Studies on the prevalence, distribution and organization of extended-spectrum β-lactamase genes and transferable (fluoro)quinolone resistance genes among Enterobacteriaceae from defined disease conditions of companion and farm animals

THESIS
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by

Anne-Kathrin Schink
from Hannover

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Supervisor: Prof. Dr. Stefan Schwarz
Supervision Group: Prof. Dr. Stefan Schwarz
Prof. Dr. Günter Klein
Prof. Dr. Peter Heisig

1st Evaluation: Prof. Dr. Stefan Schwarz,
Friedrich-Loeffler-Institut (FLI)
Institute of Farm Animal Genetics, Neustadt-Mariensee
Prof. Dr. Günter Klein,
Institute of Food Quality and Food Safety, University of Veterinary Medicine Hannover, Hannover
Prof. Dr. Peter Heisig,
Pharmaceutical Biology and Microbiology, Department of Chemistry, University of Hamburg, Hamburg

2nd Evaluation: Prof. Dr. Dik Mevius,
Faculty of Veterinary Medicine, Department of Infectious Diseases and Immunology, Utrecht University, Utrecht, The Netherlands
Central Veterinary Institute of Wageningen, Lelystad, The Netherlands

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For my family and friends,

even though they think
my work is just *bla, bla*. 
Parts of the thesis have already been published:


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**Schink, A.-K., Kadlec, K., & Schwarz, S.** (2010). Comparative analysis of the plasmid-borne $\text{bla}_{\text{CTX-M-1}}$ regions of *Escherichia coli* from different animal sources.


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Chapter 1

Introduction
Introduction

Antimicrobial agents are indispensable for the control of bacterial infections. Among the diverse classes of antimicrobial agents, β-lactam antibiotics and nowadays fluoroquinolones, which were considered as antibiotics of last resort, were frequently used in human medicine in Germany (Kern & Nink, 2011; de With et al., 2011). In veterinary medicine, little information about the application of antimicrobial agents is currently available (Schneidereit, 2011). Instead, sales figures of active pharmaceutical ingredients have been compiled for the year 2005 by the Federation of Animal Health (Bundesverband für Tiergesundheit BfT) (Schneidereit, 2008).

Treatment failure is very often due to acquired resistance determinants, which limit therapeutic options considerably.

During the last years the occurrence of extended-spectrum β-lactamases (ESBLs) and plasmid-mediated quinolone resistances (PMQR) within the family Enterobacteriaceae has gained particular attention. Members of this family can be harmless colonisers of the gut, but some of them can cause severe gastrointestinal and even extraintestinal infections in both humans and animals.

ESBLs have been first described in the early 1980s and confer resistance to β-lactam antibiotics such as penicillins, cephalosporins and monobactams. The corresponding genes have developed by point mutations from known narrow-spectrum β-lactamase genes, namely \textit{bla}$_{\text{SHV}}$ and \textit{bla}$_{\text{TEM}}$. The amino acid exchanges which resulted from the point mutations led to an expansion of the hydrolysing activity of these enzymes from penicillins to cephalosporins and monobactams. In 1989, a novel ESBL gene has been detected in an \textit{E. coli} isolate from Germany, which has been genetically unrelated to any known β-lactamase gene and designated \textit{bla}$_{\text{CTX-M-1}}$ (Bauernfeind et al., 1990). According to Livermoore et al. (2007), CTX-M ESBLs are the predominant ESBL type in Europe. Nowadays, 122 CTX-M ESBLs have been deposited in the Lahey database (http://www.lahey.org/studies/webt.htm) and represent five distinct clusters based on their amino acid sequence homology.
The first PMQR gene, \textit{qnrA}, has been described in 1998 (Martínez-Martínez et al., 1998). Qnr proteins protect the DNA-gyrase-complex, the target of quinolones and fluoroquinolones, and thus mediate resistance to quinolones and decreased susceptibility to fluoroquinolones. More \textit{qnr} genes, \textit{qnrB}, \textit{qnrC}, \textit{qnrD} and \textit{qnrS}, and subtypes thereof have been identified (Jacoby et al., 2006; Wang et al., 2009; Cavaco et al., 2009; Hata et al., 2005). Besides \textit{qnr} genes the gene \textit{aac(6')-Ib-cr}, coding for an aminoglycoside acetyltransferase, has been detected, which confers resistance to kanamycin and decreased susceptibility to ciprofloxacin and norfloxacin by acetylating their piperazinyl substituent (Robicsek et al., 2006). Two plasmid-encoded efflux pumps, QepA1 and QepA2 (Yamane et al., 2007; Cattoir et al., 2008b), have also been reported.

ESBL genes and PMQRs have been described to be colocated either on the same plasmid or on different plasmids within the same isolate (Richter et al., 2010; Dionisi et al., 2009; Müller et al., 2011; Woodford et al., 2009; Dolejska et al., 2011; Kirchner et al., 2011; Yao et al., 2011).

The information about ESBLs and PMQRs in \textit{E. coli} from diseased animals in Germany is scarce. Thus, the aims of this project were

(i) to determine the presence of ESBL and PMQR genes in \textit{E. coli} isolates from defined disease conditions of companion and farm animals,

(ii) to gain insight into the localisation and organisation of the genetic environment of the ESBL and PMQR genes, and

(iii) to obtain information about the transferability of the ESBL and PMQR genes.
Chapter 2

Analysis of $bla_{\text{CTX-M}}$-carrying plasmids from *Escherichia coli* isolates collected in the BfT-GermVet study

Anne-Kathrin Schink, Kristina Kadlec and Stefan Schwarz

Chapter 2  Analysis of $bla_{\text{CTX-M}}$ gene regions

The extent of contribution from Anne-Kathrin Schink to the article is evaluated according to the following scale:

A. has contributed to collaboration (0-33%).
B. has contributed significantly (34-66%).
C. has essentially performed this study independently (67-100%).

1. Design of the project including design of individual experiments  B
2. Performing of the experimental part of the study  C
3. Analysis of experiments  C
4. Presentation and discussion of the study in article form  C
Chapter 3

Detection of \textit{qnr} genes among \textit{Escherichia coli} isolates of animal origin and complete sequence of the conjugative \textit{qnrB19}-carrying plasmid pQNR2078

Anne-Kathrin Schink, Kristina Kadlec and Stefan Schwarz

The extent of contribution from Anne-Kathrin Schink to the article is evaluated according to the following scale:

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C. has essentially performed this study independently (67-100%).

1. Design of the project including design of individual experiments  C
2. Performing of the experimental part of the study  C
3. Analysis of experiments  C
4. Presentation and discussion of the study in article form  C
Chapter 4

Discussion
Discussion

1. Occurrence of \(\text{bla}_{\text{CTX-M}}\) genes in \textit{E. coli} from Germany and other countries

The ESBLs corresponding to the three \(\text{bla}_{\text{CTX-M}}\) genes found in this study belonged to the CTX-M-1 group. ESBLs of this group are commonly found in \textit{E. coli} isolates in Europe and represent the predominant ESBL group in some other countries (Coque et al., 2008; Livermore et al., 2007). In Germany, ESBL-producing \textit{E. coli} isolates with \(\text{bla}_{\text{CTX-M-1}}\) and \(\text{bla}_{\text{CTX-M-15}}\) genes of human and animal origin have been described (Cullik et al., 2010; Ewers et al., 2010; Schmitt et al., 2007).

The gene \(\text{bla}_{\text{CTX-M-15}}\) has been described in isolates of the worldwide distributed \textit{E. coli} type O25:H4-ST131 (Nicolas-Chanoine et al., 2008; Woodford et al., 2011). In Germany, CTX-M-15 ESBL-producing \textit{E. coli} O25b-ST131 from dogs have been found (Ewers et al., 2010). The \textit{E. coli} ST131 clonal group as well as \textit{E. coli} ST410 have shown an extended host spectrum genotype (EHSG) by their presence in humans and animals (Wieler et al., 2011). According to the MLST database (http://mlst.ucc.ie/mlst/dbs/Ecoli, last accessed 15 March 2012), \textit{E. coli} ST410 isolates have been reported in human and bovine isolates from Canada and in human isolates from Brazil, Ghana, England and Spain. \textit{E. coli} ST410 isolates have been further identified among CTX-M-15 ESBL-producing human isolates from the U.S.A. and from Brazil (Sidjabat et al., 2009; Peirano et al., 2011) as well as in Spanish isolates from humans and turkey meat (López-Cerero et al., 2011). Therefore, \textit{E. coli} ST410 isolates seem to be widely distributed among humans and animals. The \(\text{bla}_{\text{CTX-M-15}}\)-positive \textit{E. coli} isolate 913 from a dog suffering from urinary tract infection, identified in the present PhD project, also belonged to MLST type ST410 and is the first of these isolates detected in companion animals.

The \(\text{bla}_{\text{CTX-M-1}}\) ESBL-producing \textit{E. coli} isolates belonged to the novel MLST types ST1153 and ST1576. However, after we firstly described ST1153 one more porcine pathogenic \textit{E. coli} ST1153 isolate from Germany has been deposited in the MLST-database (http://mlst.ucc.ie/mlst/dbs/Ecoli, last accessed 15 March 2012), indicating
that the finding of this type of *E. coli* has not been an accidental observation. Nevertheless, *bla*<sub>CTX-M-1</sub>-carrying plasmids have been described in a multitude of *E. coli* sequence types (Ben Sallem et al., 2011; Bonnedahl et al., 2009; Cullik et al., 2010; Leverstein-Van Hall et al., 2011; Oteo et al., 2009) which is a hint towards a horizontal rather than a clonal spread.

2. The genetic environment of *bla*<sub>CTX-M</sub> genes

The *bla*<sub>CTX-M-15</sub> gene was located on an IncF plasmid (pCTX913) of ca. 50 kb in size and the two *bla*<sub>CTX-M-1</sub> genes on ca. 50 kb IncN plasmids (pCTX168 and pCTX246). The analysis of the immediately flanking regions up- and downstream of the ESBL genes on pCTX913, pCTX168 and pCTX246 revealed at least a fragment of the insertion sequence IS<sub>Ecp1</sub> 48 bp or 80 bp upstream and downstream the terminal part of *orf477*. The same genetic environment has been described for these genes before (Eckert et al., 2006; Lartigue et al., 2004). Poirel et al. (2005) proved that IS<sub>Ecp1B</sub> is able to mobilize *bla*<sub>CTX-M</sub> genes in a one-ended transposition process, which in these cases might have included the ESBL genes and the terminal part of *orf477*. The genetic environment of *bla*<sub>CTX-M-15</sub> and *bla*<sub>CTX-M-1</sub> is depicted in Figures 1 and 2 and the plasmids mentioned in the following text are listed in Table 1.

In clinical *K. pneumoniae* isolates from Nigeria, plasmids larger than 58 kb carrying *bla*<sub>CTX-M-15</sub> with the same immediately flanking 193 bp up- and 212 bp downstream have been identified (Soge et al., 2006). Furthermore, the downstream region of the gene *bla*<sub>CTX-M-15</sub> on pCTX913 with the *Δorf477-ΔtnpA* structure has been described in a wide variety of plasmids and in conjunction with different bacterial hosts. The 92,353 bp IncFII plasmid pC15-1a from a human clinical *E. coli* isolate obtained in 2002 in Canada (Boyd et al., 2004) and pYC-5b, a ca. 50 kb plasmid harboured by a human clinical *E. coli* isolate from Cameroon (Gangoue-Pieboji et al., 2005), showed the same *bla*<sub>CTX-M-15</sub> downstream region as in pCTX913. In *E. coli* isolates of the international clone O25:H4-ST131 from the UK, different plasmids have been identified and the plasmids pEK499 (117,536 bp in size and positive for
replicons FII and FIA) and pEK516 (64,471 bp in size and IncFII positive) showed the same immediate flanking regions (Woodford et al., 2009). In a Belgian study four plasmids, showing the same downstream region, were sequenced. Three were obtained from human E. coli isolates and the fourth E. coli was of equine origin. The plasmid pEC_Bactec from the equine isolate had a size of 92,970 bp and belonged to the incompatibility group IncI1. In contrast, the plasmids from isolates of human origin belonged to the replicon type FII and had a size of 73,801 bp (pEC_B24) or 118,525 bp (pEC_L8) while the third, plasmid pEC_L46, had two replicons, namely FII and FIA, and a size of 144,871 bp (Smet et al., 2010). The same was shown for IncF plasmids of varying sizes from Australia (Partridge et al., 2011) and in Germany, a human clinical E. coli ST131 isolate harboured an IncFII plasmid (pKCT407) with the $\Delta$orf477-$\Delta$tnpA structure (Cullik et al., 2010). A 220,824 bp plasmid from Swedish clinical human K. pneumonia isolates is believed to have obtained a $bla_{CTX-M-15}$ region similar to pEK499 and pC15-1a by recombination events (Sandegren et al., 2012).

Furthermore, in Acinetobacter baumannii a transposon TnAb15, which harboured an $ISEcp1-bla_{CTX-M-15}$-$\Delta$orf477-$\Delta$tnpA-$\Delta$IS26 segment, has been identified in the chromosomal DNA (Potron et al., 2011). A preferred insertion of $ISEcp1$ into the tnpA gene of Tn3-like transposons has been proposed (Smet et al., 2010), but according to Bailey et al. (2011) the Tn3-like transposon was annotated incorrectly and should be renamed as Tn2-like. The same arrangement could be found in the nucleotide sequence from a Russian Serratia liquefaciens strain (GenBank accession no. HM470254) downstream $bla_{CTX-M-22}$, emphasising tnpA of transposon Tn2 as favoured insertion site. On the IncI1 plasmid pKC390 (Cullik et al., 2010), the $\Delta$orf477 is followed by a $\Delta$mrx gene, pointing towards other insertion sites or recombination events in which the putative inverted repeat of $ISEcp1$ might play a role. However, the occurrence of the $ISEcp1-bla_{CTX-M-15}$-$\Delta$orf477-$\Delta$tnpA structure on different plasmids and in various members of the family Enterobacteriaceae of either human or animal origin from different countries underlines the impact of transposition and recombination events in the spread of the resistance gene $bla_{CTX-M-15}$. 


Table 1: **blaCTX-M**-carrying plasmids

<table>
<thead>
<tr>
<th>Plasmid(s)</th>
<th>β-lactamase</th>
<th>replicon type</th>
<th>size (bp)</th>
<th>species</th>
<th>host species</th>
<th>country</th>
<th>year of isolation</th>
<th>accession no.</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCTX913</td>
<td>blaCTX-M-15</td>
<td>F</td>
<td>~50,000</td>
<td>E. coli</td>
<td>dog</td>
<td>Germany</td>
<td>2004</td>
<td>FR828676</td>
<td>Schink et al., 2011</td>
</tr>
<tr>
<td>e.g. pJIE098</td>
<td>blaCTX-M-15</td>
<td>F</td>
<td>~145,000</td>
<td>E. coli</td>
<td>human</td>
<td>Australia</td>
<td>2005-2006</td>
<td>EU418920</td>
<td>Partridge et al., 2011</td>
</tr>
<tr>
<td>pC15-1a</td>
<td>blaCTX-M-15</td>
<td>F</td>
<td>92,353</td>
<td>E. coli</td>
<td>human</td>
<td>Canada</td>
<td>2000</td>
<td>AY458016</td>
<td>Boyd et al., 2004</td>
</tr>
<tr>
<td>pEK516</td>
<td>blaCTX-M-15</td>
<td>F</td>
<td>64,471</td>
<td>E. coli</td>
<td>human</td>
<td>United Kingdom</td>
<td>nd</td>
<td>EU935738</td>
<td>Woodford et al., 2009</td>
</tr>
<tr>
<td>pEC_B24</td>
<td>blaCTX-M-15</td>
<td>F</td>
<td>73,801</td>
<td>E. coli</td>
<td>human</td>
<td>Belgium</td>
<td>nd</td>
<td>GU371926</td>
<td>Smet et al., 2010</td>
</tr>
<tr>
<td>pKCT407</td>
<td>blaCTX-M-15</td>
<td>F</td>
<td>nd</td>
<td>E. coli</td>
<td>human</td>
<td>Germany</td>
<td>2006</td>
<td>GQ274935</td>
<td>Cullik et al., 2010</td>
</tr>
<tr>
<td>pEC_L8</td>
<td>blaCTX-M-15</td>
<td>F, FIA</td>
<td>118,525</td>
<td>E. coli</td>
<td>human</td>
<td>Belgium</td>
<td>nd</td>
<td>GU371928</td>
<td>Smet et al., 2010</td>
</tr>
<tr>
<td>pEC_L46</td>
<td>blaCTX-M-15</td>
<td>F, FIA</td>
<td>144,871</td>
<td>E. coli</td>
<td>human</td>
<td>Belgium</td>
<td>nd</td>
<td>GU371929</td>
<td>Smet et al., 2010</td>
</tr>
<tr>
<td>pEK499</td>
<td>blaCTX-M-15</td>
<td>F</td>
<td>117,536</td>
<td>E. coli</td>
<td>human</td>
<td>United Kingdom</td>
<td>nd</td>
<td>EU935739</td>
<td>Woodford et al., 2009</td>
</tr>
<tr>
<td>pUUH239.2</td>
<td>blaCTX-M-15</td>
<td>FII/FIA</td>
<td>220,824</td>
<td>K. pneumoniae</td>
<td>human Sweden</td>
<td>nd</td>
<td>CP002474</td>
<td>Sandegren et al., 2012</td>
<td></td>
</tr>
<tr>
<td>pEC_Bactec</td>
<td>blaCTX-M-15</td>
<td>I1</td>
<td>92,970</td>
<td>E. coli</td>
<td>horse</td>
<td>Belgium</td>
<td>nd</td>
<td>GU371927</td>
<td>Smet et al., 2010</td>
</tr>
<tr>
<td>pKC390</td>
<td>blaCTX-M-15</td>
<td>I1</td>
<td>nd</td>
<td>E. coli</td>
<td>human</td>
<td>Germany</td>
<td>2006</td>
<td>GQ274928</td>
<td>Cullik et al., 2010</td>
</tr>
<tr>
<td>pYC-5b</td>
<td>blaCTX-M-15</td>
<td>nd</td>
<td>~50,000</td>
<td>E. coli</td>
<td>human</td>
<td>Cameroon</td>
<td>2002</td>
<td>AY604721</td>
<td>Gangoue-Peboji et al., 2005</td>
</tr>
<tr>
<td>pMRC151</td>
<td>blaCTX-M-15</td>
<td>nd</td>
<td>&gt;58,000</td>
<td>K. pneumoniae</td>
<td>human Nigeria</td>
<td>2002-2003</td>
<td>AY995205</td>
<td>Soge et al., 2006</td>
<td></td>
</tr>
<tr>
<td>several</td>
<td>blaCTX-M-15</td>
<td>nd</td>
<td>nd</td>
<td>E. coli</td>
<td>human</td>
<td>France</td>
<td>2001</td>
<td>AM040706</td>
<td>Eckert et al., 2006</td>
</tr>
<tr>
<td>pCTX168</td>
<td>blaCTX-M-1</td>
<td>N</td>
<td>~50,000</td>
<td>E. coli</td>
<td>dog</td>
<td>Germany</td>
<td>2004</td>
<td>FN806788</td>
<td>Schink et al., 2011</td>
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<tr>
<td>pCTX246</td>
<td>blaCTX-M-1</td>
<td>N</td>
<td>~50,000</td>
<td>E. coli</td>
<td>swine</td>
<td>Germany</td>
<td>2004</td>
<td>FN806790</td>
<td>Schink et al., 2011</td>
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<td>p1204y1463</td>
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<td>N</td>
<td>~130,000</td>
<td>E. coli</td>
<td>human</td>
<td>Spain</td>
<td>2004</td>
<td>FJ235692</td>
<td>Diestra et al., 2009</td>
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<tr>
<td>pKC394</td>
<td>blaCTX-M-1</td>
<td>N</td>
<td>53207</td>
<td>E. coli</td>
<td>human</td>
<td>Germany</td>
<td>2006</td>
<td>HM138652</td>
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<td>nd</td>
<td>blaCTX-M-1</td>
<td>N</td>
<td>~40,000</td>
<td>E. coli</td>
<td>human</td>
<td>Spain</td>
<td>2001-2002</td>
<td>nd</td>
<td>Novais et al., 2007</td>
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<td>several</td>
<td>blaCTX-M-1</td>
<td>N</td>
<td>~45,000</td>
<td>E. coli</td>
<td>human, swine</td>
<td>Denmark</td>
<td>2006-2007</td>
<td>nd</td>
<td>Moodley &amp; Guardabassi, 2009</td>
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<tr>
<td>several</td>
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<td>N</td>
<td>~30,000</td>
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<td>horse</td>
<td>Czech Republic</td>
<td>2008</td>
<td>nd</td>
<td>Dolejska et al., 2011a</td>
</tr>
<tr>
<td>several</td>
<td>blaCTX-M-1</td>
<td>N</td>
<td>~40,000</td>
<td>E. coli</td>
<td>cattle</td>
<td>Czech Republic</td>
<td>2008</td>
<td>nd</td>
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</tr>
<tr>
<td>nd</td>
<td>blaCTX-M-1</td>
<td>N</td>
<td>~35,000</td>
<td>E. coli</td>
<td>mallard</td>
<td>Poland</td>
<td>2008-2009</td>
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<td>pKCT398</td>
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<td>I1</td>
<td>nd</td>
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<td>Germany</td>
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<td>GQ274931</td>
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<td>France</td>
<td>2002</td>
<td>AM003904</td>
<td>Eckert et al., 2006</td>
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</table>
On pCTX168 and pCTX246 the insertion sequence IS\textit{Ecp1} was truncated by an IS26 element. This IS26-\textit{ΔIS\textit{Ecp1}} structure was also reported on IncN plasmids from Spain and Germany and seems to be widely distributed (Diestra et al., 2009; Cullik et al., 2010).

In the downstream region of the \textit{bla}_{\text{CTX-M-15}} gene on pCTX168 and pCTX246, a truncated \textit{mph(A)}-\textit{mrx}-\textit{mphR(A)} gene cluster was detected. A complete \textit{mph(A)}-\textit{mrx}-
mphR(A) gene cluster was described by Noguchi et al. (2000) in *E. coli*. The mphR(A) gene and the terminal 55 bp of *mrx* were absent. This truncation could be due to either an interplasmid recombination event or the insertion of an intact IS*Ecp1*, because the putative recombination site resembles also a putative inverted repeat of IS*Ecp1*. Whether the IS*Ecp1*-bla<sub>CTX-M<sub>1</sub>Δorf477</sub> transposon inserted into *mrx* and an IS26 subsequently truncated the IS*Ecp1* or whether this structure had been acquired by interplasmid recombination events cannot be determined in retrospect.

**Figure 2:** (a) Schematic presentation of the genetic environment of bla<sub>CTX-M<sub>1</sub></sub> on pCTX168 and pCTX246. (b) Schematic presentation of genetic environments of bla<sub>CTX-M<sub>1</sub></sub>. The open reading frames are shown as arrows, with the arrowhead indicating the direction of transcription. IS elements are shown as boxes. Homologous regions are indicated by grey shading.
Upstream of the Δmrx gene, a complete mph(A) gene followed 45 bp afar by a complete insertion sequence IS26 with the same orientation as the IS26 upstream of blaCTX-M-1 was detected on pCTX246. The structure IS26-ΔEcp1-blaCTX-M-1-Δorf477-Δmrx-mph(A)-IS26 has been described on IncN plasmids (e.g. pKC394) from German human E. coli ST131 isolates, but the second IS26 was 3 bp upstream of mph(A), whereas on an IncI1 plasmid, pKC398, from German E. coli ST398 the second IS26 was also 45 bp upstream of mph(A) (Cullik et al., 2010). The authors have proposed a novel composite transposon, with two equally oriented IS26 and the exchange of large blaCTX-M-1-containing modules between different plasmid backbones (Cullik et al., 2010), but the IS26-ΔEcp1-blaCTX-M-1-Δorf477-Δmrx-mph(A)-IS26 structures on plasmids pKC394 and pKC398 probably developed independently because of the dissimilar spacers between mph(A) and IS26. The occurrence of the same IS26-ΔEcp1-blaCTX-M-1-Δorf477-Δmrx-mph(A)-IS26 on IncN and IncI1 plasmids might support the theory of IS26 mediated transposition events between different plasmid backbones.

However, on pCTX168 a truncated mph(A) gene was identified upstream of Δmrx and a second IS26 was not detected within the sequenced part of the plasmid. The truncation might be due to interplasmid recombination events. Despite this difference, the blaCTX-M-1 gene regions on plasmids pCTX246 and pCTX168 are otherwise related. Comparisons with sequences in the database of the National Center for Biotechnology Information (NCBI) revealed that this blaCTX-M-1 region has been identified only on plasmids of German origin so far. The corresponding E. coli isolates originated from farm and companion animals as well as from humans, pointing towards an extensive distribution in Germany. Interestingly, the animal E. coli isolates had been obtained in 2004, while the human isolates were from 2006 (Cullik et al., 2010), but whether this blaCTX-M-1 gene region has developed in animal isolates first is speculative.

The gene blaCTX-M-1 has been described in conjunction with IncN plasmids of ca. 40 kb in E. coli isolates of human origin from Spain (Novais et al., 2007). Furthermore, it was found on ca. 45 kb IncN plasmids in porcine and human E. coli isolates from Denmark (Moodley & Guardabassi, 2009), on 40 kb IncN plasmids in E. coli from
cattle as well as on 30 kb IncN plasmids of equine origin from the Czech Republic (Dolejska et al., 2011a; Dolejska et al., 2011b). A 35 kb *bla<sub>CTX-M-1</sub>*-carrying IncN plasmid has been identified in an *E. coli* isolate from a polish wild mallard (Literak et al., 2010). Thus, the *bla*<sub>CTX-M-1</sub> gene appears to be widely distributed on IncN plasmids of different sizes but there is no further information about the immediately flanking regions, except the linkage with IS<sub>Ecp1</sub> upstream of *bla*<sub>CTX-M-1</sub> on the Spanish plasmids (Novais et al., 2007).

Moreover, *bla*<sub>CTX-M-1</sub> has been identified on plasmids of various incompatibility groups. The aforementioned IncI1 plasmids have been detected aside from Germany (Cullik et al., 2010) in the Czech Republic (Dolejska et al., 2011a), in Poland (Literak et al., 2010) and in France and the Netherlands from *E. coli* isolates of healthy poultry (Girlich et al., 2007). In addition, IncI1 plasmids carrying *bla*<sub>CTX-M-1</sub> have been reported from the Netherlands in *E. coli* isolates from human, poultry and retail meat (Leverstein-van Hall et al., 2011).

**3. Occurrence of the *qnrB19* gene in Germany and in other countries**

The first detection of a *qnrB19* gene has been on plasmid pR4525 from a clinical human *E. coli* isolate from Colombia obtained in 2002 (Cattoir et al., 2008a). Later on the gene was reported in a clinical human *K. pneumoniae* isolate from the United States identified in 2007 (Endimiani et al., 2008). In a *Salmonella enterica* serovar Typhimurium isolate, obtained in 2004 from a case of human gastroenteritis in Italy, a *qnrB19* carrying plasmid has been identified (Dionisi et al., 2009). Furthermore, *qnrB19* has been detected in human *S. Typhimurium* isolates from the Netherlands (García-Fernández et al., 2009; Hammerl et al., 2010; Veldman et al., 2011) and in reptile *Salmonella* spp. isolates from Germany (Dierikx et al., 2010, Guerra et al., 2010) as well as in human *Salmonella* isolates from Korea (Jeong et al., 2011). In another study, the gene was found in human commensal *E. coli, K. pneumoniae* and *Escherichia hermannii* from Peru and Bolivia (Pallecchi et al., 2010). In Spain, a *qnrB19*-positive human *E. coli* has been isolated in 2005 (Rios et al., 2010). In
addition, qnrB19 has been identified in the Netherlands in a bovine E. coli isolate (Hordijk et al., 2011) and in equine E. coli from the Czech Republic (Dolejska et al., 2011a).

In an international collaborative retrospective study, the occurrence of PMQR genes in S. enterica and E. coli was investigated. The qnrB19 gene was detected in S. enterica isolates from humans and fowl from the Netherlands, human isolates from the UK and turkey isolates from Denmark and Finland. In Germany, qnrB19 has been identified in S. enterica isolates from food, fowl, turkeys and reptiles. In E. coli isolates, qnrB19 has been solely detected among those from Polish turkeys (Veldman et al., 2011). The qnrB19 gene has also been identified in Salmonella enterica serovar Corvallis isolates from poultry in Brazil (Ferrari et al., 2011) as well as in E. coli isolates from chicken in Nigeria (Fortini et al., 2011).

The E. coli isolates harbouring pQNR2078 and pQNR2086 originated from mares suffering from genital tract infections and were identified in 2005 in Germany. This is the first report of qnrB19 in E. coli of equine origin in Germany. The qnrB19 gene has been basically detected worldwide in different members of the family Enterobacteriaceae of human and animal origin, including commensal isolates and isolates from food and food-producing animals.

Both equine E. coli isolates had the sequence type, ST86. In the MLST-database are entries of E. coli ST86 of bovine and simian origin from Egypt and the U.S.A., respectively (http://mlst.ucc.ie/mlst/dbs/Ecoli, last accessed 15 March 2012). Furthermore, E. coli ST86 has been identified among ESBL-producing E. coli isolates from seagulls in Portugal (Simões et al., 2010).

4. The genetic environment of the qnrB19 gene

The qnrB19 gene has been described to be located on plasmids of varying sizes and replicon types, which are listed in Table 2. Figure 3 shows schematically the genetic environment of the gene qnrB19. The first qnrB19 gene described was located on the 40 kb plasmid pR4525 and organised within a 2,739 bp novel
transposon Tn2012, consisting of ISEcp1C and qnrB19 (Cattoir et al., 2008a). Unfortunately, the incompatibility group of pR4525 has not been mentioned.

Almost at the same time, an 80 kb plasmid (pLRM24) containing a large composite genetic element, designated KQ element, from a human K. pneumoniae isolate from the U.S.A. has been reported (Rice et al., 2008). The qnrB19 gene was part of the novel transposon Tn5387 of 2,966 bp in size, which was located in close proximity to the blaKPC-3 carrying transposon Tn4401. Tn5387 consists of qnrB19 and an ISEcp1, which showed a single basepair exchange in comparison to the ISEcp1 of Tn2012. The region between ISEcp1 and qnrB19 showed 100% identity in both transposons, but the sequence upstream of qnrB19 differed. In Tn2012 qnrB19 was 157 bp afar from the end of the putative inverted repeat of ISEcp1, whereas in Tn5387 it was 383 bp. The first 155 bp showed 100% identity, including 14 bp of the putative inverted repeat of Tn2012.

On the IncL/M-like plasmid p61/9 from a S. enterica isolate from Italy, the transposon Tn2012 was followed downstream by an IS26 bracketed region containing blaSHV-12. The authors had proposed that the acquisition of Tn2012 on p61/9 was due to an illegitimate recombination, because the 5 bp target site duplication after the second right inverted repeat was absent (Dionisi et al., 2009). However, the ISEcp1 showed the same exchange in the nucleotide sequence as the one in Tn5387 and the first 246 bp upstream qnrB19 were also identical, whereas the similarity with Tn2012 ended 155 bp upstream qnrB19. This raised the question whether Tn2012 had been inserted into p61/9. A closer look at the sequence revealed that the last homologous base pairs of p61/9 with Tn5387 resembled a putative inverted repeat of ISEcp1 with 8 of 14 base pairs identity (Poirel et al., 2005). The complete homologous region started and ended with inverted repeats and was flanked by 5 bp direct repeats. Thus, another putative transposon containing qnrB19 seems to be present on p61/9. (Fig.3b)
Table 2: *qnrB19*-carrying plasmids

<table>
<thead>
<tr>
<th>plasmid(s)</th>
<th>replicon type</th>
<th>size (bp)</th>
<th>species</th>
<th>host species</th>
<th>country</th>
<th>year of isolation</th>
<th>accession no.</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pQNR2078</td>
<td>N</td>
<td>42,379</td>
<td><em>E. coli</em></td>
<td>horse</td>
<td>Germany</td>
<td>2004</td>
<td>HE613857</td>
<td>Schink et al., 2012</td>
</tr>
<tr>
<td>pR4525</td>
<td>nd</td>
<td>~40,000</td>
<td><em>E. coli</em></td>
<td>human</td>
<td>Colombia</td>
<td>2002</td>
<td>EU523120</td>
<td>Cattoir et al., 2008a</td>
</tr>
<tr>
<td>pLRM24</td>
<td>nd</td>
<td>~80,000</td>
<td><em>K. pneumoniae</em></td>
<td>human</td>
<td>U.S.A.</td>
<td>2007</td>
<td>EU624315</td>
<td>Rice et al., 2008</td>
</tr>
<tr>
<td>p61/9</td>
<td>L/M</td>
<td>nd</td>
<td><em>S. enterica</em></td>
<td>human</td>
<td>Italy</td>
<td>2004</td>
<td>FJ790886</td>
<td>Dionisi et al., 2009</td>
</tr>
<tr>
<td>pSGI15</td>
<td>ColE-like</td>
<td>2699</td>
<td><em>S. enterica</em></td>
<td>human</td>
<td>Netherlands</td>
<td>nd</td>
<td>FN428572</td>
<td>Hammerl et al., 2009</td>
</tr>
<tr>
<td>pECY6-7</td>
<td>ColE-like</td>
<td>2699</td>
<td><em>E. coli</em></td>
<td>human</td>
<td>Peru</td>
<td>2005</td>
<td>GQ374156</td>
<td>Pallecchi et al., 2010</td>
</tr>
<tr>
<td>pECC14-9</td>
<td>ColE-like</td>
<td>3071</td>
<td><em>E. coli</em></td>
<td>human</td>
<td>Bolivia</td>
<td>2005</td>
<td>GQ374157</td>
<td>Pallecchi et al., 2010</td>
</tr>
<tr>
<td>several</td>
<td>ColE_{Tp} (n=7)</td>
<td>2700-4500</td>
<td><em>Salmonella spp.</em></td>
<td>reptile</td>
<td>Germany</td>
<td>2000-2008</td>
<td>nd</td>
<td>Guerra et al., 2010</td>
</tr>
<tr>
<td>pSR132</td>
<td>nd</td>
<td>&gt;23,000</td>
<td><em>E. coli</em></td>
<td>human</td>
<td>Italy</td>
<td>2007-2008</td>
<td>GU074393</td>
<td>Richter et al., 2010</td>
</tr>
<tr>
<td>p013.1IncR</td>
<td>R</td>
<td>~40,000</td>
<td><em>E. coli</em></td>
<td>cattle</td>
<td>Netherlands</td>
<td>nd</td>
<td>HM146784</td>
<td>Hordijk et al., 2011</td>
</tr>
<tr>
<td>several</td>
<td>N (n=2)</td>
<td>~40,000</td>
<td><em>E. coli</em></td>
<td>horse</td>
<td>Czech Republic</td>
<td>2008</td>
<td>nd</td>
<td>Dolejska et al., 2011a</td>
</tr>
</tbody>
</table>

Discussion
Figure 3: (a) Schematic presentation of the genetic environment of \textit{qnrB19} on pQNR2078 and pQNR2086. (b) Schematic presentation of genetic environments of \textit{qnrB19}. The open reading frames are shown as arrows, with the arrowhead indicating the direction of transcription. IS elements are shown as boxes. Homologous regions are indicated by grey shading.
The 2699 bp ColE-like plasmid pSGI15, harboured by a human *S. enterica* isolate from the Netherlands, has been described in 2009 (Hammerl et al., 2010). The immediate flanking regions of *qnrB19*, 245 bp upstream and 225 bp downstream, showed 100% identity with Tn5387 and the *qnrB19* region on p61/9. This plasmid exhibited 100% identity with pECY6-7, which had been identified in a study on human commensal *E. coli* isolates from Peru and Bolivia, as well as in *K. pneumoniae* and *E. hermannii* (Pallecchi et al., 2010). In the same study, the structurally related ColE-like plasmid pECC14-9 of 3071 bp, which carried an identical *qnrB19* region, was described. Hence ColE-like plasmids carrying *qnrB19* seem to have widely spread geographically and within the Enterobacteriaceae. This is underlined by the finding of small ColE<sub>Tp</sub> plasmids, carrying *qnrB19* from *Salmonella* spp. of reptile origin isolated in Germany (Guerra et al., 2010)

The *qnrB19* gene has been also identified within an IS<sub>CR1</sub> complex class 1 integron in an *E. coli* isolate from Italy (Richter et al., 2010). The sequence deposited in the database showed 100% identity with Tn5387 in the first 221 bp immediately up- and 225 bp downstream of *qnrB19*.

From the Netherlands, the 40 kb plasmid p013.1IncR, harboured by a bovine *E. coli* isolate, has been reported. This plasmid showed a similar upstream region of *qnrB19* in comparison to the aforementioned plasmid p61/9. However, instead of an insertion sequence ISE<sub>Ecp1</sub> another IS26 was located 44 bp downstream *qnrB19* on this IncR plasmid (Hordijk et al., 2011). The upstream region of *qnrB19* on pQNR2078 and pQNR2086 showed also similarity with p61/9 and 140 bp downstream of *qnrB19*, a second IS26 in the same orientation was identified. These 140 bp had 100% identity with Tn2012 and Tn5387. The insertion of IS26 results in 8 bp direct repeats in the target side either flanking a single IS26 or if two direct repeated IS26 act as a transposon flanking both (Iida et al., 1984). As the 8 bp immediately up- and downstream of the two IS26 were entirely different, the formation of this region was probably not due to integration of IS26 but to recombination events involving these insertion sequences. However such a region might act as a transposon in the future. The dissimilar spacers between *qnrB19* and
the downstream IS26 on pQNR2078/2086 and p013.1IncR point towards an independent development of these genetic structures.

In the Czech Republic, 40 kb conjugative IncN plasmids carrying solely qnrB19 have been isolated from *E. coli* of equine origin (Dolejska et al., 2011a). These plasmids resembled in size the 42,379 bp plasmid pQNR2078 from equine *E. coli* sequenced in the present PhD study. Therefore, such plasmids seem to be widely distributed among *E. coli* isolates from horses.

Remarkably, the qnrB19 gene was the only resistance gene on plasmids pQNR2078/2086. In other studies qnrB19 has been co-located with other resistance determinants like ESBL genes. On the 40 kb plasmid pR4525, bla$_{CTX-M-12}$ and bla$_{SHV-12}$ have been located in addition to qnrB19 and in another study a bla$_{SHV-12}$ gene has been detected on a qnrB19-carrying plasmid (Cattoir et al., 2008a; Rios et al., 2010). These findings indicate that the genetic environment of the qnrB19 genes is involved in ongoing alterations due to transposition and recombination events and similar structures might be found in different members of the family Enterobacteriaceae.

5. Plasmids in Enterobacteriaceae

The spread of resistance genes is on one hand due to successful clones, which could emerge all over the world, and on the other hand mediated by horizontal gene transfer in which plasmids play an important role. Plasmids are classified in incompatibility groups and 27 Inc groups have been described in Enterobacteriaceae so far (Carattoli, 2011).

Plasmids of the IncF family are widespread among Enterobacteriaceae but also restricted to them (Carattoli, 2011). They play a major role in the dissemination of bla$_{CTX-M-15}$ genes and are frequently found in association with the successful *E. coli* clone O25:H4-ST131, but also in other *E. coli* lineages (Carattoli, 2009; López-Cerero et al., 2011; Partridge et al., 2011; Smet et al., 2010; Woodford et al., 2009). In addition, the gene *aac(6')-Ib-cr* has been described to be co-located on these
plasmids. Plasmid pCTX913 carried an \textit{aac(6\textprime)-Ib-cr} gene as well (unpublished data), indicating that it was closely related to these widespread and successful plasmids.

Among the plasmid families, the IncN type is widely distributed among Enterobacteriaceae worldwide (Carattoli, 2009) and exhibits a broad host range (Krishnan & Iyer, 1988). IncN plasmids have been reported carrying different resistance genes e.g. \textit{bla}_{CTX-M-1}, \textit{bla}_{CTX-M-3}, \textit{bla}_{CTX-M-15} and \textit{bla}_{CTX-M-65} (Cullik et al., 2010; Dolejska et al., 2011a; Dolejska et al., 2011b; Literak et al., 2010; Marcadé et al., 2009; Moodley & Guardabassi, 2009; Novais et al., 2007) and also different \textit{qnr} genes like \textit{qnrB2}, \textit{qnrB19} and \textit{qnrS1} (García-Fernández et al., 2009). A plasmid multilocus sequence typing scheme has been established to categorise IncN plasmids in different sequence types based on the nucleotide sequence of selected loci within the genes \textit{repA}, \textit{traJ} and \textit{korA} in order to discriminate IncN plasmids more precisely (García-Fernández et al., 2011). IncN plasmids carrying \textit{bla}_{CTX-M-1} and exhibiting pMLST type ST1 have been identified in \textit{E. coli} isolates from human and animal isolates from Greece, Italy, Denmark, the Netherlands, Germany and the UK (http://pubmlst.org/plasmid/, last accessed 15 March 2012).

The plasmids pCTX168 and pCTX246 also belonged to ST1 and have been deposited as ESBL-248 (id 568) and ESBL-249 (id 569), respectively, in the PubMLST database. In contrast, one of the aforementioned \textit{bla}_{CTX-M-1}-carrying IncN plasmids, pKC394, from German human \textit{E. coli} isolates with a similar genetic arrangement immediately up- and downstream of \textit{bla}_{CTX-M-1} (Cullik et al., 2010) as pCTX246 has been completely sequenced and revealed the pMLST type ST8. The same pMLST type was identified for the \textit{bla}_{CTX-M-65} carrying IncN plasmid pKC396 (García-Fernández et al., 2011). ST1 differs from ST8 in three nucleotides within \textit{repN} and in one nucleotide within \textit{traJ}. The finding of similar genetic environments of \textit{bla}_{CTX-M-1} on different IncN plasmids points towards gene transfer either by recombination events or by transposition between different IncN plasmid backbones as already proposed (Cullik et al., 2010). Another possibility would be the alteration of the pMLST specific loci on the plasmid itself.

The pMLST type ST8 had been determined for plasmids pQNR2078/2086 as well and the nucleotide sequence of pQNR2078 had a remarkable overall identity of 99.0
% with plasmid pKC396. Both sequences differed only in the resistance gene region and within their iterons. It is possible that the IncN pMLST ST8 backbone acquired different resistance gene regions by independent recombination events potentially involving IS26.

Furthermore, a human *S. Typhimurium* isolate from the Netherlands harbourered an IncN-ST8 plasmid that carried *qnrB19* (García-Fernández et al., 2009). This observation indicated a wide distribution of those plasmids geographically and within the family Enterobacteriaceae. However, no further information about the size of the plasmid and the nucleotide sequence is available and therefore it would be too speculative to conclude that it could be the same plasmid as pQNR2078/2086.

The localisation of ESBL and *qnr* genes on related IncN plasmids and linked to insertion sequences like ISEcp1 and especially IS26 is a cause of concern as IncN plasmids have been described to be easily transmitted between different *E. coli* lineages of human and animal origin and among members of different species within the family Enterobacteriaceae (Krishnan & Iyer, 1988; Moodley & Guardabassi, 2009). Moreover, similar genetic structures and IS elements facilitate homologous recombination events, resulting in a genetic environment of the *bla*$_{CTX-M-1}$ and the *qnrB19* genes that represents an appropriate basis for further dissemination.

### 6. Animal reservoirs?

It has been stated that the transmission of ESBLs is more likely due to plasmid-mediated horizontal gene transmission than due to the expansion of bacterial clones (Carattoli, 2008). The transmission of IncN plasmids carrying *bla*$_{CTX-M-1}$ from *E. coli* isolates of porcine origin to different *E. coli* lineages found among farmworkers (Moodley, 2009) and the *in situ* conjugation of *bla*$_{TEM-52}$ carrying IncI1 plasmids from poultry to human commensal *E. coli* (Smet et al., 2011) support this theory. As *qnr* genes have been detected on the same plasmid types, they have theoretically similar transmission potentials and the finding of an IncN-ST8 plasmid, which carried
qnrB19, in a human S. Typhimurium isolate (García-Fernández et al., 2009) might point towards plasmid transmission.

There are several reports of bla_{CTX-M} and qnr genes in commensal and pathogenic bacteria of livestock (Aarestrup et al., 2006; Agerso et al., 2011; Blanc et al., 2006; Briñás et al., 2005; Dolejska et al., 2011b; Fortini et al., 2011; Girlich et al., 2007; Gonçalves et al., 2010; Kirchner et al., 2011; Liu et al., 2008; Meunier et al., 2006; Richter et al., 2010; Rodríguez-Martínez et al., 2001; Smet et al., 2008; Veldman et al., 2011; Yao et al., 2011) and companion animals (Carattoli et al., 2005; Costa et al., 2004; Costa et al., 2008; Dolejska et al., 2011b; Maddox et al., 2011a; Shaheen et al., 2011; Wittum et al., 2010). These findings underline that ESBL-producing and qnr-positive isolates are involved in animal diseases. However, the reports of these genes in isolates from healthy animals are of concern, because they could enable unnoticed transmission of resistance determinants. Transmission between animals in the same barn has been described for horses from the UK, and thus document the spread of bacteria with resistance genes in the animal population (Maddox, 2011b). In addition, such genes have been detected in bacterial isolates from wild animals, indicating another potential reservoir of these resistance genes in animals (Guenther et al., 2011; Literak et al., 2010).

Community-acquired infections with ESBL-producing E. coli have been reported and the authors proposed that retail meat could be a source of such isolates, which then could cause infections, colonise the human gut or donate resistance plasmids (Doi et al., 2010). This theory is supported by a study from the Netherlands in which bla_{CTX-M-1} genes have been identified on IncI1-ST7 plasmids within E. coli ST58 and ST117 isolates from poultry, retail meat and human patients suggesting transmission of ESBL-producing E. coli through the food chain (Leverstein-van Hall et al., 2011). Companion animals live nowadays in close contact with their owner and receive intensive care, thus facilitating the transmission of bacteria from humans to animals and vice versa (Guardabassi et al., 2004; Wieler et al., 2011). The occurrence of CTX-M-15 ESBL-producing E. coli of the O25:H4-ST131 group among dogs from the Netherlands, France, Denmark, Spain and Germany, as well as in a horse (Ewers et al., 2010) points towards clonal transmission from human to animals, which could
also be possible for other bacteria, such as *E. coli* ST410, besides plasmidic spread of resistance genes. The occurrence of ESBL and *qnr* genes in wild animals might be due to ingestion of contaminated human waste via e.g. wastewater (Martínez, 2009) or land-application of livestock manure (Guenther et al., 2011; Kümmerer, 2009). Yellow-legged gulls feeding at a city dump in France harboured ESBL-producing Enterobacteriaceae (Bonnedahl et al., 2009). Recently, human waste-associated CTX-M-1 and CTX-M-15 ESBL-producing *E. coli* from Antarctic water samples have been identified underlining the human impact on environmental contamination with resistant bacteria (Hernández et al., 2012).

Several authors stated the presence of an animal reservoir after detecting ESBL genes in animal isolates (Bonnedahl et al., 2009; Geser et al., 2011; Girlich et al., 2007; Leverstein-van Hall et al., 2011; Smet et al., 2008). Moreover, a reservoir in healthy humans has been considered (Ben Sallem et al., 2011). Nevertheless, the occurrence of related plasmids and distinct clones in humans as well as in animals points towards transmission between different host species, although there is no proof for a stable reservoir among animals so far.

7. Concluding remarks

The number of ESBL-producers as well as *qnr*-positive isolates among the 417 *E. coli* isolates of the BfT-GermVet study was low.

The ESBL genes *bla*<sub>CTX-M-1</sub> and *bla*<sub>CTX-M-15</sub> and the PMQR gene *qnrB19* were detected in *E. coli* isolates from diseased animals in Germany. Several reports described the co-localisation of ESBL and PMQR genes within the same isolate and on the same plasmid (Dionisi et al., 2009; Dolejska et al., 2011a; Kirchner et al., 2011; Müller et al., 2011; Richter et al., 2010; Woodford et al., 2009; Yao et al., 2011). In contrast, the porcine and canine isolates, harbouring pCTX168 and pCTX246 with *bla*<sub>CTX-M-1</sub>, were not positive for *qnr* or *aac(6′)-Ib-cr* genes and the equine isolates, harbouring pQNR2078 and pQNR2086 with *qnrB19*, did not produce an ESBL. Solely one of the five investigated plasmids pCTX913 from a canine *E. coli*
isolate carried an $aac(6')-lb-cr$ and a $bla_{CTX-M-15}$ gene. These findings and the in depth analysis of the plasmids harbouring these genes contribute to the knowledge of the distribution and organisation of such resistance genes.
Chapter 5

Summary
Summary

Anne-Kathrin Schink Studies on the prevalence, distribution and organization of extended-spectrum β-lactamase genes and transferable (fluoro)quinolone resistance genes among Enterobacteriaceae from defined disease conditions of companion and farm animals

Extended-spectrum β-lactamase (ESBL)- and Qnr-protein-producing *Escherichia coli* isolates have gained considerable public attention during recent years. However, information about such isolates from diseased animals in Germany is scarce. Thus, the aims of this study were (i) to determine how often and which subtypes of ESBL and *qnr* genes are present in *E. coli* from defined disease conditions of companion and farm animals and (ii) to gain insight into the location and organization of the resistance genes. For this, the *E. coli* isolates collected all over Germany in the BfT-GermVet study were used.

In the BfT-GermVet study, 417 *E. coli* isolates from diseased dogs/cats (n = 228), horses (n = 102), and swine (n = 87) were tested for their susceptibility to 24 antimicrobial agents by broth microdilution. To identify potential ESBL-producers, all 100 ampicillin-resistant *E. coli* isolates from this collection were subjected to an initial screening for cefotaxime resistance and subsequent phenotypic confirmatory tests. In a second part of the project, all *E. coli* isolates were screened for *qnr* genes. The ESBL and *qnr* genes were detected by specific PCR assays and the complete resistance genes including their flanking regions were cloned and sequenced. Plasmids were transferred by conjugation and transformation into *E. coli* recipients and subjected to PCR-based replicon typing. One *qnrB19*-carrying plasmid was sequenced completely. Multilocus sequence typing (MLST) was performed for the ESBL-producing and for the *qnr*-positive *E. coli* isolates.
Solely three *E. coli* isolates showed an ESBL phenotype. The canine isolate 913 from a urinary tract infection harboured a bla_{CTX-M-15} gene and the *E. coli* isolates from canine pneumonia (isolate 168) and porcine metritis-mastitis-agalactia syndrome (isolate 246) bla_{CTX-M-1} genes. The gene *qnrB19* was detected in two *E. coli* isolates (2078 and 2086) from mares suffering from genital tract infections.

MLST showed that isolate 913 had the sequence type ST410, while isolates 168 and 246 belonged to the novel sequence types ST1576 and ST1153, respectively. Isolates 2078 and 2086 were assigned to ST86.

Isolate 913 harboured the ca. 50 kb IncF plasmid pCTX913. This plasmid carried the bla_{CTX-M-15} gene and an aac(6')-Ib-cr gene, which confers resistance to kanamycin and reduced susceptibility to ciprofloxacin. Furthermore, pCTX913 conferred resistance to gentamicin and tetracycline. Upstream of bla_{CTX-M-15} the insertion sequence IS_{Ecp1} was present and downstream a truncated orf477 and a fragment of a transposase gene *tnpA*.

The two bla_{CTX-M-1} ESBL genes were located on structurally related plasmids of ca. 50 kb which did not confer any other resistance properties. PCR-based replicon typing identified both plasmids, designated pCTX168 and pCTX246, to belong to IncN. Plasmid pCTX168 was conjugative. The bla_{CTX-M-1} upstream and the immediate downstream regions of pCTX168 and pCTX246 were similar whereas the sequences further downstream of bla_{CTX-M-1} differed. In the upstream region, a fragment of the insertion sequence IS_{Ecp1}, truncated by an IS_{26} element, was detected. In the downstream region, a fragment of orf477 and a truncated *mrx* gene were identified on both plasmids. On plasmid pCTX246, a complete mph(A) gene and another IS_{26} element were seen further downstream of the Δmrx gene, while on plasmid pCTX168 the mph(A) gene was truncated.

The *qnrB19*-carrying conjugative plasmids pQNR2078 and pQNR2086 belonged also to IncN, were indistinguishable by restriction analysis and did not carry other resistance genes. The *qnrB19* gene was flanked by copies of the insertion sequence IS_{26} located in the same orientation. The completely sequenced plasmid, pQNR2078, had a size of 42,379 bp and exhibited 47 open reading frames. Except for the resistance gene region, the remaining part of pQNR2078 showed 99%
nucleotide sequence identity to the $bla_{CTX-M-65}$-carrying plasmid pKC396 from human E. coli.

The $bla_{CTX-M-15}$ gene region as identified on the IncF plasmid pCTX193 has been detected in diverse plasmid backgrounds in different members of the Enterobacteriaceae from human and animal sources isolated in several countries. In addition, the gene $bla_{CTX-M-1}$ gene in combination with the IS26-$\Delta$ISEcp1-structure, the truncated $mph(A)$-$mrk$-$mphR(A)$ gene cluster which is followed by a second IS26, linked to IncN plasmids has been described in a German human clinical E. coli ST131 isolate, but not in isolates of animal origin in Germany so far.

The gene $qnrB19$ gene was detected in a new genetic environment on conjugative IncN plasmids and for the first time in an E. coli isolate of equine origin in Germany.

Despite the fact, that the number of $bla_{CTX-M}$ and $qnr$ genes among E. coli from the BiT-GermVet study was low, the results of this study showed that the integration of insertion sequences and interplasmid recombination events accounted for the structural variability of the $bla_{CTX-M}$ gene regions and the formation of the genetic environment of $qnrB19$ on pQNR2078. Furthermore, IncN plasmids, which carry $bla_{CTX-M-1}$ or $qnrB19$ genes, might be involved in the dissemination of these resistance genes among Enterobacteriaceae of animal and human origin.
Zusammenfassung
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Anne-Kathrin Schink

Untersuchungen zu Vorkommen, Verbreitung und Organisation von Genen für β-Laktamasen mit erweitertem Wirkungsspektrum und transferablen (Fluor)Chinolon-Resistenzgenen bei Enterobacteriaceae aus definierten Krankheitsprozessen von Kleintieren und Nutztieren

In der jüngeren Vergangenheit sind *Escherichia coli*-Isolate, die Gene für β-Laktamasen mit erweitertem Wirkungsspektrum (extended-spectrum β-lactamase; ESBL) und/oder Qnr-Proteine tragen und so Resistenz gegenüber Penicillinen, Cephalosporinen und Monobaktamen beziehungsweise (Fluor)Chinolononen zeigen, zunehmend in den Fokus des öffentlichen Interesses gelangt. Da es über diese Gene bei *E. coli*-Isolaten von erkrankten Tieren in Deutschland nur wenig Informationen gibt, waren die Ziele dieser Studie festzustellen (i) wie häufig und welche Gene für ESBLs und Qnr-Proteine bei *E. coli* Isolaten von erkrankten Kleintieren und Nutztieren zu finden sind und (ii) die genetische Lokalisation und Organisation dieser Gene zu untersuchen. Hierzu wurde das Stammkollektiv der deutschlandweit durchgeführten BfT-GermVet Studie verwendet.

In der BfT-GermVet Studie wurden für 417 *E. coli*-Isolate von erkrankten Hunden/Katzen (n = 228), Pferden (n = 102) und Schweinen (n = 87) minimale Hemmkonzentrationen gegenüber 24 antimikrobiellen Wirkstoffen mittels Bouillonmikrodilution bestimmt. Von diesen 417 *E. coli*-Isolaten zeigten 100 Resistenz gegenüber Ampicillin. Um potentielle ESBL-Produzenten zu identifizieren, wurden zusätzlich die minimalen Hemmkonzentrationen für Cefotaxim bestimmt und anschließend phänotypische Bestätigungstests durchgeführt. Alle *E. coli*-Isolate wurden auf das Vorhandensein von *qnr*-Genen untersucht. Die ESBL- und *qnr*-Gene wurden mittels spezifischer PCR-Assays detektiert und die vollständigen Gene inklusive ihrer flankierenden Regionen kloniert und sequenziert. Plasmide wurden
über Konjugation oder Transformation in *E. coli*-Empfängerstämme transferiert und mit PCR-basiertem Replikontypisierung charakterisiert. Für die ESBL-produzierenden und *qnr*-positiven *E. coli*-Isolate wurde Multilocus-Sequenztypisierung (MLST) durchgeführt.


Isolat 913 beinhaltete das ca. 50 kb IncF-Plasmid pCTX913 mit den Genen *bla<sub>CTX-M-15</sub>* und *aac(6')-Ib-cr* – letzteres Gen vermittelt Resistenz gegenüber Kanamycin und verminderte Empfindlichkeit gegenüber Ciprofloxacin. Des Weiteren vermittelte pCTX913 auch Resistenz gegenüber Gentamicin und Tetrazyklin. In der stromaufwärts von *bla<sub>CTX-M-15</sub>* gelegenen Region befand sich die Insertionssequenz ISEcp1 und stromabwärts ein Fragment von *orf477* sowie ein Fragment eines Transposasegens tnpA.

Die *bla<sub>CTX-M-1</sub>* ESBL-Gene waren auf strukturell ähnlichen ca. 50 kb-großen Plasmiden der Inkompatibilitätsgruppe IncN lokalisiert (pCTX168 und pCTX246), die neben der β-Laktamresistenz keine weiteren Resistzenzen aufwiesen. Das Plasmid pCTX168 erwies sich als konjugativ. Der Bereich stromaufwärts von *bla<sub>CTX-M-1</sub>* war bei beiden Plasmiden gleich und bestand aus einem Fragment der Insertionssequenz ISEcp1, welche von einer Insertionssequenz des Typs IS26 unterbrochen wurde. Stromabwärts des *bla<sub>CTX-M-1</sub>*-Genes wurden ein Fragment des offenen Leserahmens *orf477* und ein partiell deletiertes *mrx*-Gen ebenfalls auf beiden Plasmiden gefunden. Weiter stromabwärts unterschieden sich die Sequenzen der beiden Plasmide dagegen voneinander. Auf pCTX246 wurde ein vollständiges
mph(A)-Gen und ein weiteres IS26 identifiziert, während auf pCTX168 das Gen mph(A) partiell deletiert war.


References


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