of seven) isolates carried one or two other genes encoding β-lactam resistance, two to four genes encoding resistance to aminoglycosides or trimethoprim and apparently also other resistance determinants were present. Five isolates were resistant to up to 11 antimicrobials including the fluoroquinolones. Such multidrug resistance in pathogenic bacteria severely limits therapeutic options. This is a serious concern for veterinary medicine but also for human medicine since direct transfer of ESC-resistant isolates from animals to humans has been documented (Arlet et al., 2006).

Conjugal experiments to transfer antimicrobial resistance are usually performed with reference strains (*E. coli* K12) or strains adapted for conjugation (Hasman et al., 2005). In the present study, in vitro conjugation was performed between clinical isolates. Resistance to antibiotics which were not observed in the recipients (to ceftiofur and ceftazidime in *Salmonella* S57, S58, S60) was detected in the transconjugants. However, the plasmid profiles of the recipients and the transconjugants were identical. A possible explanation for this might be that transferred resistance determinants were located on a low-copy plasmid which was not detected. In the present study, *K. pneumoniae* isolates did not transfer their resistance determinants by conjugation but in a previous study, Leverstein-van Hall et al. (2002) reported clinical human *K. pneumonia* and *K. oxytoca* isolates which could transfer their integrons and TEM-1, SHV-1 genes to *E. coli* K12. Together, these data indicate the ease of the spread of multiple resistance determinants among Enterobacteriaceae. Companion animals, i.e. horses, are an underreported source of TGC-positive Enterobacteriaceae.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vetmic.2007.04.027.

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