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OCCURRENCE OF ANTIBIOTIC RESISTANCE GENES IN Enterobacteriaceae ISOLATES FROM HORSES AND COMPANION ANIMALS IN AUSTRIA

DIPLOMA THESIS
to receive the honour
MAGISTRA MEDICINAE VETERINARIAE
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Occurrence of Antibiotic Resistance Genes in *Enterobacteriaceae* Isolates from Horses and Companion Animals in Austria

1. Introduction

1.1. *Enterobacteriaceae*

*Enterobacteriaceae* are a large group of related bacteria living in soil, water and decaying matter, and are also common occupants of both human’s and animal’s intestines. They are acquired through contaminated food or water, and are the major cause of diarrheal illnesses. (Hany et al., 2013).

They are gram-negative facultative anaerobic rods, which ferment a wide range of sugars and are oxidase-negative, but for the *Plesiomonas* species which are oxidase-positive (Quinn et al., 2011, Salerno et al., 2007). Currently the family contains 53 genera and 301 species (http://www.bacterio.net, last accessed 24.09.2013).

There are three major groups to which they can be assigned: Non-pathogens are *Enterobacteriaceae* with no known pathogenic significance for animals such as *Erwinia*. As they occur naturally in faeces and in the environment, they can contaminate clinical samples. Opportunistic pathogens, like *Proteus*, *Morganella*, *Edwardsiella* and *Serratia* species, may cause disease in other parts of the body than the alimentary tract. (Quinn et al., 2011)

In between there are pathogens which were considered as opportunistic in former times and are now ranked among the pathogens, also called ESKAPE pathogens, comprising following bacteria: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumonia*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa* and *Enterobacter spp.* (Pendleton et al., 2013).

These pathogens are a major cause of noscomial infections and show high rates of resistance making treatment complicated, especially in intensive care units. (Rice, 2010)

Finally there are the major animal pathogens causing both enteric and systemic disease. Examples are *Escherichia coli*, *Salmonella* species and *Yersinia* species. Growth on MacConkey agar is one of their differentiation criteria as *Enterobacteriaceae* are not inhibited by the bile salts in the medium. (Quinn et al., 2011)

*Enterobacteriaceae* in companion animals and horses possibly play an important role in zoonotic transmission of bacterial resistance genes to humans. (Gandolfi-Decristophoris et al., 2013).
1.2. β-lactamases

Resistance to antibacterial drugs has become an increasingly important problem both in animals and humans. Genes encoding resistance mechanisms such as β-lactamases arise as an inevitable consequence of the selective pressure produced by the use of antimicrobial agents in animal husbandry and human medicine. There are various ways for bacteria to reduce susceptibility to antibiotics like increased drug efflux, drug inactivation, drug-target-modification and decreased membrane permeability. Plasmids and transposons containing integron sequences play an important role in transmission and acquisition of resistance genes and account for their fast spread between different bacterial genera and species. This transfer is particularly common in Enterobacteriaceae, anaerobes and Pseudomonas species colonizing the intestinal tract. (Quinn et al., 2011)

The substrate of β-lactamases are the bactericidal β-lactams including penicillins, cephalosporins, carbapenems and monobactams. β-lactams act on the bacterial cell wall by binding and inhibiting PBP’s (Penicillin Binding Proteins), which catalyze the D-ala D-ala crosslinkages of the peptidoglycan wall. (Rawat et al., 2010)

β-lactamases are enzymes which inactivate β-lactam antibiotics based on the cleavage of the amino bond in the β-lactam ring. They differ widely in their substrate specificity with some acting more rapidly on penicillins, others on cephalosporins. β-lactamase genes, called bla-genes, are often localized on plasmids, transposons or gene cassettes, which favours their dissemination. (Schwarz et al., 2006)

They can be grouped by two different classification systems. (Schwarz et al., 2006)

The Ambler classification groups them according to structural differences i.e. amino-acid sequence is used to determine molecular phylogeny. They are divided into four classes (A, B, C and D). Class A, C and D are active-site serine β-lactamases with a serine residue in the catalytic center. Class A was the originally specified serine β-lactamase. Class C and D were discovered later and had little sequence similarity to class A or each other. Members of class C are also named AmpC β-lactamases, whereas class D enzymes are known as OXA β-lactamases. Class B comprises metallo-β-lactamases, which require a bivalent metal ion, zinc, for activity. (Hall et al., 2005, Ambler, 1980)
The functional classification system of Bush et al. from 1989 groups β-lactamases according to substrate and inhibitor profiles and was updated in 2010. Three major groups are differentiated with group 1 (class C) containing cephalosporinases, group 2 (classes A and D) including broad-spectrum, inhibitor-resistant, and extended-spectrum β-lactamases and serine carbapenemases and group 3 containing metallo-β-lactamases. (Bush et al., 1995, Schwarz et al., 2006, Bush et al., 2010)

Group 1 β-lactamases hydrolyze all β-lactams except carbapenems and are not inhibited by clavulanic acid. AmpC β-lactamases belong to this group and are widespread in gram-negative bacteria with ampC genes usually located on the chromosome but also on plasmids. (Schwarz et al., 2006, Bush et al., 2010)

Group 2 β-lactamases are generally inhibited by active site-directed β-lactamase inhibitors and belong to molecular classes A or D. They are further subgrouped as follows:

Enzymes of subgroup 2a inactivate only penicillins.

Subgroup 2b enzymes are broad-spectrum β-lactamases, such as TEM-1 and SHV-1, with the ability to hydrolyze penicillins and early cephalosporins, such as cephaloridine and cephalothin. (Schwarz et al., 2006, Bush et al., 2010)

Extended-spectrum β-lactamases (ESBL) are comprised in subgroup 2be and mostly belong to the TEM, SHV, CTX-M and OXA families conferring resistance to oxyimino-cephalosporins, for example cefotaxime and ceftazidime, and monobactams. Less common are members of the PER, VEB and GES ESBL families. (Schwarz et al., 2006, Bush et al., 2010)

Enzymes of subgroup 2a, 2b and 2be are all sensitive to inhibition by β-lactamase inhibitors, like clavulanic acid and tazobactam. (Schwarz et al., 2006, Bush et al., 2010)

Subgroup 2br enzymes are broad-spectrum β-lactamases with the same spectrum of activity as subgroup 2b but with acquired resistance to clavulanic acid and related inhibitors. TEM enzymes that combine an extended spectrum with relative resistance to clavulanic acid inhibition have been classed into subgroup 2ber and are also called CMT (complex mutant TEM) β-lactamases. (Schwarz et al., 2006, Bush et al., 2010)

Subgroup 2c includes carbenicillin-hydrolyzing β-lactamases which are inhibitor-sensitive and enzymes of subgroup 2ce are defined as extended-spectrum carbenicillinases.
Enzymes of subgroup 2d, known as OXA enzymes, include cloxacillin- and oxacillin-hydrolyzing β-lactamases, whereas the spectrum of subgroup 2de enzymes also covers oximino-β-lactams and of subgroup 2df additionally carbapenems. (Schwarz et al., 2006, Bush et al., 2010)

Cephalosporinases belonging to subgroup 2e, possess extended-spectrum cephalosporin-hydrolyzing activities and are inhibited by clavulanic acid or tazobactam. Subgroup 2f represents serine-carbapenemases, which are better inhibited by tazobactam than clavulanic acid. Examples are plasmid-encoded GES- and KPC-enzymes. (Schwarz et al., 2006, Bush et al., 2010)

Group 3 enzymes are metallo-β-lactamases inactivating all β-lactams except monobactams. They are insensitive to classical β-lactamase inhibitors, but sensitive to metal ion chelators such as EDTA (Ethylenediaminetetraacetic acid) or dipicolinic acid. They belong to structural group B and need divalent zinc cations at their active site. Important examples are the VIM- and IMP-enzymes. (Schwarz et al., 2006, Bush et al., 2010)

1.2.1. Emergence of antibiotic resistance mechanisms

Resistance to β-lactams may be the result of the production of β-lactamases, impermeability of the outer membrane by non-expression of porin-genes, extrusion of antibiotics through efflux-pumps and target modification (β-lactam-insensitive PBPs). These modalities may occur singly or in different combinations. (Rawat et al., 2010)

Serine-based β-lactamase diversity is enhanced by the fact that they are found in bacteria living in a wide variety of environments and thus being subject to different selective pressures. (Bush et al., 2010) Selective pressure leads to the continuous adaption of resistance mechanisms aided by various molecular strategies. These include expansion of the substrate spectrum by amino acid substitutions in certain enzymes as was the case with ESBLs which originated from broad-spectrum TEM- and SHV-type β-lactamases. Acquisition of genes encoding ESBLs from environmental bacteria is particularly significant. Examples are the CTX-M-type β-lactamases from *Kluyvera* spp., which now arise in *Enterobacteriaceae*. There are also the many mechanisms for horizontal gene transfer of *bla* genes by plasmids, integrons and insertion sequences as for instance the transfer of the integron-located *ampC* gene from *Citrobacter freundii* to *Klebsiella* spp. Another molecular strategy is high-level
expression of intrinsic bla genes like bla<sub>ampC</sub> or bla<sub>OXA</sub> caused by promoter mutations, modifications in regulatory genes and integration of insertion sequences containing an efficient promoter. (Pfeifer et al., 2010)

Penicillins, cephalosporins and carbapenems are important treatment regimens for infectious diseases. β-lactamases are the most common resistance mechanism to these antimicrobial agents and presence and characterization of them therefore plays a critical role in appropriate treatment selection. (Bush et al., 2010)

1.2.2. AmpC β-lactamases

AmpC enzymes were the first β-lactamases reported to destroy penicillin. They belong to class C in the Ambler structural classification and group 1 in Bush et al.’s functional classification system (Jacoby et al., 2009).

AmpC β-lactamases are clinically important cephalosporinases encoded on the chromosomes of many of the Enterobacteriaceae and a few other organisms, where they mediate resistance to cephalothin, cefazolin, cefoxitin, most penicillins, and β-lactamase-inhibitor β-lactam combinations. Transmissible plasmids with acquired genes for AmpC β-lactamases can explain resistance in Enterobacteriaceae lacking or poorly expressing a chromosomal ampC gene, such as Escherichia coli, Klebsiella pneumoniae, and Proteus mirabilis. In some bacterial strains AmpC enzymes are inducible, causing overexpression and resistance in initially susceptible isolates. (Jacoby et al., 2009) Organism identification is indicative of AmpC production in organisms with inducible chromosomal AmpC β-lactamases as these organisms readily mutate to develop resistance during therapy with β-lactam antibiotics other than the carbapenems, penems or zwitterionic (sometimes referred to as fourth-generation cephalosporins (e.g., cefpime).

Detection of an AmpC β-lactamase in organisms, which lack a chromosomal AmpC β-lactamase, like Klebsiella spp., Salmonella spp., Citrobacter koseri, or Proteus mirabilis is confirmatory for plasmid-mediated AmpC. (Thomson, 2010)

So far six families of plasmid-mediated AmpC β-lactamases have been identified by grouping them based on their chromosomal origin. The six families comprise ACC (origin Hafnia alvei), FOX (origin Aeromonas caviae), DHA (origin Morganella morganii), CIT (origin Citrobacter freundii), and EBC (origin Enterobacter cloacae). Phenotypically these
families cannot be differentiated and multiplex PCR or microarray should be used. (Pérez-Pérez et al., 2002, Fosse et al., 2003, Bogaerts et al., 2011, Cuzon et al., 2012)

The nomenclature of the \( \text{bla}_{\text{ampC}} \) genes is somewhat inconsistent and is on the one hand based on the resistance conferred to cephemycins (CMY), cefoxitin (FOX), moxalactam (MOX) and latamoxef (LAT) and on the other hand results from the type of \( \beta \)-lactamase as AmpC-type (ACT) and Ambler class C (ACC), from the site of discovery as Miriam Hospital in Providence, R.I. (MIR-1) or Dhahran hospital in Saudi Arabia (DHA) or from the patient (Bilal) who provided the original sample (BIL-1). (Phillipon et al., 2002)

The first plasmid-mediated AmpC \( \beta \)-lactamase to be found was CMY-1 (MOX-group) in Saudia Arabia in 1989, followed by CMY-2 (CIT-group) in Greece and MIR-1 (EBC-group) in the United States. The first gene of the FOX-family, FOX-1, was detected 1994 in Argentina, of the DHA-family it was DHA-1 in 1997 in Saudi Arabia and ACC-1 of the ACC-group was first determined in Germany in 1999. (Jacoby et al., 2009)

Plasmid-mediated AmpC genes have been found around the world and were mostly detected in enterobacteria. CMY-2 of the CIT-group seems to be the most common plasmid-mediated AmpC \( \beta \)-lactamase. (Jacoby et al., 2009)

Multi-drug resistance is often found in isolates containing plasmid-encoded AmpC- \( \beta \)-lactamases. However plasmid-mediated AmpC \( \beta \)-lactamases appear to be less common than ESBLs. (Thomson, 2010)

1.2.3. Extended Spectrum \( \beta \)-Lactamases (ESBLs)

ESBLs are enzymes capable of hydrolysing penicillins, broad-spectrum cephalosporins and monobactams, and are generally derived from TEM and SHV-type enzymes, broad-spectrum \( \beta \)-lactamase progenitors. The most important difference between ESBLs and their broad-spectrum \( \beta \)-lactamase progenitors is the ability of ESBLs to hydrolyze 3rd and 4th-generation cephalosporins resulting from mutations of the original genes leading to amino acid substitutions. (Pfeifer et al., 2010) ESBLs are often located on plasmids that are transferable from strain to strain and between bacterial species. (Rupp et al., 2003)

With the exception of OXA-type enzymes (which are class D enzymes), the ESBLs are of molecular class A. In the Bush-Jacoby-Medeiros classification scheme they belong to group 2be or group 2d (OXA-type). Group 2d shares most of the properties with group 2be, but
differs in being inhibitor resistant.

ESBLs are derived from group 2b \(\beta\)-lactamases (for example TEM-1, -2, SHV-1) and some differ from their progenitor by only one amino acid, which nonetheless leads to changed enzymatic activity.

There are four enzyme families, which are currently regarded as the most common ESBLs among Enterobacteriaceae, namely TEM (Temoneira)-type \(\beta\)-lactamases, SHV (Sulphhydryl variable) -type \(\beta\)-lactamases, CTX (ceftaximase) -M-type \(\beta\)-lactamases and OXA (oxacillinase) -type \(\beta\)-lactamases (Ewers et al., 2011).

They show similar behaviour but differ in their evolutionary histories. The mutants of TEM and SHV \(\beta\)-lactamases, with about 178 SHV- and 214 TEM-variants as stated by the Lahey clinical database, were dominant during the 1990’s (Dallenne et al., 2010, http://www.lahey.org/Studies/, last accessed 07.11.2013). During the past decade rapid and massive spread of the CTX-M enzymes, the name deriving from cefotaxime resistance, was described. They are divided into five subgroups with currently 148 variants as assigned by the Lahey clinic database (http://www.lahey.org/Studies/, last accessed 07.11.2013 ).

They seem to be the most prevalent ESBLs in Enterobacteriaceae in Europe. (Dallenne et al., 2010, Pfeifer et al., 2010)

The OXA-type \(\beta\)-lactamases (group 2d) are so named because of their oxacillin-hydrolyzing abilities. They predominantly occur in Pseudomonas aeruginosa.

A variety of other \(\beta\)-lactamases (PER, VEB, GES, BES, TLA, SFO, IBC groups) which are plasmid-mediated or integron-associated class A enzymes have also been discovered. (Rawat et al., 2010)

Antibacterial choice is often complicated by multi-drug-resistance. Many ESBL-producing organisms also express AmpC \(\beta\)-lactamases and may be co-transferred with plasmids mediating aminoglycoside resistance. In addition, there is an increasing association between ESBL production and fluoroquinolone resistance. Currently, carbapenems are regarded as the drugs of choice for treatment of infections caused by ESBL-producing organisms.

Unfortunately, use of carbapenems has been associated with the emergence of carbapenem-resistant bacterial species such as Stenotrophomonas sp. or Pseudomonas sp. (Rupp et al., 2003)
1.2.3.1. CTX-M ESBLs

CTX-M ESBLs are divided into five phylogenetic groups; group 1, 2, 8, 9 and 25, with each group having a natural ancestor in a chromosomal gene derived from the *Kluyvera* spp., especially *Kluyvera ascorbata* and *Kluyvera georgiana*. Prevalence of CTX-M groups differs in each country. (Woodford et al., 2006)

The first CTX-M ESBL was discovered in Japan in 1986 in a cefotaxime-resistant *E. coli* strain isolated from the fecal flora of a laboratory dog, which was used for pharmacokinetic studies of β-lactam antibiotics. Since then this mechanism of resistance has spread to different countries and is now a major concern in certain areas, such as South America, the Far East and Eastern Europe. CTX-M groups 1, 2 and 9 seem to be the most widespread. (Bonnet et al., 2004)

The simultaneous observation of CTX-M enzymes in nosocomial and community strains from multiple geographic locations is consistent with their emergence from a widespread reservoir, owing to independent genetic events, like the mobilization of *bla*<sub>CTX-M</sub> genes from the chromosomes of environmental *Kluyvera* bacteria. (Bonnet et al., 2004)

This process could also explain the delayed expansion of CTX-M enzymes, as opposed to the explosive emergence of TEM and SHV ESBLs since 1985, which derived from widespread plasmid-mediated enzymes. (Bonnet et al., 2004)

The success of the CTX-M type ESBLs is caused by several factors including low fitness cost imposed by CTX-M production, high selective pressure by use of β-lactam antibiotics and fluoroquinolones and efficient capture and dispersal of *bla*<sub>CTX-M</sub> genes by mobile genetic elements. (D’Andrea et al., 2013)

1.2.3.2. SHV- and TEM-ESBLs

Plasmid-mediated SHV- and TEM-ESBLs are derived from mutations in the broad-spectrum TEM-1 and SHV-1 genes by one or more amino acid substitution around the active site. (Jain et al., 2008, Bradford et al., 2001)

The name ‘TEM’ is a contraction of Temoniera, the name of a patient from whom resistant bacteria was isolated. In contrast, ‘SHV’ is a contraction of sulphydryl variable: a description of the biochemical properties of this β-lactamase.

The first plasmid-mediated β-lactamase in gram-negatives, TEM-1, was described in the early
1960s. Originally found in a single strain in *E. coli*, it spread worldwide to other species of *Enterobacteriaceae*. This was facilitated by TEM-1 being plasmid and transposon mediated. (Bradford et al., 2001)

The first reported SHV β-lactamase had a narrow spectrum of activity. By the accumulation of point mutations at sites that affect the active site of the enzyme, a family of derivatives of SHV-1 has evolved. Derivatives of SHV-1 either have an extended spectrum of activity, capable of inactivating third-generation cephalosporins or are resistant to β-lactamase inhibitors. (Heritage et al., 1999)

**1.2.3.3. OXA-1- and OXA-2-group ESBLs**

OXA-1- and OXA-2-group ESBLs are named after their oxacillin-hydrolyzing abilities. Hydrolysis rates for oxacillin and cloxacillin greater than 50% than that for benzylpenicillin characterize them. They belong to the molecular class D in the Ambler classification scheme and are functionally classified into subgroup 2d. (Paterson et al., 2005)

They predominantly occur in *Pseudomonas aeruginosa* but have been detected in many other gram-negative bacteria. The most common OXA-type β-lactamase is OXA-1. (Paterson et al., 2005)

The *bla*OXA-1 group comprises *bla*OXA-1, -4, -30, -31 and *bla*OXA-47 genes, the *bla*OXA-2 group comprises *bla*OXA-2, -3, -15, -32, -34 and *bla*OXA-53 genes. (Hasman et al., 2005)

Not all OXA enzymes are ESBLs, OXA enzymes of group 2d are oxacillinases and of group 2df carbapenemases. (Bush et al., 2010)

**1.2.3.4. PER ESBLs**

PER ESBLs stand for *Pseudomonas* extended resistance type β-lactamases. The PER-type ESBLs are among the most efficient β-lactamases, able to hydrolyze broad-spectrum cephalosporins, but are susceptible to clavulanic acid inhibition. They represent a distinct class A cephalosporinase phenotype so far occurring mainly in South America and Europe. (Paterson et al., 2003, Paterson et al., 2005)

The PER-1 β-lactamase was first discovered in strains of *P. aeruginosa* isolated from patients

PER-2, which has 86% amino acid homology with PER-1, was found among S. enterica serovar Typhimurium strains in Argentina.

PER-1 was found mainly in Turkey and PER-2 in South America. (Bradford et al., 2001)

1.2.3.5. VEB ESBLs

VEB-1 (Vietnamese extended spectrum β-lactamase) was first described in an E. coli strain isolated in a French hospital from the pus of a 4-month-old Vietnamese boy in 1996. (Farajnia et al., 2012) Subsequently it was also found in a P. aeruginosa isolate from a patient from Thailand. (Bradford et al., 2001)

It confers resistance to extended-spectrum cephalosporins and aztreonam.

bla \_\text{\text{VEB}} \text{ \text{genes only show significant homology at the DNA level with } \text{\text{bla}} \_\text{\text{PER-1}} \text{ \text{and } \text{\text{bla}} \_\text{\text{PER-2}}.}

Some β-lactamase genes not only have a plasmid location, but may be encoded in gene cassettes that are present in the variable region of integrons. (Poirel et al., 1999) In most cases bla \_\text{\text{VEB}} \text{ \text{is located on a class I integron. (Zong et al., 2009) bla \_\text{\text{VEB}} \text{ \text{genes are further assumed to be colocalized on the same class I integron as } \text{\text{bla}} \_\text{\text{OXA}} \text{ \text{genes. (Kiratisin et al., 2008) }}

VEB-1 was also found to be encoded on a large (>100-kb) transferable plasmid found in a Klebsiella pneumoniae isolate, indicating easy horizontal gene transfer. (Poirel et al., 1999)

1.2.3.6. GES ESBLs

GES-1 ESBL (Guiana extended-spectrum β-lactamase) was first isolated in Klebsiella pneumonia in 1998 in France from a rectal swab of a girl who was previously hospitalized in French Guiana. (Poirel et al., 2000)

GES, VEB and PER are clustered together within a class A ESBL subgroup.

bla \_\text{\text{GES}} \text{ \text{genes confer an extended-spectrum cephalosporin resistance profile antagonized by the addition of clavulanic acid and tazobactam. (Poirel et al., 2000) }}

More GES variants have been identified. Some of them, such as GES-2, GES-4, GES-5, or GES-6, are characterized by their extended spectrum toward carbapenems. These enzymes have been detected in Enterobacteriaceae (K. pneumoniae, Enterobacter cloacae,
or *Escherichia coli* and *P. aeruginosa*, with a growing geographical dissemination (including South Africa, South Korea, France, Greece, Canada, China, and Brazil) in the last few years. (Viedma et al., 2009)

1.3. Fluoroquinolone resistance

The first quinolone, nalidixic acid, was introduced into clinical use in 1962. In the mid 1980s ciprofloxacin, which was particularly effective against gram-negative bacteria, first became available. (Strahilevitz et al., 2009) Parallel to increased quinolone utilization, quinolone resistance emerged and also plasmid-mediated resistance was discovered. (Jacoby, 2009) Since the introduction of fluoroquinolones, resistance of the *Enterobacteriaceae* to these agents has become common, widespread, and generally nonclonal. This implies that fluoroquinolone resistance has emerged independently many times. Higher-level resistance probably emerged by selection of organisms with low-level quinolone resistance, which were able to survive quinolones at therapeutic levels. So multiple resistance mutations could have arisen sequentially, leading for example to accumulation of mutations in the bacterial enzymes targeted, e.g. DNA gyrase and topoisomerase IV. (Strahilevitz et al., 2009)

1.3.1. *qnr* genes

*qnr* genes are defined as naturally occurring alleles encoding a pentapeptide repeat protein that confers reduced susceptibility to nalidixic acid or fluoroquinolones. Alleles are grouped into the same family, if they show a difference in nucleotides or derived amino acids of less than 30%. Alleles within these families differ in at least one amino acid. (Strahilevitz et al., 2009)

*qnr* proteins are formed by two domains of pentapeptide repeats (tandem 5-amino-acid repeats) separated by a single amino acid, usually glycine. There are five known *qnr* gene families; *qnrA, qnrB, qnrS, qnrC* and *qnrD*. (Strahilevitz et al., 2009)

The first *qnr*, named after quinolone resistance, gene was isolated in Alabama in 1994 in *Klebsiella pneumonia*. (Jacoby, 2009, Strahilevitz et al., 2009)
Their mechanism of resistance is to prevent inhibition of DNA gyrase and topoisomerase IV by ciprofloxacin and other fluoroquinolones. The original qnr gene was named qnrA. The origin of qnrA is most likely the chromosome of an aquatic bacterium, Shewanella algae. (Jacoby, 2009)

qnrB was found in Klebsiella pneumonia in India (Jacoby, 2009) and has a broader repertoire of variants than qnrA and qnrS. (Strahilevitz et al., 2009)

qnrS was discovered in Shigella flexneri in Japan. Genes with more than 80% similarity to qnrS have been found in the genome of a marine organism, Vibrio splendidus (Jacoby, 2009)

qnrC was isolated from a clinical strain of Proteus mirabilis from Shanghai, China, which transferred low-level quinolone resistance. (Strahilevitz et al., 2009)

qnrD was first detected in four Salmonella enterica isolates obtained from humans in the Henan Province of China. (Strahilevitz et al., 2009)

qnr genes are often linked to ESBLs or AmpC β-lactamases. (Jacoby, 2009)

1.3.2. qepA

The qepA gene encodes an efflux pump that extrudes quinolones. It was discovered on a plasmid in an E. coli strain from a urine specimen from an inpatient in Japan, in 2002. Phylogenetic analysis showed that it belongs to the 14-transmembrane-segment family transporters of gram-positive Actinomycetales. (Strahilevitz et al., 2009)

1.3.3. aac(6')-ib-cr

aac(6')-ib-cr is a plasmid-determined variant of aminoglycoside acetyltransferase, which can modify agents, such as ciprofloxacin, by acetylation of its piperazinyl substituent. So resistance to two unrelated antimicrobial agent classes, the aminoglycosides and the fluoroquinolones, is provided by this gene. (Strahilevitz et al., 2009, Jacoby, 2009)

It confers resistance to tobramycin, amikacin, and kanamycin. The increase in MIC (minimal inhibitory concentration) conferred by aac(6')-ib-cr is smaller than that conferred by qnr proteins. As predicted by its specific quinolone target, it is selective only for agents with piperziny1 secondary amines. (Strahilevitz et al., 2009)
1.4. Project description
The aim of the present study was to screen *Enterobacteriaceae* isolates from horses and companion animals for the occurrence of ESBL-, *ampC*- and quinolone-resistance genes by PCR and to compare the presence of these resistance genes between the individual species. The DNA isolates were studied for following resistance genes depending on their phenotypic profile, which was determined in a parallel diploma thesis; AmpC-families ACC, FOX, MOX, DHA, CIT and EBC, CTX-M, SHV, TEM, OXA-1, OXA-2, VEB, PER, GES, *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *aac(6')-Ib-cr* and *qepA*. 
2. Materials and Methods

2.1. Bacterial isolates

were isolated from 150 different samples, which were submitted for bacteriological analysis to the Unit of Clinical Microbiology and Infection Biology, Institute for Bacteriology, Mycology and Hygiene of the University of Veterinary Medicine, Vienna between 2010 and 2012 (Table 1).

*Enterobacteriaceae* were included in the present study if phenotypical testing hinted presence of ESBLs, AmpC β-lactamases and/or quinolon resistance genes.

57 of the *Enterobacteriaceae* isolates originated from dogs (38%), 56 from horses (37.3%), 14 from cats (9.3%), 6 from birds (4%), 4 from rats (2.7%), 3 from guinea pigs (2%) and two from each of these animal species; frogs and tortoises (1.3% each). Single samples were derived from a monkey, a donkey, a cheetah, a boid and a skate (each 0.7%).

Bacterial isolates were derived from various samples, including faeces, wound swabs, organ biopsies, abdominocentesis fluid, pus from a hoof abscess and other material.

The *Enterobacteriaceae* isolates examined in the present study characterized either by species specific PCR for *E. coli* or using API 10S, which was performed in parallel a study.

In all 101 *E. coli*, 16 *Citrobacter* sp., 12 *Klebsiella* sp., 12 *Enterobacter* sp., 5 *Salmonella* sp., 2 *Serratia* sp., 1 *Pantoea* sp. and 1 *Shigella* sp. DNA extraction was performed in the course of another diploma thesis and DNA samples were kept frozen at -25°C until further analysis.
2.2. PCR for detection of resistance genes

Simplex (monoplex) PCR was used for detection of most resistance genes, whereas multiplex PCR was used for detection of the AmpC-families.

PCR was performed in a MultiGene™ Gradient PCR Thermal Cycler W, Labnet International, Inc., with PCR conditions summarized in Table 2.

The final volume for the PCR reactions was 20µl composed of 7µl of water, 10µl of Ready Mix Taq, 1µl of each forward and reverse primer in a concentration of 10pmol/µl and 1µl of isolated DNA.

A ReadyMix™ Taq PCR Reaction Mix with MgCl₂ from Sigma-Aldrich (20 mM Tris-HCl, pH 8.3, 100 mM KCl, 3 mM MgCl₂, 0.002 % gelatin, 0.4 mM dNTP mix (dATP, dCTP, dGTP, TTP), stabilizers, 0.06 units Taq DNA Polymerase/µL) was used in all the PCRs.

PCR products were separated by gel electrophoresis in 1x Tris-borate-EDTA buffer (TBE) in the „Profile SUB 2025“ electrophoresis chamber from Schleicher & Schuell and with BioRad PowerPac Basic from BioRad as a power supply.

Gel slots were loaded with 6 µl of each PCR-product together with 5µl of Gene Ruler™ 100bp Plus DNA ladder, (Thermo Scientific, Massachusetts, USA), in a concentration of 0.5 µg/µl. Gel electrophoresis was conducted at approximately 160 V for generally forty minutes in a 2% agarose gel.

Gels were stained with ethidium bromide in a concentration of 0.5 µg per ml for 20 minutes and afterwards destained in water for 10 minutes.

Visualization and photographing of bands was done with a UV-transilluminator, Bio Rad, and the software „Quantity One“.
Table 1: Origin, characteristics and year of isolation of bacterial isolates used in this study with phenotypes for AmpC- (AMPC), ESBL-β-lactamases (ESBL), Quinolon-resistance-genes (Q)

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>bla and qnr genes</th>
<th>Number of isolates</th>
<th>Species</th>
<th>Year of isolation</th>
<th>Source</th>
<th>Material</th>
</tr>
</thead>
<tbody>
<tr>
<td>AmpC (n=16)</td>
<td>n.d.</td>
<td>10</td>
<td>E.coli (n=4), Enterobacter (n=4), Citrobacter (n=2)</td>
<td>2011 (n=1), 2012 (n=9)</td>
<td>dog (n=2), bird (n=3), frog (n=1), cat (n=3), snake (n=1), skin (n=1), intestine (n=1), ovipositor (n=1), tonsils (n=1)</td>
<td>faeces (n=3), choanal swab (n=1), liver (n=2), nasal irrigation (n=1), intestine (n=2), urine (n=1)</td>
</tr>
<tr>
<td>CIT</td>
<td>6</td>
<td>E.coli (n=4), E. coli (n=2)</td>
<td>2011</td>
<td>1</td>
<td>faeces, intestine</td>
<td></td>
</tr>
<tr>
<td>ESBL (n=1)</td>
<td>SHV</td>
<td>1</td>
<td>E.coli</td>
<td>2011</td>
<td>dog</td>
<td>pleural swab, faeces, intestine</td>
</tr>
<tr>
<td>ESBL/AmpC (n=1)</td>
<td>MOX</td>
<td>1</td>
<td>E.coli</td>
<td>2011</td>
<td>cat</td>
<td>wound (n=1), lungs (n=1), air sac (n=2), trachea (n=2)</td>
</tr>
<tr>
<td>AmpC-Q (n=17)</td>
<td>n.d.</td>
<td>3</td>
<td>E.coli (n=1), Salmonella choleraesuis (n=3), Citrobacter braakii (n=1), Serratia odorifera (n=1), Citrobacter braakii (n=1), E. coli</td>
<td>2010 (n=1), 2012 (n=2)</td>
<td>cat, horse, ape</td>
<td>wound (n=1), lungs (n=1), air sac (n=2), trachea (n=2)</td>
</tr>
<tr>
<td>CIT</td>
<td>6</td>
<td>E.coli</td>
<td>2010</td>
<td>1</td>
<td>faeces (n=1), vagina (n=1), synovial fluid (n=1), wound (n=3), sartorial fluid (n=1), tissue biopsy (n=1), uterus (n=1), wound (n=3), seroma (n=1)</td>
<td>synovial fluid</td>
</tr>
<tr>
<td>CIT, qnrB</td>
<td>2</td>
<td>Klebsiella pneumonia</td>
<td>2010</td>
<td>1</td>
<td>faeces (n=1), vagina (n=1), synovial fluid (n=1), wound (n=3), sartorial fluid (n=1), tissue biopsy (n=1), uterus (n=1), wound (n=3), seroma (n=1)</td>
<td>synovial fluid</td>
</tr>
<tr>
<td>CIT, qnrB, qnrS</td>
<td>2</td>
<td>Klebsiella pneumonia</td>
<td>2010</td>
<td>1</td>
<td>faeces (n=1), vagina (n=1), synovial fluid (n=1), wound (n=3), sartorial fluid (n=1), tissue biopsy (n=1), uterus (n=1), wound (n=3), seroma (n=1)</td>
<td>synovial fluid</td>
</tr>
<tr>
<td>CIT, qnrS</td>
<td>1</td>
<td>E. coli</td>
<td>2010</td>
<td>1</td>
<td>faeces (n=1), vagina (n=1), synovial fluid (n=1), wound (n=3), sartorial fluid (n=1), tissue biopsy (n=1), uterus (n=1), wound (n=3), seroma (n=1)</td>
<td>synovial fluid</td>
</tr>
<tr>
<td>MOX, CIT, qnrB, qnrS</td>
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<td>Klebsiella pneumonia</td>
<td>2010</td>
<td>1</td>
<td>faeces (n=1), vagina (n=1), synovial fluid (n=1), wound (n=3), sartorial fluid (n=1), tissue biopsy (n=1), uterus (n=1), wound (n=3), seroma (n=1)</td>
<td>synovial fluid</td>
</tr>
<tr>
<td>ESBL, Q (n=61)</td>
<td>CTX-M, OXA-1, SHV, qnrB, qnrS</td>
<td>3</td>
<td>E.coli (n=5), Klebsiella pneumonia (n=1)</td>
<td>2010 (n=1), 2011 (n=2), 2010 (n=2), 2011 (n=4)</td>
<td>dog (n=3), horse (n=3)</td>
<td>wound (n=1), lungs (n=1), air sac (n=2), trachea (n=2)</td>
</tr>
<tr>
<td>CTX-M, OXA-1, TEM, qnrS, qnrS</td>
<td>3</td>
<td>Enterobacter cloacae (n=1), E. coli (n=2)</td>
<td>2010 (n=1), 2011 (n=2)</td>
<td>dog (n=2), horse (n=1)</td>
<td>synovial fluid (n=1), wound (n=2)</td>
<td></td>
</tr>
<tr>
<td>CTX-M, OXA-1, qnrB, qnrS</td>
<td>1</td>
<td>Enterobacter cloacae</td>
<td>2010</td>
<td>1</td>
<td>synovial fluid (n=1), wound (n=2)</td>
<td></td>
</tr>
<tr>
<td>TEM, qnrB, qnrS</td>
<td>1</td>
<td>Enterobacter cloacae</td>
<td>2010</td>
<td>1</td>
<td>synovial fluid (n=1), wound (n=2)</td>
<td></td>
</tr>
<tr>
<td>CTX-M, TEM</td>
<td>11</td>
<td>E.coli</td>
<td>2010 (n=2), 2011 (n=9)</td>
<td>dog (n=2), cat (n=1), horse (n=7), ape (n=1)</td>
<td>faeces (n=1), liver (n=1), air sac (n=1), muzzle (n=1), ear swab (n=1)</td>
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</tr>
</tbody>
</table>

1. not determined
Table 1: Origin, characteristics and year of isolation of bacterial isolates used in this study with phenotypes for AmpC (AMPC), ESBL-β-lactamases (ESBL), Quinolon-resistance-genes (Q)

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>hla and qnr genes</th>
<th>Number of isolates</th>
<th>Species</th>
<th>Year of isolation</th>
<th>Source</th>
<th>Material</th>
</tr>
</thead>
<tbody>
<tr>
<td>OXA-1, SHV, qnrB</td>
<td>1</td>
<td>Klebsiella pneumoniae</td>
<td>2011</td>
<td>dog</td>
<td>peritoneal fluid</td>
<td></td>
</tr>
<tr>
<td>CTX-M, SHV</td>
<td>6</td>
<td>E. coli (n=5), Enterobacter cloacae (n=1)</td>
<td>2010 (n=2), 2011 (n=1)</td>
<td>dog (n=1), horse (n=1), rat</td>
<td>peritoneal fluid (n=1), trachea (n=2), wound (n=2), faeces (n=1)</td>
<td></td>
</tr>
<tr>
<td>CTX-M, OXA-1, qnrS</td>
<td>1</td>
<td>E. coli</td>
<td>2011, 2012</td>
<td>dog</td>
<td>wound, faeces</td>
<td></td>
</tr>
<tr>
<td>CTX-M, OXA-1, SHV, qnrB, TEM</td>
<td>2</td>
<td>E. coli</td>
<td>2011, 2012</td>
<td>dog (n=1), horse (n=2)</td>
<td>faeces (n=1), wound (n=2)</td>
<td></td>
</tr>
<tr>
<td>CTX-M, OXA-1, TEM</td>
<td>2</td>
<td>E. coli, Klebsiella pneumoniae</td>
<td>2011, 2012</td>
<td>dog</td>
<td>faeces, knee implant</td>
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</tr>
<tr>
<td>CTX-M, OXA-1, SHV, TEM, qnrB</td>
<td>1</td>
<td>E. coli</td>
<td>2010</td>
<td>dog</td>
<td>faeces</td>
<td></td>
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<tr>
<td>CTX-M, TEM, qepS</td>
<td>1</td>
<td>Klebsiella pneumoniae</td>
<td>2012</td>
<td>dog</td>
<td>knee implant</td>
<td></td>
</tr>
<tr>
<td>CTX-M, SHV, TEM</td>
<td>6</td>
<td>Pantoea sp. (n=1), E. coli (n=2), Citrobacter freundii (n=1), Citrobacter braakii (n=1), Enterobacter cloacae (n=1)</td>
<td>2010 (n=1), 2011 (n=4), 2012 (n=1)</td>
<td>horse</td>
<td>trachea (n=2), wound (n=3), nasal swab (n=1)</td>
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</tr>
<tr>
<td>CTX-M, OXA-1, SHV, TEM</td>
<td>1</td>
<td>Citrobacter freundii</td>
<td>2012</td>
<td>horse</td>
<td>air sac</td>
<td></td>
</tr>
<tr>
<td>CTX-M, SHV, TEM, qnrB</td>
<td>1</td>
<td>E. coli</td>
<td>2010</td>
<td>horse</td>
<td>brain</td>
<td></td>
</tr>
<tr>
<td>CTX-M, TEM, SHV, TEM, qnrB, qnrS</td>
<td>2</td>
<td>E. coli</td>
<td>2011</td>
<td>donkey, horse</td>
<td>wound</td>
<td></td>
</tr>
<tr>
<td>SHV, qnrS</td>
<td>2</td>
<td>E. coli</td>
<td>2012</td>
<td>horse</td>
<td>placenta</td>
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</tr>
<tr>
<td>OXA-1, SHV, qnrB, qnrS</td>
<td>1</td>
<td>E. coli</td>
<td>2012</td>
<td>horse</td>
<td>air sac</td>
<td></td>
</tr>
<tr>
<td>CTX-M, OXA-1, SHV, TEM, qnrS</td>
<td>1</td>
<td>E. coli</td>
<td>2012</td>
<td>horse</td>
<td>air sac</td>
<td></td>
</tr>
</tbody>
</table>
Table 1: Origin, characteristics and year of isolation of bacterial isolates used in this study with phenotypes for AmpC- (AMPC), ESBL-β-lactamases (ESBL), Quinolon-resistance-genes (Q)

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>bla and qnr genes</th>
<th>Number of isolates</th>
<th>Species</th>
<th>Year of isolation</th>
<th>Source</th>
<th>Material</th>
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</thead>
<tbody>
<tr>
<td>Q (n=45)</td>
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<td>Klebsiella pneumonia (n=2), E. coli (n=26), Enterobacter cloacae (n=1), Citrobacter freundii (n=1), Shigella (n=1), Salmonella choleraesuis (n=1)</td>
<td>2010 (n=7), 2011 (n=23), 2012 (n=2)</td>
<td>rat (n=2), dog (n=14), guinea pig (n=3), cat (n=3), bird (n=1), cheetah (n=1), horse (n=8)</td>
<td>faeces (n=8), wound (n=8), semen (n=1), skin (n=4), urine (n=2), nose discharge (n=1), hoof (n=1), intestine (n=2), uterus (n=1), vagina (n=2), sinus (n=2)</td>
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<tr>
<td></td>
<td>qnrB</td>
<td>6</td>
<td>E. coli</td>
<td>2010 (n=1), 2011 (n=5)</td>
<td>dog (n=2), bird (n=1), horse (n=3)</td>
<td>skin (n=1), intestine (n=1), urine (n=1), wound (n=2), sinus (n=1)</td>
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<tr>
<td></td>
<td>qnrS</td>
<td>7</td>
<td>E. coli (n=6), Klebsiella pneumoniae (n=1)</td>
<td>2011</td>
<td>dog (n=4), cat (n=2), horse (n=1)</td>
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</tr>
<tr>
<td>AmpC/ESBL-Q (n=7)</td>
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<td>dog</td>
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<td>CTX-M, OXA-1, CIP, qnrB</td>
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<td>dog</td>
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<td>E. coli</td>
<td>2011</td>
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<td></td>
<td>TEM</td>
<td>1</td>
<td>Citrobacter freundii</td>
<td>2012</td>
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<td>E. coli</td>
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<td>E. coli</td>
<td>2011</td>
<td>horse</td>
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</tbody>
</table>

Note: Material includes faeces, wound, skin, intestine, urethra, vagina, nose discharge, hoof, sinus, uterus, and trachea.
<table>
<thead>
<tr>
<th>bla and qnr genes</th>
<th>primer</th>
<th>product size</th>
<th>Initial denaturation</th>
<th>number of cycles</th>
<th>Denaturation</th>
<th>Annealing</th>
<th>Extension</th>
<th>Final extension</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTX-M-forward</td>
<td>TCTTCCGAAATAAGGAAATCCC</td>
<td>909 bp</td>
<td>5' at 95°C</td>
<td>30</td>
<td>40° at 95°C</td>
<td>40° at 55°C</td>
<td>1' at 72°C</td>
<td>7 at 72°C</td>
<td>Kiratisin et al., 2008.</td>
</tr>
<tr>
<td>CTX-M-reverse</td>
<td>CGGTTCGCTATTACAAAC</td>
<td>706 bp</td>
<td>5' at 94°C</td>
<td>30</td>
<td>30° at 94°C</td>
<td>30° at 55°C</td>
<td>60° at 72°C</td>
<td>10' at 72°C</td>
<td>Sturenburg et al., 2004</td>
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<tr>
<td>SHV-r</td>
<td>GATGTTGTCATTCGCTCGCC</td>
<td>909 bp</td>
<td>5' at 94°C</td>
<td>30</td>
<td>30° at 94°C</td>
<td>30° at 55°C</td>
<td>60° at 72°C</td>
<td>10' at 72°C</td>
<td>Dierikx et al., 2012</td>
</tr>
<tr>
<td>TEM-r</td>
<td>GCGGAAACCTACATTTA</td>
<td>964 bp</td>
<td>5' at 94°C</td>
<td>30</td>
<td>30° at 94°C</td>
<td>30° at 55°C</td>
<td>60° at 72°C</td>
<td>10' at 72°C</td>
<td>Dierikx et al., 2012</td>
</tr>
<tr>
<td>OXA-1-f</td>
<td>AGAAAACATAAAATACGTTAG</td>
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<td>5' at 94°C</td>
<td>30</td>
<td>30° at 94°C</td>
<td>30° at 55°C</td>
<td>60° at 72°C</td>
<td>10' at 72°C</td>
<td>Dierikx et al., 2012</td>
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<tr>
<td>OXA-1-r</td>
<td>TGGTGTATGTAAGCTGCTC</td>
<td>706 bp</td>
<td>5' at 94°C</td>
<td>30</td>
<td>30° at 94°C</td>
<td>30° at 55°C</td>
<td>60° at 72°C</td>
<td>10' at 72°C</td>
<td>Dierikx et al., 2012</td>
</tr>
<tr>
<td>OXA-2-f</td>
<td>ACGATAAGACGCTGCTGCA</td>
<td>601 bp</td>
<td>5' at 94°C</td>
<td>30</td>
<td>30° at 94°C</td>
<td>30° at 55°C</td>
<td>60° at 72°C</td>
<td>10' at 72°C</td>
<td>Dierikx et al., 2012</td>
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<tr>
<td>OXA-2-r</td>
<td>ATYCTGTTGGCTCTGATCTAATA</td>
<td>346 bp</td>
<td>3' at 94°C</td>
<td>25</td>
<td>30° at 94°C</td>
<td>30° at 64°C</td>
<td>1' at 72°C</td>
<td>7 at 72°C</td>
<td>Pérez-Pérez et al., 2001</td>
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<tr>
<td>ACC-f</td>
<td>AACAGCTGCTGCAGGGCCG</td>
<td>520 bp</td>
<td>3' at 94°C</td>
<td>25</td>
<td>30° at 94°C</td>
<td>30° at 64°C</td>
<td>1' at 72°C</td>
<td>7 at 72°C</td>
<td>Pérez-Pérez et al., 2001</td>
</tr>
<tr>
<td>ACC-r</td>
<td>TTCGCCGCATCATCTCCAGC</td>
<td>500 bp</td>
<td>3' at 94°C</td>
<td>25</td>
<td>30° at 94°C</td>
<td>30° at 64°C</td>
<td>1' at 72°C</td>
<td>7 at 72°C</td>
<td>Pérez-Pérez et al., 2001</td>
</tr>
<tr>
<td>FOX-f</td>
<td>AACATGGGATACGTGTGCA</td>
<td>405 bp</td>
<td>3' at 94°C</td>
<td>25</td>
<td>30° at 94°C</td>
<td>30° at 64°C</td>
<td>1' at 72°C</td>
<td>7 at 72°C</td>
<td>Pérez-Pérez et al., 2001</td>
</tr>
<tr>
<td>FOX-r</td>
<td>CGAAGCGCTACGCCGCTGG</td>
<td>462 bp</td>
<td>3' at 94°C</td>
<td>25</td>
<td>30° at 94°C</td>
<td>30° at 64°C</td>
<td>1' at 72°C</td>
<td>7 at 72°C</td>
<td>Pérez-Pérez et al., 2001</td>
</tr>
<tr>
<td>MOX-f</td>
<td>GCTGCTCCAGGAGCATGATC</td>
<td>302 bp</td>
<td>3' at 94°C</td>
<td>25</td>
<td>30° at 94°C</td>
<td>30° at 64°C</td>
<td>1' at 72°C</td>
<td>7 at 72°C</td>
<td>Pérez-Pérez et al., 2001</td>
</tr>
<tr>
<td>MOX-r</td>
<td>CACATTGATACGTTGATGTC</td>
<td>302 bp</td>
<td>3' at 94°C</td>
<td>25</td>
<td>30° at 94°C</td>
<td>30° at 64°C</td>
<td>1' at 72°C</td>
<td>7 at 72°C</td>
<td>Pérez-Pérez et al., 2001</td>
</tr>
<tr>
<td>DHA-f</td>
<td>AACATTGATGACGGCTGGTG</td>
<td>302 bp</td>
<td>3' at 94°C</td>
<td>25</td>
<td>30° at 94°C</td>
<td>30° at 64°C</td>
<td>1' at 72°C</td>
<td>7 at 72°C</td>
<td>Pérez-Pérez et al., 2001</td>
</tr>
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<td>DHA-r</td>
<td>CGAAGCACTTGGCATGCTG</td>
<td>302 bp</td>
<td>3' at 94°C</td>
<td>25</td>
<td>30° at 94°C</td>
<td>30° at 64°C</td>
<td>1' at 72°C</td>
<td>7 at 72°C</td>
<td>Pérez-Pérez et al., 2001</td>
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<td>3' at 94°C</td>
<td>25</td>
<td>30° at 94°C</td>
<td>30° at 64°C</td>
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<td>7 at 72°C</td>
<td>Pérez-Pérez et al., 2001</td>
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<td>3' at 94°C</td>
<td>25</td>
<td>30° at 94°C</td>
<td>30° at 64°C</td>
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<td>7 at 72°C</td>
<td>Pérez-Pérez et al., 2001</td>
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<td>30° at 94°C</td>
<td>30° at 64°C</td>
<td>1' at 72°C</td>
<td>7 at 72°C</td>
<td>Pérez-Pérez et al., 2001</td>
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<td>25</td>
<td>30° at 94°C</td>
<td>30° at 64°C</td>
<td>1' at 72°C</td>
<td>7 at 72°C</td>
<td>Pérez-Pérez et al., 2001</td>
</tr>
<tr>
<td>bla and qnr genes</td>
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<td>product size</td>
<td>initial denaturation</td>
<td>number of cycles</td>
<td>denaturation</td>
<td>annealing</td>
<td>extension</td>
<td>final extension</td>
<td>reference</td>
</tr>
<tr>
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<td>----------------------</td>
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<td>--------------</td>
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<td>----------------------</td>
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<tr>
<td>VEB-f</td>
<td>GATAGGAGTACAGACATATG</td>
<td>914 bp</td>
<td>5' at 94°C</td>
<td>30</td>
<td>30° at 94°C</td>
<td>30° at 52°C</td>
<td>1' at 72°C</td>
<td>10' at 72°C</td>
<td>Kiratisin et al., 2008</td>
</tr>
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<td>VEB-r</td>
<td>TTTAATCCAAATAGTTATCCACG</td>
<td>864 bp</td>
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<td>30</td>
<td>30° at 94°C</td>
<td>30° at 56°C</td>
<td>1' at 72°C</td>
<td>10' at 72°C</td>
<td>Kiratisin et al., 2008</td>
</tr>
<tr>
<td>GES-f</td>
<td>ATGGCCTCATCAGCACC</td>
<td>927 bp</td>
<td>5' at 94°C</td>
<td>30</td>
<td>30° at 94°C</td>
<td>30° at 48°C</td>
<td>1' at 72°C</td>
<td>10' at 72°C</td>
<td>Kiratisin et al., 2008</td>
</tr>
<tr>
<td>GES-r</td>
<td>CTATTTTGCGGTGCTAGG</td>
<td>516 bp</td>
<td>5' at 94°C</td>
<td>30</td>
<td>30° at 94°C</td>
<td>30° at 53°C</td>
<td>1' at 72°C</td>
<td>10' at 72°C</td>
<td>Dierikx et al., 2012</td>
</tr>
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<td>PER-f</td>
<td>ATGAAATGCATCACAAATGG</td>
<td>469 bp</td>
<td>5' at 94°C</td>
<td>30</td>
<td>30° at 94°C</td>
<td>30° at 53°C</td>
<td>1' at 72°C</td>
<td>10' at 72°C</td>
<td>Dierikx et al., 2012</td>
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<td>PER-r</td>
<td>TCAATCCGGGACTCTGAT</td>
<td>482 bp</td>
<td>5' at 94°C</td>
<td>34</td>
<td>45° at 94°C</td>
<td>45° at 55°C</td>
<td>45° at 72°C</td>
<td>10' at 72°C</td>
<td>Park et al., 2006</td>
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<td>aac (6')-lb-cr-f</td>
<td>TGGCGATGCTCTATGAGTGGCTA</td>
<td>417 bp</td>
<td>5' at 94°C</td>
<td>30</td>
<td>30° at 94°C</td>
<td>30° at 53°C</td>
<td>1' at 72°C</td>
<td>10' at 72°C</td>
<td>Dierikx et al., 2012</td>
</tr>
<tr>
<td>aac (6')-lb-cr-r</td>
<td>CTCGAAATGTCTGCGTGCTTA</td>
<td>499 bp</td>
<td>5' at 94°C</td>
<td>30</td>
<td>1' at 96°C</td>
<td>1' at 60°C</td>
<td>1' at 72°C</td>
<td>5' at 72°C</td>
<td>Yamane et al., 2007</td>
</tr>
<tr>
<td>qepA-f</td>
<td>GCA GGT CCA GCA GCG GGT AG</td>
<td>584 bp</td>
<td>5' at 94°C</td>
<td>30</td>
<td>30° at 94°C</td>
<td>30° at 53°C</td>
<td>1' at 72°C</td>
<td>10' at 72°C</td>
<td>Wang et al., 2009</td>
</tr>
<tr>
<td>qepB-r</td>
<td>CTT CCT GCC CGA GTA TCG TG</td>
<td>199 bp</td>
<td>1' at 96°C</td>
<td>30</td>
<td>1' at 96°C</td>
<td>1' at 60°C</td>
<td>1' at 72°C</td>
<td>5' at 72°C</td>
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</tr>
<tr>
<td>qnrA-f</td>
<td>GAT CGG CAA AGG TTA GGT CA</td>
<td>417 bp</td>
<td>5' at 94°C</td>
<td>30</td>
<td>30° at 94°C</td>
<td>30° at 53°C</td>
<td>1' at 72°C</td>
<td>10' at 72°C</td>
<td>Dierikx et al., 2012</td>
</tr>
<tr>
<td>qnrB-f</td>
<td>GAT GTT GAA AGC CAG AAA GG</td>
<td>469 bp</td>
<td>5' at 94°C</td>
<td>30</td>
<td>30° at 94°C</td>
<td>30° at 53°C</td>
<td>1' at 72°C</td>
<td>10' at 72°C</td>
<td>Dierikx et al., 2012</td>
</tr>
<tr>
<td>qnrS-f</td>
<td>ACG ACA CCT GGT AGT TGT CC</td>
<td>516 bp</td>
<td>5' at 94°C</td>
<td>30</td>
<td>30° at 94°C</td>
<td>30° at 53°C</td>
<td>1' at 72°C</td>
<td>10' at 72°C</td>
<td>Dierikx et al., 2012</td>
</tr>
<tr>
<td>qnrS-r</td>
<td>TAA ATT GGC ACC CTG TAG GC</td>
<td>584 bp</td>
<td>5' at 94°C</td>
<td>30</td>
<td>30° at 94°C</td>
<td>30° at 53°C</td>
<td>1' at 72°C</td>
<td>10' at 72°C</td>
<td>Dierikx et al., 2012</td>
</tr>
<tr>
<td>qnrC-f</td>
<td>GGGTTGACATTCAATGAAGCT</td>
<td>666 bp</td>
<td>5' at 94°C</td>
<td>30</td>
<td>30° at 94°C</td>
<td>30° at 50°C</td>
<td>30° at 72°C</td>
<td>10' at 72°C</td>
<td>Wang et al., 2009</td>
</tr>
<tr>
<td>qnrC-r</td>
<td>TCCACTTTGAGGTTTCT</td>
<td>417 bp</td>
<td>5' at 94°C</td>
<td>30</td>
<td>30° at 94°C</td>
<td>30° at 49°C</td>
<td>30° at 72°C</td>
<td>10' at 72°C</td>
<td>Veldman et al., 2011</td>
</tr>
<tr>
<td>qnr-D-f</td>
<td>CGGGGAAATAGAAGTAAAAT</td>
<td>not described</td>
<td>5' at 94°C</td>
<td>30</td>
<td>30° at 94°C</td>
<td>30° at 49°C</td>
<td>30° at 72°C</td>
<td>10' at 72°C</td>
<td>Veldman et al., 2011</td>
</tr>
<tr>
<td>qnr-D-r</td>
<td>TATCGGTGAAATATACACC</td>
<td>not described</td>
<td>5' at 94°C</td>
<td>30</td>
<td>30° at 94°C</td>
<td>30° at 49°C</td>
<td>30° at 72°C</td>
<td>10' at 72°C</td>
<td>Veldman et al., 2011</td>
</tr>
</tbody>
</table>
3. Results

Between 2010 and 2012 150 isolates were phenotypically classified as resistant to β-lactam antibiotics or fluoroquinolones according to Clinical and Laboratory Standards Institute guidelines, 2013.

In 2010 30 isolates matched the inclusion criteria, in 2011 it were 82 isolates and in 2012 38 were included.

41 samples were determined to have an AmpC phenotype. Using multiplex-PCR assay for detection of the tested plasmid-mediated-ampC-gene-families in 20 none of these could be observed. In the remaining 21 isolates following gene families were determined: bla genes of the CIT-family (n=18), of the MOX-family (n=2) and one isolate showed a combination of genes from the CIT- and MOX-family.

An ESBL phenotype was displayed in 71 of the 150 isolates. 56 isolates tested positive for blaCTX-M-genes by simplex PCR. Furthermore 21 isolates were found to harbor blaOXA-1, 32 specimens blaSHV and 41 isolates blaTEM. No blaOXA-2, blaVEB, blaGES and blaPER genes were detected.

115 out of 150 isolates were resistant to ciprofloxacin. Out of these PCR revealed one qepA gene, 30 qnrB and 27 qnrS genes. In none of the samples qnrA, qnrC, qnrD or aac-6'-lb-cr were found. Various combinations of resistance genes were summarized in Table 1 and other features in Table 3-6.
Table 3: Number of resistance genes found in specimens of each year

<table>
<thead>
<tr>
<th>Year of isolation</th>
<th>CTX-M</th>
<th>OXA-1</th>
<th>SHV</th>
<th>TEM</th>
<th>CIT</th>
<th>MOX</th>
<th>qnrB</th>
<th>qnrS</th>
<th>qepA</th>
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</thead>
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<tr>
<td>2010 (n=30)</td>
<td>14</td>
<td>5</td>
<td>5</td>
<td>8</td>
<td>3</td>
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<td>9</td>
<td>5</td>
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</tr>
<tr>
<td>2011 (n=82)</td>
<td>35</td>
<td>11</td>
<td>18</td>
<td>26</td>
<td>2</td>
<td>3</td>
<td>16</td>
<td>17</td>
<td>0</td>
</tr>
<tr>
<td>2012 (n=38)</td>
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<td>5</td>
<td>9</td>
<td>7</td>
<td>14</td>
<td>0</td>
<td>5</td>
<td>5</td>
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</table>

Table 4: Combination of ESBL and AmpC resistance genes with fluoroquinolone resistance genes

<table>
<thead>
<tr>
<th>Fluoroquinolone resistance gene</th>
<th>CTX-M (n=56)</th>
<th>OXA-1 (n=21)</th>
<th>SHV (n=32)</th>
<th>TEM (n=41)</th>
<th>CIT (n=19)</th>
<th>MOX (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>qnrB (n=30)</td>
<td>15</td>
<td>13</td>
<td>11</td>
<td>8</td>
<td>6</td>
<td>1</td>
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<tr>
<td>qnrS (n=27)</td>
<td>7</td>
<td>9</td>
<td>8</td>
<td>8</td>
<td>4</td>
<td>1</td>
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<tr>
<td>qepA (n=1)</td>
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Table 5: Number of resistance genes found in different bacterial species

<table>
<thead>
<tr>
<th>Bacterial species</th>
<th>CTX-M</th>
<th>OXA-1</th>
<th>SHV</th>
<th>TEM</th>
<th>CIT</th>
<th>MOX</th>
<th>qnrB</th>
<th>qnrS</th>
<th>qepA</th>
</tr>
</thead>
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<tr>
<td>Escherichia coli (n=101)</td>
<td>43</td>
<td>15</td>
<td>20</td>
<td>28</td>
<td>4</td>
<td>2</td>
<td>18</td>
<td>21</td>
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<tr>
<td>Klebsiella sp. (n=12)</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>5</td>
<td>1</td>
<td>8</td>
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<td>Enterobacter sp. (n=12)</td>
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\(^1\) number of isolates in this year
\(^2\) number of specimens containing this gene
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<th>animal species</th>
<th>CTX-M</th>
<th>OXA-1</th>
<th>SHV</th>
<th>TEM</th>
<th>CIT</th>
<th>MOX</th>
<th>qnrB</th>
<th>qnrS</th>
<th>qepA</th>
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<td>dog (n=57)</td>
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<td>3</td>
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<td>21</td>
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<td>8</td>
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4. Discussion

A variety of resistance genes was found within tested bacteria isolated from seven animal species.

In the present study most AmpC β-lactamase producing isolates were found to harbor genes of the CIT-group (19 out of 22). Enzymes of the CIT-group have the broadest geographic spread and seem to occur more often than enzymes of other groups (Jacoby et al., 2009), which was also the case in the present study.

CIT was reported to occur in Enterobacteriaceae isolates in most European countries as following studies show. The Norwegian Norm-Vet report 2011 described only cases of $\text{bla}_{\text{CMY-2}}$ (CIT-group), the most common AmpC β-lactamase and one $\text{bla}$ gene belonging to the DHA-group in human and animal specimens, similarly the Swedish SVARM report 2012 accounts only cases of $\text{bla}_{\text{CMY-2}}$ mainly found in $E. coli$ isolates from poultry, as well as the Danish Danmap report 2012, in which solely $\text{bla}$ genes of the CIT-group were detected. (NORM-VET 2011, SWEDRES/SVARM 2012, DANMAP 2012) In Switzerland a study conducted between 2006 and 2007 in human hospitals found a prevalence of $\text{bla}_{\text{ampC}}$ genes of 0.16% in Enterobacteriaceae without inducible chromosomal ampC genes and found enzymes of the DHA-group and CIT-group, whereby $\text{bla}_{\text{CIT}}$ genes were clearly predominant. (Adler et al., 2008) Another study conducted during the years 2007, 2008 and 2009 on Enterobacteriaceae isolates from companion animals and horses in the Netherlands detected eleven plasmid-mediated ampC genes, all belonging to the CIT-group (Dierikx et al., 2012). In Germany a study on 22679 Salmonella isolates showed six isolates to carry $\text{bla}_{\text{CIT}}$ genes. (Rodriguez et al., 2009)

A combination of $\text{bla}_{\text{CIT}}$ and $\text{bla}_{\text{MOX}}$ was also detected in the present study. It was suggested that expression of more than one plasmid-mediated AmpC β-lactamase might not be possible due to the inability to accurately detect multiple AmpC β-lactamases or the possibility that bacterial cells can only accommodate one AmpC β-lactamase and still be viable pathogens. (Pérez-Pérez et al., 2001) However in more recent studies plasmids cocarrying genes of the CIT- and DHA-group were reported. (Tamang et al., 2012)

Enterobacteriaceae isolates carrying ampC genes often contained multiple other resistance genes in the present study. PCR outcomes for one of the isolates suggest that it harbored a
combination of CIT, CTX-M, OXA-1 and qnrB genes. Several isolates harbored ampC and qnr genes alike (4 Klebsiella sp., 2 E. coli, 1 Serratia sp.). ampC genes were found in specimens from 8 different animal species and 5 bacterial species. Isolates from dogs (n=6) and horses (n=5) were predominant.

In 19 isolates exhibiting an AmpC phenotype no ampC genes were found. This could be due to testing for plasmid-mediated AmpC β-lactamases but not chromosomal ones. An AmpC resistance phenotype can also be caused by chromosomal bla_{ampC} genes, which are not origins of plasmid-mediated AmpC β-lactamases. (Pérez-Pérez, 2001) That could explain E. coli isolates (n=10) showing AmpC phenotypes without positive PCR results. Hyperproduction of the chromosomal ampC gene in these specimens seems to be the most likely conclusion. (Jacoby et al., 2009, Pérez-Pérez, 2001)

No plasmid-mediated ampC genes could be detected in AmpC isolates of Citrobacter sp. (n=5) and Enterobacter sp. (n=4), which are bacterial species serving as origins for plasmid-mediated ampC genes and therefore not fitting the explanation given for the E. coli specimens. While the chromosomal gene of Citrobacter freundii is proposed to be the origin of the CIT group, the one from Enterobacter cloacae gave rise to the EBC group of plasmid-mediated β-lactamases. However other chromosomal bla_{ampC} genes of Citrobacter sp. and Enterobacter sp. could cause an AmpC phenotype in derepressed mutants. Derepressed mutants are bacterial organisms constitutively overexpressing chromosomal genes. (Jacoby et al., 2009, Pérez-Pérez, 2001)

There is also the possibility of not detecting newly discovered AmpC β-lactamases like bla_{CPE-1}, which was described in 2004 likely originating from Citrobacter freundii, whereas the primers designed for multiplex PCR derive from a publication of Pérez-Pérez in 2001. (Nakano et al., 2004)

bla_{ampC} genes of the MOX-group have been found rarely in Northern Europe as opposed to some countries in Asia like India (Parveen et al., 2012), Turkey, where bla genes of the MOX-group were found to be predominant (Yilmaz et al., 2013), Japan, where bla_{MOX-1} was isolated in 1991 (Horii T. et al., 1994) and bla_{CMY-9} in 1995 (Doi et al., 2002), Taiwan (bla_{CMY-8}) and South Korea (bla_{CMY-1}, bla_{CMY-10}, bla_{CMY-11}) (Doi et al., 2002). A bla_{MOX} gene was detected in Europe in a Greek patient in 1997 (bla_{MOX-2}) (Raskine et al., 2002). AmpC β-lactamases of the MOX-group are apparently distributed more in the South of Europe and
Asia, so that spread of resistance genes with the bringing in of companion animals, like cats and dogs, from Southern countries should also be considered.

ESBL-producing *Enterobacteriaceae* have become one of the major problems in human medicine, especially in terms of nosocomial infections and therefore investigations are urgently needed to quantify the significance of horses and companion animals as sources of infection and to identify the role of these often multiresistant bacteria in disease of pets and horses. (Ewers et al., 2011) Multiresistance in a bacterial isolate is defined as the presence of three or more resistance genes or mutations, all of which are affecting different antimicrobial classes or subgroups. (Schwarz et al., 2010)

Several countries document the use of antimicrobial agents in animals, especially livestock. (Ewers et al., 2011) However only few countries provide detailed information about the prescriptions for dogs, cats and horses. Surveillance studies like DANMAP and SVARM confirmed β-lactam antibiotics as the most commonly prescribed antibiotics in companion animals. (Ewers et al., 2011)

During the 1990’s predominance of TEM- and SHV-ESBLs was reported, whereas nowadays CTX-M enzymes are found most often. (Coque et al., 2008) A predominance of CTX-M ESBLs was also found in the present study for the year 2011 (Table 3), where specimens were collected for the whole duration of the year, therefore leading to the most representative results. 42.7% of the specimens tested positive for *bla*<sub>CTX-M</sub>-genes, 31.7% for *bla*<sub>TEM</sub>-genes and 21.9% for *bla*<sub>SHV</sub>-genes. This shows that in companion animal and horse isolates from Austria CTX-M ESBLs seem to be the most commonly occurring type of ESBL.

One of the main concerns is also co-selection of ESBLs with other resistance genes, for example fluoroquinolone, aminoglycoside and sulfonamide resistance genes, often leading to the development of multiresistant bacteria and the danger of coselection of one resistance gene with another one. (Coque et al., 2008)

33.3% of the specimens recognized as AmpC β-lactamase producers also contained a *qnrB* and/or a *qnrS* gene, this was also the case for 32% of the *bla*<sub>CTX-M</sub> positive specimens, for 24.4% of the *bla*<sub>TEM</sub> positive ones, for 43.8% of the *bla*<sub>SHV</sub> positive isolates and for 71.4% of the specimens with resistance genes of the OXA-1 group.

Studies showed that clonal expansion of *Enterobacteriaceae* with the ability to acquire
plasmids with multiple ESBLs was favoured as these clones are especially successful. (Coque et al., 2008)

There are a multitude of factors contributing to the dissemination of resistance genes although with different importance, to name just a few there is antibiotic overuse in humans and animals, hospital cross-infection, the food chain, trade and human migration. Generally ESBLs seem to be far less common in Europe than in Latin America and Asia/Pacific regions but more frequent than in North America. (Coque et al., 2008) An EARSS (European Antimicrobial Resistance Surveillance System) study conducted in 2011 revealed a high proportion of *Klebsiella pneumoniae* isolates (>50%) to be resistant to third generation cephalosporins (indicating ESBL production) in Lithuania, Poland, Slovakia, Hungary, Greece and Bulgaria and also 25%-<50% of resistant *E. coli* isolates in Slovakia, in comparison to the other European countries like Austria, Germany and several others (data was not available for every country). Most published studies also indicate that the prevalence of ESBL isolates is lower in most northern European countries, for example Norway, Sweden, Finland, Ireland and the United Kingdom compared to the southern and eastern ones. (Coque et al., 2008, EARS-Net 2011)

*E. coli* isolates from the German resistance monitoring programme, GERM-Vet, showed the *blaCTX-M* gene of group 1 to be the predominant ESBL gene among *E. coli* isolates from diseased animals in Germany. (Schink et al., 2013)

A study conducted in Tyrol on ten cats and two dogs, mainly kept in animal shelters, showed that 75% of the isolates belonged to the CTX-M-1-group, 8% to the CTX-M-2-group and 17% to the CTX-M-9-group and that one isolate was positive for both CTX-M-1 and CTX-M-9. Another interesting finding of this study was that sequence types detected by MLST in several dogs and cats were previously reported to occur in severe human infection, indicating transmission between humans and companion animals. (Franiek et al., 2012)

In the present study the most commonly occurring ESBL genes in dogs were the CTX-M group with 37.7%, *blaTEM* with 24.5% and the OXA-1 group with 22.6%. A study conducted in China from 2007 to 2008 among 240 *E. coli* isolates recovered from healthy and sick dogs showed a high prevalence of ESBLs and identified the CTX-M group to be the most common one (Sun et al., 2010). Two studies conducted in Europe from 2008 to 2009 and 2010 found the CTX-M group and *blaSHV* in *E. coli* specimens and genes of the CTX-M group in
Citrobacter freundii isolates of dogs to be the most prevalent. (Ewers et al., 2011) bla<sub>SHV</sub> in the present study was responsible for 15% of the ESBL phenotypes in dog specimens with bla<sub>CTX-M</sub>, bla<sub>TEM</sub> and the OXA-1 group being found more often. In the Netherlands 38 dog specimens collected between 2007 and 2009 showed a typical phenotype for ESBLs and ampC β-lactamases, of these 17 tested positive for bla<sub>TEM</sub>, 10 for bla<sub>CTX-M-1</sub> group, 4 for bla<sub>CTX-M-9</sub> group and 3 for bla<sub>OXA-1</sub>. (Dierikx et al., 2012) The study by Dierikx et al. shows similar results to the present study in so far that the two most commonly found ESBL genes in dogs in their study and the present one are bla<sub>TEM</sub> and the bla<sub>CTX-M</sub>. (Dierikx et al., 2012)

In cats the ESBL genes in the present study were of the bla<sub>CTX-M</sub> group (2 out of 3), the other ESBL gene found was bla<sub>TEM</sub>. In the Netherlands a total of 11 ESBL resistance genes were found within 14 feline bacterial isolates containing ESBL genes of following origin, 5 bla<sub>TEM</sub> genes, 2 of the bla<sub>CTX-M-1</sub> group and one each of bla<sub>OXA-1</sub>, bla<sub>SHV</sub>, the bla<sub>CTX-M-9</sub> and the bla<sub>CTX-M-2</sub> group. (Dierikx et al., 2012) In Rome, Italy, PCR screening for ESBL genes in E. coli isolates from companion animals provided following results; three cat specimens contained bla<sub>CTX-M</sub> group 1 and bla<sub>TEM</sub>, in 17 dog specimens 15 bla<sub>TEM</sub>, 13 bla<sub>CTX-M</sub> group 1, 4 bla<sub>SHV</sub> and 2 ampC genes of the CIT-group were found and there was also an isolate from a rat, found dead in one of the kennels, screened, which contained an ampC gene of the CIT-group and a bla<sub>CTX-M</sub> group 1 gene. (Carattoli et al., 2005)

The results of the present study show a high accordance with the one from Carattoli et al.; cat isolates in the present study tested positive for bla<sub>CTX-M</sub> and bla<sub>TEM</sub> as well, dog isolates also mainly contained the ESBL genes mentioned and ampC genes of the CIT-group prevailed (8 of the CIT-group as compared to 2 of the MOX-group). bla<sub>OXA</sub> genes were not tested for by Carattoli et al., but it is indicated in the study that the observed resistance phenotype cannot be completely explained by the identified β-lactamase genes, so that presence of other β-lactamas es is likely. (Carattoli et al., 2005)

Horses are basically regarded as food-producing animals in Austria and most other countries restricting the use of antimicrobial agents for treatment, but they may likewise be classified as companion animals, allowing veterinarians to treat them with a broader variety of substances. (Ewers et al., 2011)

In the present study the most commonly found ESBLs in horse isolates were CTX-M type
ESBLs (in 55.4% of the horse isolates), SHV-ESBLs (in 37.5%) and TEM-ESBLs (in 46.4%). Only *bla*<sub>AMP</sub> genes of the CIT-group were detected, which is consistent with the findings of the following studies.

A study conducted in 2003 to 2005 on seven multiresistant *Enterobacteriaceae* isolates from horses, four *Escherichia coli* and three *Klebsiella pneumoniae*, found them to carry *bla<sub>CTX-M-1</sub>* (5 out of 7 isolates), *bla<sub>CIT</sub>* (1 out 7), *bla<sub>TEM</sub>* (4 out of 7) and/or *bla<sub>SHV</sub>* (3 out of 7) genes. (Vo et al., 2007) Another study investigated seven *Enterobacteriaceae* isolates from horses between 2007 and 2009 and determined 9 of them to harbor *bla<sub>CTX-M</sub>-genes of group 1, two of group 9 and one of group 2, furthermore 8 isolates contained *bla<sub>TEM</sub>*. No *bla<sub>SHV</sub>* genes were found. (Dierikx et al., 2011)

An outbreak of salmonellosis led to the closure of one of the largest equine hospitals in the United States. *Salmonella enterica* isolates obtained from equine patients there from July 2003 to May 2004 were positive for *bla<sub>CIT</sub>* and *bla<sub>SHV</sub>* genes by PCR. (Rankin et al., 2005) In the present study the same three groups of ESBL and AmpC genes (*bla<sub>TEM</sub>* and *bla<sub>SHV</sub>* and *bla<sub>CIT</sub>* were carried by the 5 *Salmonella sp.* isolates from horses.

2 isolates of extraintestinal pathogenic *E. coli* from horses, collected between October 1999 and December 2007 in Australia, also conferred resistance through *bla<sub>CIT</sub>*. (Gibson et al., 2010)

A study conducted on 4 *Citrobacter freundii* horse specimens isolated in Europe from 2008 to 2010 revealed them to harbor *bla<sub>CTX-M</sub>-genes of group 1. (Ewers et al., 2011)

In the present study all 4 *Citrobacter freundii* horse specimens tested positive for *bla<sub>TEM</sub>* and *bla<sub>SHV</sub>* genes and two of them for *bla<sub>CTX-M</sub>*.

However the results of the present study with *Enterobacteriaceae* from horses mainly carrying resistance genes belonging to CTX-M, TEM and SHV ESBLs as well as to the AmpC β-lactmase CIT-group is consistent with other studies conducted in Europe, the United States and Australia.

33% of the isolates from 149 human patients from the southeast of Austria contained CTX-M genes and the most commonly encountered CTX-M genes there were of the *bla<sub>CTX-M-1</sub>* (38 out of 49) and *bla<sub>CTX-M-9</sub>* (11 out of 49) group. (Eisner et al., 2006) *bla<sub>CTX-M</sub>* occurs to be the most prevalent ESBL in human and animal patients in Austria.

Less prevalent ESBLs like *bla<sub>VEB</sub>, bla<sub>PER</sub>* and *bla<sub>GES</sub>*, which can be either plasmid-mediated or
encoded on the chromosome, were not found in any of the tested isolates. This was not surprising as these \textit{bla} genes occur only rarely in Europe and have mainly been reported in Asia and South America. \textit{bla}_{GES} has been predominantly found in environmental samples and all three resistance genes seem to occur especially often in \textit{Acinetobacter baumanii}. (Poirel et al., 2012) Also the majority of reports regarding ESBL enzymes in companion animals reflect those ESBLs which are predominant in human samples of the investigated geographical regions suggesting that due to the often smaller sample size of veterinary studies only the most frequent enzymes are detected while larger sample sizes would possibly also show less frequent variants and \textit{bla}_{VEB}, \textit{bla}_{PER} and \textit{bla}_{GES} have so far not been found in isolates of human patients from Austria. (Ewers et al., 2011)

In France 31 bacterial isolates from humans from 2006 were screened and only one contained a VEB-type ESBL, none \textit{PER}- or \textit{GES}-type ESBLs (Dallenne et al., 2010), likewise a study conducted in Thailand from 2004 to 2005 found no \textit{bla}_{GES} or \textit{bla}_{PER} genes, but found \textit{bla}_{VEB} to be present in ESBL-producing \textit{Klebsiella pneumonia} and \textit{E. coli} isolates (Kiratisin et al., 2008).

\textit{Bla}_{PER} has mainly been found in gram-negative bacilli, such as \textit{Pseudomonas aeruginosa}, \textit{Acinetobacter} spp., and \textit{Alcaligenes faecalis}; however, it has also recently been found in \textit{Enterobacteriaceae} (Bae et al., 2011). 1991 a \textit{bla}_{PER} gene was found in \textit{Klebsiella pneumonia} from a hospitalized patient in France (Nordmann et al., 1993). Production of \textit{PER} enzymes was also reported in Turkey in \textit{Salmonella typhimurium} isolates from two hospitals (Vahaboglu et al., 1995) and in Northern Italy \textit{bla}_{PER} genes have been found in \textit{Proteus mirabilis} isolated during 1997 and 1998 in a hospital (Pagani et al., 2002). In 2004 \textit{PER} production in a urinary isolate of \textit{Providencia rettgeri} in Ankara, Turkey was reported (Bahar et al., 2004).

In Austrian companion animals and horses in the present study none of these resistance genes was found and overall they seem to be still more seldom in Europe than other ESBLs.

Fluoroquinolone resistance screening in the present study lead to following findings; none of the \textit{qnrA}, \textit{qnrC}, \textit{qnrD} and \textit{aac(6')-Ib-cr} genes were found, whereas one isolate harboring the \textit{qepA} gene was found. Plasmid-mediated fluoroquinolone resistance in isolates examined during the present study was conveyed by \textit{qnrS} and/or \textit{qnrB} genes.
qnrC and qnrD genes have mainly been found in China, one in a clinical *Proteus mirabilis*
isoalte in 2006 and the other in isolates of *Salmonella enterica* spp. of humans from 2006 to
2007, newer studies report the qnrD gene to occur in Nigeria as well. (Wang et al., 2009,
Cavaco et al., 2008, Ogbolu et al., 2011)

Plasmid-mediated qnrA genes were found in the United States, Asia, but also in Europe, for
example in France and the Netherlands. (Strahlevitz et al., 2009) In a study conducted in the
Netherlands three qnrA genes were detected in *E. cloacae* isolates of dogs. (Dierikx et al.,
2012) In Austria qnrA has been found in an *E. coli* isolate from a human patient in Innsbruck.
(Huemer et al., 2011) However in the present study no qnrA gene was found in animal
isolates.

*aac(6')-Ib-cr* is an aminoglycoside acetyltransferase and may be even more prevalent than
qnr genes especially in *E. coli* strains. It has been reported to occur worldwide; in the United
States, South America, Australia as well as Europe, where it was found for example in *E. coli*
isolates collected between 2000 and 2006 in France, Portugal, Spain and Switzerland, in *E.
coli* and *K. pneumoniae* isolates in Germany (2006-2007), in Slovenia, the United Kingdom,
Hungary and Bulgaria. (Strahlevitz et al., 2009) It was likewise found in 16 out of 197 *E. coli*
isolates in North-East Italy. (Frasson et al., 2011) In companion animals it is also present as a
study from China shows, where 19 out of 101 animal isolates, including several dog and cat
specimens, carried an *aac(6')-Ib-cr* gene. (Ma et al., 2008)

qepA together with *blaTEM* and a *blaCTX-M* gene of group 1 was detected in the present study
in an *E. coli* isolate from 2012 derived of the faeces of a dog and to the best of the author’s
knowledge it is the first report of a qepA gene in a companion animal in Austria. It has been
previously found in companion animals. In China 16 *E. coli* strains derived from dogs, pigs,
ducks and partridges contained qepA. (Ma et al., 2008)

In the vast majority of surveys qnr genes were more prevalent in *Klebsiella* spp. and
*Enterobacter* spp. than in *E. coli*. (Strahlevitz et al., 2009) In the present study qnr genes
were found in 30,7% of *E. coli* specimens, in 25% of the *Enterobacter* spp. and in 75% of the
*Klebsiella* spp., indicating that at least for *Klebsiella* spp. this was true also in the present
study, although results could be biased by an unbalanced distribution of *Enterobacteriaceae*
isolates, as isolates included in the present study belonged mainly to *E. coli* (101 of 150
specimens).
In the present study the only two qnr genes found were qnrB and qnrS with a slightly higher number of isolates carrying qnrB (30) than qnrS (27) genes. These two genes were also found in the Netherlands in two cat specimens (qnrS) and a specimen from a turtle (qnrB). (Dierikx et al., 2012)

Another study on plasmid-mediated fluoroquinolone resistance in Salmonella enterica and E. coli isolates from humans, food producing animals, reptiles, animal feed, food and the environment revealed a dominance of qnrB and qnrS compared to qnrA and aac(6’)-Ib-cr in 13 European countries (Veldman et al., 2011). qnrS genes were found in E. coli isolates from Poland, Finland, the Netherlands and Denmark in food-producing animals and in Salmonella enterica isolates from 7 different countries including Germany and Italy, as well as several others. qnrB was detected in E. coli isolates from turkeys in Poland and in Salmonella enterica isolates from pigs in the Czech Republic, fowl in Germany, Spain and the Netherlands, reptiles in Germany and turkeys in Spain, Finland, Denmark and Germany (Veldman et al., 2011). Summarizing the results of this study it becomes obvious that qnrB and qnrS genes are widespread in European countries (Veldman et al., 2011) and this supports the findings of the present study.

Plasmid-mediated quinolone resistance (PMQR) genes are often cocarried with ESBL or ampC genes on the same plasmids, which is concerning, because selection through antibiotic treatment favours bacterial strains carrying multiple resistance genes. qnrB is often associated with blaCTX-M, blaSHV and blaTEM genes, whereas qnrS genes are not that likely to be associated with other resistance genes and can be found more often on small and nonconjugative plasmids or in combination with blaTEM. (Strahlevitz et al., 2009)

77% of the qnrB and 70% of the qnrS positive isolates were also positive for an ESBL and or an ampC gene. In the present study the difference of qnrB and qnrS concerning cocarriage with ampC and/ or ESBL genes in the same isolate seems not to be noticeable. 40% of the qnrB and 26% of the qnrS genes were were associated with blaCTX-M and 43% of the qnrB and 33% of the qnrS genes with blaOXA. qnr genes in the present study occurred mainly in isolates testing positive for blaCTX-M and/ or blaOXA genes.
5. Conclusion

Monitoring of resistance genes in companion animals and horses seems to be highly important as these animal species are exposed to similar antimicrobial agents as humans during treatments and are also usually in close contact with their owners, especially companion animals, whose social role has changed in the last decades.

Companion animals and horses harbor a variety of different β-lactam resistance genes also shown to occur in humans. Therefore the animal population should be considered as a reservoir for exchanging antimicrobial resistance determinants in bacterial populations and as having a high zoonotic potential.

Inferred from the results of the present study resistance genes in Enterobacteriaceae seem not to occur species specific and there is no predominance of a certain resistance gene in a specific animal species, although the number of specimens would have to be higher to ascertain this fact. In order to choose appropriate antimicrobial treatment bacterial culturing with species identification and susceptibility testing of site-specific isolates should be performed.
6. Summary

Plasmid-mediated resistance genes in *Enterobacteriaceae* of animals pose a serious threat as these genes can thereby accumulate in bacterial cells resulting in multiresistant strains of bacteria which become complicated or even impossible to treat with the known antimicrobial agents. As transmission of resistance genes between humans and animals has been documented, it is especially worrisome to find resistant *Enterobacteriaceae* in companion animals and horses as they live in close contact with their owners and are treated with the same classes of antibiotics used in human medicine.

Therefore the aim of the present study was to perform PCR screening on *Enterobacteriaceae* isolates from companion animals and horses, showing a resistance phenotype as defined by CLSI criteria 2013, for the occurrence and identification of ESBL, *ampC* and fluoroquinolone resistance genes.

Resistant bacterial strains were isolated from specimens of 13 different animal specimens collected between 2010 and 2012.

Various resistance genes as well as combinations of these were found with the predominant ESBLs being *bla*<sub>CTX-M</sub>, *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub> and *bla*<sub>OXA</sub>.

*bla*<sub>CTX-M</sub> genes were clearly predominant compared to the other ESBL genes.

*ampC* genes of the CIT- and MOX-type, as well as fluoroquinolone resistance genes, namely *qnrS*, *qnrB* and *qepA* were found.

Following resistance genes were not detected; *bla*<sub>VEB</sub>, *bla*<sub>GES</sub>, *bla*<sub>PER</sub>, *bla*<sub>OXA-2</sub>, *qnrA*, *qnrC*, *qnrD* and *aac(6')-Ib-cr*.

The prudent use of antimicrobial agents in small animal and horse clinics is of great importance and national surveillance and monitoring of resistance genes should not merely focus on food-producing animals, but also on companion animals and horses.
7. Zusammenfassung

Resistenz-Gene gegen Antibiotika spielen, sowohl in der Human-, als auch in der Veterinärmedizin, eine wichtige Rolle, da Infektionen mit bakteriellen Erregern, die ESBL-, ampC- oder qnr-Resistenz-Gene oder eine Kombination mehrerer dieser Gene enthalten, nur schwer therapierbar sind.

Resistenz-Gene können auf unterschiedliche Art und Weise zwischen Bakterien übertragen werden, wobei Plasmide hierbei eine wichtige Rolle spielen. 


Die Ergebnisse dieser Studie zeigen, bezüglich ESBL Resistenz-Genen, einen hohen Anteil an blaCTX-M, blaTEM, blaSHV und blaOXA-1-positiven Isolaten, während blaVEB, blaGES, blaPER und blaOXA-2 nicht gefunden werden konnten.


Aus dieser und ähnlichen Studien ist ersichtlich, dass verantwortungsvoller Einsatz von Antibiotika bei Haustieren und Pferden, sowie nationales und internationales Resistenzmonitoring, wichtig ist um die Wirksamkeit bedeutender Antibiotikagruppen zu erhalten.
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